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Bioactivity of honey: phenolic composition, antioxidant trends and carcinoma cell lines effects through digestion

Ceren Mutlu [a,*], Zeynep Demir [b], Volkan Aylanc [a,c], Aysun Özkan [b], Mustafa Erbaş [d]

[a] CIMO, LA SusTEC, Instituto Politécnico de Bragança, Campus de Santa Apolónia, 5300-253 Bragança, Portugal
[b] Biology Department, Science Faculty, Akdeniz University, Antalya, Türkiye
[c] LAQV-REQUIMTE, Departamento de Química e Bioquímica, Faculdade de Ciências, Universidade do Porto, 4169-007, Porto, Portugal
[d] Food Engineering Department, Engineering Faculty, Akdeniz University, Antalya, Türkiye

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ABSTRACT

Honey is not only a food source but also a valuable substance for health and medicine, owing to its complex composition and bioactive properties. However, there is very limited information on the changes in the active compounds of honey during digestion and their antioxidant capacity and effect on cancer cells at the end of digestion. Herein, we investigate the dynamic changes in their bioactive compound composition and antioxidant activity during in vitro gastrointestinal digestion and the effect of digested honey on several cancer cell lines, after determining the antimicrobial and anti-inflammatory effects of mono- and multifloral kinds of honey. The tested raw honey samples exhibited higher anti-inflammatory properties (36 %–80 %) with increasing total phenolic content (78–132 mg GAE/100 g), along with significant antimicrobial activity against *E. coli* (6–9 mm) and *S. aureus* (6–14 mm) bacterial species. The findings showed that total phenolic and flavonoid contents increased significantly during digestion, with a peaking value of 258 mg GAE/100 g in the intestinal phase, while TEAC and CUPRAC analysis exhibited variable trends depending on the digestion stage. Moreover, the concentration of compounds such as gallic, syringic, caffeic, *p*-coumaric, trans-cinnamic acid, and methyl-3,4,5-trihydroxybenzoate identified by HPLC-DAD showed some fluctuations at different stages of digestion. Cytotoxicity analysis revealed that digested honey samples, particularly those with higher phenolic content, exhibited pronounced antiproliferative effects on cancer cells at higher concentrations, with minimal effects on healthy cells. These findings underscore the importance of honey's bioactive compounds, their transformation during digestion, and their potential health benefits.

1. Introduction

With its rich bioactive compounds, honey possesses numerous health-promoting properties. Both in vivo and in vitro studies have demonstrated that honey has been used since ancient times as an effective wound-healing agent, a nutritious natural food, and a source of various health benefits, including antioxidant, anti-inflammatory, anti-cancer, and antimicrobial activities (Cilla et al., 2022; Gośliński et al., 2021; O Sullivan et al., 2013; Seraglio et al., 2021).

Bioactivity refers to the capacity of a compound to produce biological effects, including antioxidant, antimicrobial, and anti-inflammatory actions (Dima et al., 2020), and the bioactivity of honey is attributed to its diverse bioactive compounds, including phenolic acids, flavonoids, methylglyoxal, hydrogen peroxide, enzymes, bee defensin, amino acids, organic acids, and minerals (Ávila et al., 2019; Cilla et al., 2022; Magoshi et al., 2023; Seraglio et al., 2017). Among these, polyphenols have been particularly emphasized due to their significant contribution to honey's biological activity and numerous research have focused on elucidating the relationship between polyphenols and the health-promoting effects of honey (Ávila et al., 2019; Seraglio et al., 2017).

Phenolic compounds are secondary metabolites primarily synthesized by plants via the phenylpropanoid pathway, starting from the phenylalanine or tyrosine amino acids. Through this pathway, these amino acids are deaminated into cinnamic acids, and hydroxyl groups are subsequently attached to the phenyl ring, resulting in a diverse range of phenolic compounds such as benzoic acids (C6-C1), cinnamic acids (C6-C3), and flavonoids (C6-C3-C6) (Castellano et al., 2012). In honey, the primary source of phenolic compounds is nectar, and their

* Corresponding author.
E-mail address: ceren@ipb.pt (C. Mutlu).

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concentration and composition can vary significantly depending on geographical and climatic conditions, bee species, production processes, and storage conditions (Cilla et al., 2022; Seraglio et al., 2021). The quantity and composition of phenolic compounds vary considerably for monofloral and multifloral blossom honey and honeydew honey produced from different botanical origins and geographical areas worldwide. Nevertheless, the extent to which honey exhibits its health-promoting effects is influenced not only by the concentration and variety of these compounds but also by their bioaccessibility during gastrointestinal digestion. Bioaccessibility refers to the portion of an ingested bioactive compound that is released from the food matrix and becomes available for absorption across the epithelial layer of the gastrointestinal tract (Dima et al., 2020). Numerous studies have revealed that bioactive compounds undergo significant changes during digestion due to factors such as enzymatic activity, ion interactions, and variations in pH value. These changes directly affect the bioaccessibility and bioavailability of these compounds (Cianciosi, Forbes-Hernández, Afrin, et al., 2020; Cilla et al., 2022; Magoshi et al., 2023; O Sullivan et al., 2013; Seraglio et al., 2017; Seraglio et al., 2021). The growing importance of bioaccessibility and bioavailability has been emphasized in several studies, highlighting their relevance not only for researchers but also for broader societal interests. To evaluate the alterations in the quantity, composition, and biological activity of these compounds, both in vitro and in vivo gastrointestinal digestion models have been performed (Seraglio et al., 2021). While the composition of honey, the factors influencing its components, and its health benefits have been extensively studied, research focusing on the changes and effects that occur during honey digestion remains limited. As highlighted in several studies, it is crucial to consider this situation and conduct further investigations in this area (Cianciosi, Forbes-Hernández, Giampieri, et al., 2020; Cilla et al., 2022; Magoshi et al., 2023; O Sullivan et al., 2013; Seraglio et al., 2017; Seraglio et al., 2021). In this study, we focused our efforts on investigating the antimicrobial and anti-inflammatory properties of various honeys while examining the dynamic changes in their phenolic content, composition, and antioxidant activity during different stages of in vitro gastrointestinal digestion. Moreover, we evaluated the cytotoxic effects of digested honey on lung, epidermal, colon, and liver cancer cell lines, as well as on healthy cells. To the best of our knowledge, this is the first study to explore the cytotoxic impact of digested honey on lungs, epidermal, and liver cancer cells. These findings provide comprehensive insights into the potential health-promoting properties and therapeutic applications of honey.

2. Material and methods

2.1. Material

Six honey samples from different botanical origins and geographical locations from Türkiye were used in this study. Detailed information regarding these samples is provided in Table 1. The chemicals with analytical and chromatographical grades, and microorganisms, media, and other materials used in microbiological analyses and bioaccessibility experiments were obtained in high purity from Honeywell (USA), Isolab Laborgeräte GmbH (Germany), and Merck (Germany). The cancer cell lines used in the study included A549 (ATCC CCL-185™, passage no: 73–75), H1299 (ATCC CRL-5803™, passage no: 67–69),

Table 1
Botanical origins and geographical locations of honey samples.

Samples	Dominant Pollen (>%45)	Seconder Pollen (%16–45)	Minor Pollen (%3–15)	Trace Pollen (<%3)	Origin	Location
H1	*Teucrium* spp.:56.45	Cistaceae: 17.74	Fabaceae: 9.67 Apiaceae: 3.22 *Rumex* spp.: 3.22	Asteraceae: 1.61 *Onobrychis* spp.: 1.61 Lamiaceae: 1.61 Liliaceae: 1.61 Papaveraceae: 1.61 Rosaceae: 1.61	Monofloral (*Teucrium* spp.)	Isparta
H2	–	–	Brassicaceae: 14.66 Lamiaceae: 14.66 Asteraceae: 13.33 Fabaceae: 12 *Lotus* spp.: 12 *Astragalus* spp.: 10.66 *Anchusa* spp.: 9.33 *Sanguisorba* spp.: 5.33 *Centaurea* spp.: 13.04	*Cephalaria* spp.: 2.66 Rosaceae: 2.66 Berberidaceae: 1.33 *Salix* spp.: 1.33	Multifloral	Erzurum
H3	–	Rosaceae: 43.47 Asteraceae: 17.39	Lamiaceae: 13.04 Poaceae: 8.69 Cistaceae: 4.34	–	Multifloral	Karaman
H4	*Astragalus* spp.: 75	Apiaceae: 25	–	–	Monofloral (*Astragalus* spp.)	Van
H5	Apiaceae: 63.21	–	*Trifolium* spp.: 9.19 Cistaceae: 8.04 *Hedysarum* spp.: 4.59 *Centaurea* spp.: 3.44	*Echium* spp.: 2.29 Poaceae: 2.29 Rosaceae: 2.29 *Onobrychis* spp.: 2.29 Asteraceae: 1.14 Brassicaceae: 1.14	Monofloral (Apiaceae)	Denizli
H6	–	*Trifolium* spp.: 25.92	Asteraceae: 14.81 *Salix* spp.: 14.81 Cistaceae: 11.11 Ericaceae: 11.11 *Xanthium* spp.: 7.40 Apiaceae: 3.70 *Castanea sativa*: 3.70 Poaceae: 3.70 *Teucrium* spp.: 3.70	–	Multifloral	Samsun

A431 (ATCC CRL-1555™, passage no: 94–96), Caco-2 (ATCC HTB-37™, passage no: 36–38), Hep-G2 (ATCC HB-8065™, passage no: 66–68), and Bj (ATCC CRL-2522™, passage no: 82–84). These cell lines were sourced from the American Type Culture Collection (ATCC, Rockville, USA) and supplied via the Molecular Biology Department of Akdeniz University, Türkiye.

2.2. Total phenolic and flavonoid content analyses

Total phenolic and flavonoid content analyses were carried out with the undigested and digested honey samples. For the total phenolic content analysis of undigested honey samples, 1 g of sample was extracted with 80 % methanol at 40 °C for 2 h using horizontal shaking. The extract was then centrifuged at 7100×*g* for 10 min. Subsequently, 0.5 mL of the supernatant was mixed with 2.5 mL of Folin-Ciocalteu reagent (10 %) and 2 mL of sodium carbonate solution (7.5 %). After that, the samples were kept at 50 °C for 5 min in a water bath and cooled to room temperature. In the analysis of digested samples, 0.5 mL of digesta, taken from oral, gastric, and intestinal stages, was used for the analysis. The absorbance was detected spectrophotometrically at 760 nm against the blank which includes 80 % methanol solution instead of sample using a Cary 60 UV–Vis spectrophotometer (Agilent, USA). Results were calculated by gallic acid standard curve as mg GAE/100 g (Škerget et al., 2005).

For the total flavonoid content analysis of undigested honey samples, 1 g of sample was extracted with 50 % methanol at 25 °C for 24 h using horizontal shaking (Mouhoubi-Tafinine et al., 2016). The extract was then centrifuged at 7100×*g* for 10 min. Subsequently, 2 mL of supernatant was mixed with 2 mL of methanolic aluminum chloride (2 %) solution. In the analysis of digested samples, 2 mL of digesta was used for the analysis. After a 30-min incubation period to allow the reaction to occur, the absorbance of the samples was measured spectrophotometrically at 415 nm against the blank which includes 50 % methanol solution instead of sample. Results were calculated by quercetin standard curve as mg QE/100 g (Bueno-Costa et al., 2016).

2.3. Antioxidant activity analysis

The antioxidant activity of undigested and digested honey samples was assessed using the Trolox equivalent antioxidant capacity (TEAC) and Cupric Reducing Antioxidant Capacity (CUPRAC) methods. For the antioxidant activity analyses of undigested honey samples, they were extracted with a phosphate buffer solution (5 mM, pH 7.4) at 25 °C for 30 min using horizontal shaking. The extract was then centrifuged at 7100×*g* for 10 min. For the TEAC assay, 5, 10, 10, 15, and 20 μL of the supernatant were mixed with $ABTS^{\bullet+}$ solution (prepared to an absorbance of 0.700 ± 0.02 at 734 nm) to achieve a total reaction volume of 1 mL. In the analysis of digested samples, 5, 10, 10, 15, and 20 μL of the diluted digesta with phosphate buffer solution were used for the analysis. After a 6 min incubation period, the absorbance of the samples was measured spectrophotometrically at 734 nm against the phosphate buffer solution (Mutlu & Erbas, 2023). The CUPRAC antioxidant activity of the samples was determined by mixing of 1 mL of copper chloride (10 mM), 1 mL of neocuproine (7.5 mM), and 1 mL of ammonium acetate (pH 7.0), along with an appropriate volume of the undigested sample extract (x mL) and water (1.1–x mL). In the analysis of digested samples, the related volume of the diluted digesta (x mL) were used for the analysis. The sample volume was adjusted based on the absorbance, ensuring a range of 0.20–0.40 at 450 nm. After a 30-min incubation period to allow the reaction, the absorbance was measured spectrophotometrically at 450 nm against the blank which includes phosphate buffer solution instead of sample (Apak et al., 2007). Antioxidant activity results were calculated by the Trolox standard curve as μmol TE/g.

2.4. Antimicrobial activity analysis

Escherichia coli ATCC 25922 and *Staphylococcus aureus* ATCC 25923 strains were used to assess the antibacterial activity of undigested honey samples. These bacterial strains were cultured in a sterilized nutrient broth medium at 37 °C for 24 h. After incubation, the optical densities of the bacterial cultures were measured using a spectrophotometer at a wavelength of 630 nm. The cultures were then diluted with sterile Ringer's solution to achieve a cell density of 10^7–10^8 CFU/mL and incubated in nutrient agar medium for 2 h (Osés et al., 2016).

For the antibacterial analysis, 100 μL of honey sample extracts (1 g/mL) (Alotibi et al., 2018), prepared by mixing with 70 % ethanol and incubating at 80 rpm for 24 h at room temperature, were diffused onto sterile discs. These discs were then incubated with the bacterial cultures at 37 °C for 24 h. Following incubation, the diameters of the inhibition zones around the discs were measured (in mm), and the results were expressed as the average of these measurements. In the analysis, a sample-free disc prepared with 70 % ethanol and a standard disc containing ampicillin (10 μg/mL) were used as controls (Osés et al., 2016).

2.5. Anti-inflammatory activity analysis

The anti-inflammatory activity of undigested honey samples was evaluated based on their potential to inhibit hyaluronidase enzyme activity. Honey samples were extracted with distilled water (0.40 g/mL) at 25 °C for 24 h using horizontal shaking. The extract was then centrifuged at 7100×*g* for 10 min, and 100 μL of the supernatant was mixed with 200 μL of hyaluronidase enzyme (310 U/mL) and 200 μL of phosphate buffer solution (0.2 M, pH 7.0), containing 77 mM sodium chloride and 0.01 % bovine serum albumin. After incubating the mixture at 37 °C for 10 min, 200 μL of trisodium phosphate solution (0.3 M, pH 5.35) containing 0.03 % hyaluronic acid sodium salt was added. The resulting mixture was incubated at 37 °C for 45 min to allow the hyaluronidase enzyme to act on the hyaluronic acid. Following incubation, 2 mL of acetate buffer (24 mM sodium acetate–79 mM acetic acid–0.1 % bovine serum albumin, pH 3.75) was added to the mixtures, which were then allowed to stand at room temperature for 10 min. Enzyme activity was inhibited by heating the samples at 100 °C for 1 min, followed by cooling on ice for 2 min. A mixture containing 100 μL of ddH_2O instead of honey extract was used as a control sample. The absorbance of the samples was measured spectrophotometrically at 600 nm, and the results were expressed as the inhibition rate (%) relative to the control sample (Kolayli et al., 2016).

2.6. Phenolic composition analysis

In the phenolic composition analysis, the undigested honey samples were extracted with acidified ddH_2O (pH 2.0) at a 1:4 *w/v* ratio by ultrasonication for 30 min. The extract was then centrifuged at 6200×*g* for 10 min, and the supernatant was passed through a pre-conditioned (with 6 mL of methanol and 3 mL of ddH_2O) solid-phase extraction (SPE) C18 cartridge (Chromafix C18, Macherey-Nagel, Germany). Sugars remaining in the cartridge were removed by washing with 15 mL of acidified ddH_2O (pH 2.0). Phenolic compounds were then eluted into tubes by passing 6 mL of methanol through the cartridge and kept under vacuum at 35 °C until the methanol was completely evaporated. For the analysis of undigested honey samples, the digesta was passed through the pre-conditioned SPE C18 cartridge, and the remaining stages were conducted following the same procedure. The resulting residue was dissolved in methanol, filtered through a 0.22 μm filter into vials, and 20 μL of the extract was injected into the HPLC-DAD system for analysis (Gašić et al., 2014; Marshall et al., 2014).

Phenolic compounds were separated using a C18 column (3.0 × 150 mm, 2.7 μm, InfinityLab Poroshell 120 EC-C18, Agilent, USA). The analysis conditions were applied as described by Marshall et al. (2014). Detector wavelengths were set to 280, 320, and 370 nm, and the results

were expressed as mg/100 g of sample by using curves of standard compounds.

2.7. In vitro gastrointestinal digestion

For in vitro digestion, simulated oral (SOF), gastric (SGF), and intestinal (SIF) fluids were prepared according to the method described by Minekus et al. (2014). In oral digestion stage, 12 mL of SOF (pH 7.0) and 75 μL of calcium chloride (0.3 mol/L) was added into 3 g of honey. NaOH (1 M) was used (x μL) to adjust pH to 7.0, and water was added to the mixture (2925-x μL). Oral digestion was performed at 37 °C for 2 min with constant shaking at 100 rpm. After oral digestion, 7 mL of the oral bolus was taken for analyses, and the remaining oral bolus was subjected to gastric digestion by adding 6 mL of SGF, 4 μL of calcium chloride, and 1.28 mL of pepsin. HCl (1 M) was used (x μL) to adjust pH to 3.0, and water was added to the mixture (716-x μL). Gastric digestion was performed at 37 °C for 2 h with constant shaking at 100 rpm. After gastric digestion, 8 mL of the gastric chyme was taken for analyses, and the remaining gastric chyme was subjected to intestinal digestion by adding 4.4 mL of SIF, 16 μL of calcium chloride, 2 mL of pancreatin, and 1 mL of bile salt. NaOH (1 M) was used (x μL) to adjust pH to 7.0, and water was added to the mixture (584-x μL). Intestinal digestion was done at 37 °C for 2 h with constant shaking at 100 rpm. In addition, the control sample was prepared by using ddH_2O instead of honey, and the digestion procedure was also applied to this sample.

Samples taken from each stage of digestion were centrifuged at 7100×*g* for 10 min and filtered through a 0.22 μm membrane filter. The prepared samples were stored at −40 °C for subsequent analyses, including total phenolic content, total flavonoid content, phenolic composition, antioxidant activity, and cytotoxicity (Minekus et al., 2014; Ozdal et al., 2019).

2.8. Cytotoxicity analysis

The cytotoxic effects of digested honey samples on lung (A549, H1299), epidermis (A431), column (Caco-2), and liver (Hep-G2) cancer cells, and fibroblast (Bj) cells were evaluated by CellTiter-Blue® Cell Viability Assay, following the method reported by Mutlu et al. (2024). Cytotoxicity analysis was performed using H3 and H6-coded honey samples. These samples were selected based on their higher total phenolic and flavonoid content after in vitro digestion. Additionally, they exhibited high CUPRAC antioxidant activity results.

2.9. Statistical analysis

Chemical analyses were performed in duplicates, while antimicrobial, anti-inflammatory, and cytotoxicity analyses were conducted in four replicates. The data were evaluated using analysis of variance (ANOVA) and the Duncan Multiple Comparison Test via the SAS statistical software (SAS Statistical Software, v.9.00, USA). The two-tailed *t*-test was employed for comparisons between the two groups. Significance levels of the *p*-value are denoted as follows: 0.01 to 0.05 (*), 0.001 to 0.01 (**), and < 0.001 (***). Results are presented as "mean ± standard error," based on the dry matter of the samples.

3. Results and discussion

3.1. Antimicrobial and anti-inflammatory activity of honey

Antimicrobial and anti-inflammatory analysis results are presented in Fig. 1A and B, respectively. The antibacterial activity of honey is attributed to its reducing sugar content, high viscosity, osmotic pressure, low pH, low water activity, and the presence of hydrogen peroxide, phenolic compounds, and the lysozyme enzyme. These properties are known to vary depending on the nectar source (Libonatti et al., 2014). Among the samples, H1 demonstrated the highest inhibitory effect against both bacterial strains. Additionally, all honey samples, except H1 (*Teucrium* spp.) and H4 (*Astragalus* spp.), exhibited a nearly identical ($p > 0.05$) antimicrobial effect against *E. coli* ATCC 25922 and *S. aureus* ATCC 25923. Besides, the inhibitory effects of H1 and H4 on *S. aureus* ATCC 25923 were notably higher and were statistically significant ($p < 0.05$). It has been previously reported that the double cell wall structure composed of lipopolysaccharides and proteins in Gram-negative bacteria provides them greater resistance to antimicrobial agents compared to Gram-positive bacteria (Velásquez et al., 2017). Previous studies on various floral honey samples reported inhibition zones ranging from 0.00 to 8.00 mm against *E. coli* ATCC 25922 and from 4.00 to 18.00 mm against *S. aureus* ATCC 25923, findings that align with our results (Ulusoy et al., 2010; Vică et al., 2021).

The anti-inflammatory activity of honey operates through multiple mechanisms, including the inhibition of reactive oxygen species formation, leukocyte infiltration, matrix metalloproteinase-9 production in keratinocytes, cyclooxygenase-2 enzyme activity, and nitric oxide synthase expression (Hadagali & Chua, 2014). In this study, the anti-inflammatory activity of honey samples ranged from 35.96 % to 79.78 %. Consistent with the antimicrobial activity results, H1 honey exhibited the highest anti-inflammatory effect. This result explains why honey has been used on wounds since ancient times to prevent inflammation induced by microorganisms. Previous studies have reported that the anti-inflammatory activity of honey samples at a concentration of 0.75 g/mL changed in the range of 12.00–85.00 % (Alevia et al., 2021) and 46.60–54.20 % (Osés et al., 2022). Although the concentration of the samples in this study (0.4 g/mL) differs from those used in the reported studies (0.75 g/mL), it has been noted in another study that the anti-inflammatory activity values of honey samples did not significantly change with increasing concentrations beyond 0.02 g/mL (da Silva et al., 2016). Additionally, the variations among samples are attributed to differences in the levels of glucose oxidase, hydrogen peroxide, gluconic acid, methylglyoxal, and phenolic compounds in honey (Hadagali & Chua, 2014; Miguel et al., 2017).

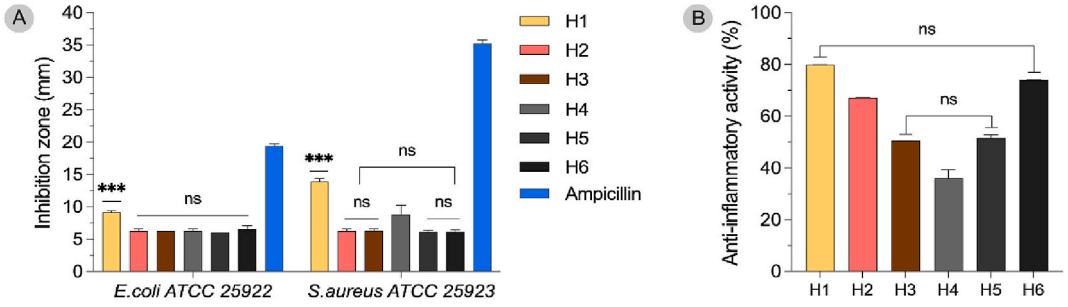

Fig. 1. The results, evaluated by two-tailed *t*-test, of (A) antimicrobial based on disc diffusion method and (B) anti-inflammatory potentials based on hyaluronidase inhibition method of honey samples (H1, H2, H3, H4, H5, and H6).

3.2. Total phenolic and flavonoid content

The results of the variance analysis revealed significant differences between the honey samples, as well as notable effects of digestion stages on phenolic and flavonoid contents ($p < 0.01$), and there were no detected total phenolic and flavonoid contents in oral, gastric, and intestinal stage control samples. The honey sample labeled as H3 exhibited the highest total phenolic and flavonoid content than those of the other samples with values of 132.07 mg GAE/100 g and 7.30 mg QE/100 g, respectively (Fig. 2A and B). Previous studies have reported that the total phenolic content of honey samples from various locations and botanical sources ranged between 1.00 and 106.46 mg GAE/100 g (Albu et al., 2022; Fernández-Estellé et al., 2023; Kivrak et al., 2016), while the flavonoid content ranged from 0.44 to 9.24 mg QE/100 g (Albu et al., 2022; Kivrak et al., 2016). These findings suggest that the phenolic content of honey is strongly influenced by its botanical origin, serving as a key indicator for determining both the botanical and geographical origins of honey (Becerril-Sánchez et al., 2021).

The phenolic and flavonoid contents of the samples increased by an average of 2.50 and 4.95 times, respectively, during digestion, reaching their highest levels at the end of intestinal digestion. Notably, the phenolic and flavonoid contents of honey samples coded H3 and H6 were significantly higher than those of other samples (Fig. 2A and B). The stability and concentration of phenolic compounds have been reported to vary depending on the pH conditions of the gastric and intestinal environments. These compounds may either be preserved during digestion or become more susceptible to digestive conditions depending on the complexes they form with minerals and proteins in the food matrix (Alevia et al., 2021; Seraglio et al., 2017).

It has been emphasized that the bioaccessibility of phenolic compounds is limited by their interactions with macromolecules such as proteins and polysaccharides. Digestive enzymes contribute to the hydrolysis of matrix macronutrients and the breakdown of the food matrix, thereby enhancing the release of polyphenols and flavonoids (Ketnawa et al., 2022). During gastric digestion, the acidic environment helps stabilize phenolic compounds while disrupting their interactions with proteins, thus promoting their release via pepsin enzymatic activity (Li et al., 2023). In the intestinal phase, protein hydrolysis and phenolic release continue through the catalytic action of the pancreatin enzyme (Diep et al., 2022).

Seraglio et al. (2017) demonstrated that phenolic acids and flavonoids in some honeydew honey blends were affected to varying degrees during the gastric and intestinal digestion stages. These changes included both increases and decreases in the concentration of specific compounds. It has also been suggested that phenolic compounds exhibit greater stability under stomach conditions compared to intestinal conditions, with their concentrations potentially increasing. These variations are closely linked to the composition of the sample matrix and the specific conditions of digestion (Seraglio et al., 2017). Furthermore, Alevia et al. (2021) reported a significant increase in the total phenolic content of honey samples after in vitro digestion. This increase was attributed to the digestive effects on phenolic compounds and chemical interactions between the Folin–Ciocalteu reagent and sugars. Similarly, Franco-Ulloa et al. (2024) emphasized that sugars in honey can interfere with the Folin-Ciocalteu reagent used to determine total phenolic content (Franco-Ulloa et al., 2024). Although the Folin-Ciocalteu method has some limitations, as highlighted by various researchers, it remains widely used as a reference method in honey-related studies (Lawag et al., 2023). Despite potential interference, monosaccharides like fructose and glucose, which make up a large portion of honey's sugar content, do not undergo significant changes during digestion. Therefore, any interference in digested samples is expected to be similar to that in raw samples. Besides, it has been noted that the method used to quantify flavonoids is more specific than the Folin-Ciocalteu method (Alevia et al., 2021). The total flavonoid content showed a significant increase, which paralleled the rise observed in the total phenolic content. Thus, it can be concluded that digestion resulted in a clear increase in the phenolic compound content.

In addition to honey, various studies on different food sources have reported significant increases in phenolic content following in vitro digestion. It has been observed that the total phenolic content of certain cereals and legumes increased approximately 5-fold (from 52.2 mg/100 g to 263.3 mg/100 g) and 3-fold (from 98.4 mg/100 g to 324.3 mg/100 g), respectively, after in vitro digestion. In addition, similar trends were also observed in some fruits, vegetables, and chocolate (Koehnlein et al., 2016). These increases were attributed by Koehnlein et al. (2016) to the

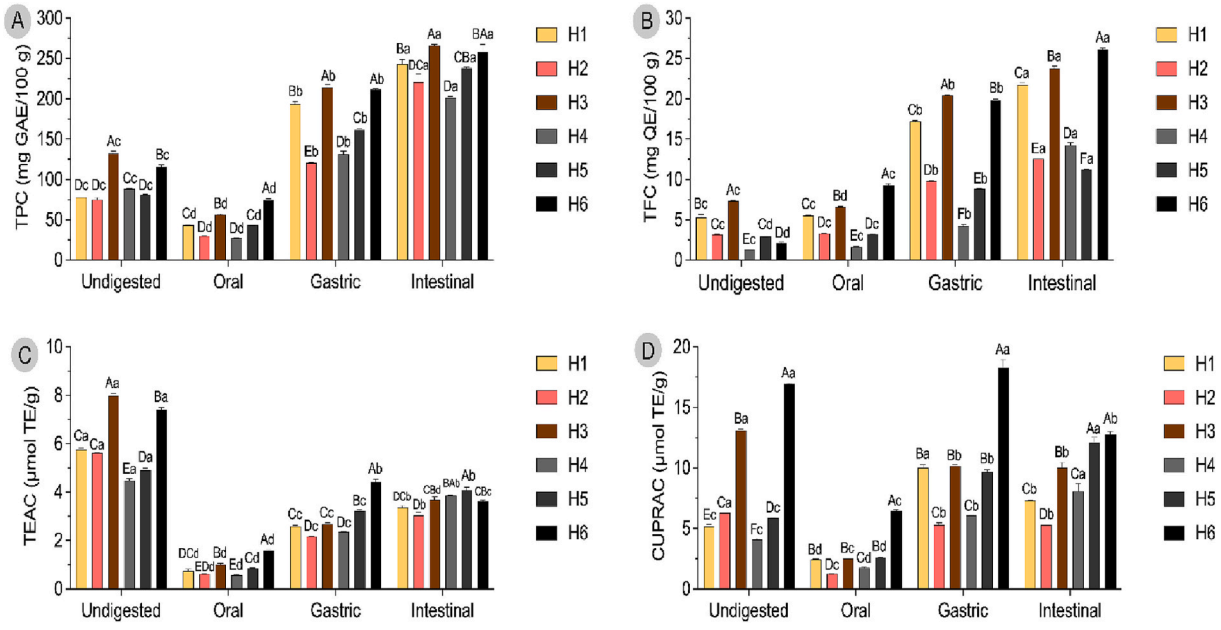

Fig. 2. Changes in total phenolic (A) and flavonoid (B) content and TEAC (C) and CUPRAC (D) antioxidant activities of honey samples during in vitro digestion. The capital letters (A, B, etc.) indicate the differences between the averages of samples, while the lowercase letters (a, b, etc.) represent the differences between the averages of digestion stages for each sample.

hydrolysis of phenolic compounds conjugated with proteins and/or carbohydrates by digestive enzymes. Chen et al. (2014) investigated the effects of in vitro digestion on the total phenolic content and antioxidant activity of 33 fruit samples. They reported that the total phenolic content of fruits such as apple, banana, cantaloupe, grape, grapefruit, nectarine, pear, sugarcane, tomato, and watermelon increased at varying rates after digestion. In particular, sugarcane varieties exhibited 2- to 4-fold increases. These results were associated to the possibility that digestive conditions may induce structural changes in polyphenol compounds, thereby altering their chemical properties, bioaccessibility, bioavailability, and biological activity (Chen et al., 2014). Fawole and Opara (2016) also examined the stability of total phenolic compounds in pomegranate co-products during in vitro digestion. They reported that the total phenolic contents of both juice and marc were higher in digested samples compared to their undigested counterparts. In pomegranate peel samples, the highest concentration of total phenolics was observed during the gastric phase, followed by a decrease in the intestinal phase. This increase was attributed to the acidic hydrolysis of phenolic glycosides and the formation of flavylium cations, which are known to accumulate under acidic conditions (Fawole & Opara, 2016).

3.3. Antioxidant activity of honey

The differences in antioxidant activity among the honey samples, and across the various stages of digestion were statistically significant ($p < 0.01$). The TEAC and CUPRAC antioxidant activities of the samples ranged from 4.49 to 7.99 μmol TE/g and 4.08 to 16.94 μmol TE/g, respectively, with the highest antioxidant activity values observed in samples coded H3 and H6, consistent with their total phenolic content (Fig. 2C and D). Numerous studies have emphasized the strong association between the antioxidant activity of honey and its phenolic content. Gośliński et al. (2021) demonstrated a strong correlation ($R^2 \geq 0.90$) between antioxidant activity and total phenolic and flavonoid content through a multidimensional comparative analysis of various phenolic compounds (Gośliński et al., 2021). Previous studies have reported that the TEAC antioxidant activities of different honey samples vary widely, ranging from 1.61 to 34.73 μmol TE/g (Ávila et al., 2019), while CUPRAC antioxidant activities range from 0.38 to 23.80 μmol TE/g (Kaygusuz et al., 2016; Saroğlu et al., 2023). This wide variation is attributed to differences in phenolic compounds, proteins, amino acids, organic acids, and glucose oxidase levels in the nectar source (Saroğlu et al., 2023).

The TEAC and CUPRAC antioxidant activity values of the samples during digestion exhibited significant variations ($p < 0.01$), and there were no detected antioxidant activities in oral, gastric, and intestinal stage control samples. In general, the antioxidant activity of digested samples was lower compared to undigested samples (Fig. 2C and D). However, an increase in CUPRAC antioxidant activity values was observed at the end of digestion in samples coded H1, H4, and H5. Additionally, the maximum antioxidant activity levels for samples coded H1 and H6 were reached during the gastric digestion phase. It has been reported that low pH conditions enhance the stability of various antioxidant compounds, such as caffeic acid, chlorogenic acid, gallic acid, catechin, and quercetin, while higher pH levels can alter the chemical structure of these compounds, thereby reducing their antioxidant capacity. During digestion, these stability changes may occur, but phenolic compounds, proteins, vitamins, and organic acids can also be released from the food matrix through the activity of digestive enzymes, resulting in variations in antioxidant activity (Cianciosi, Forbes-Hernández, Giampieri, et al., 2020). Alevia et al. (2021) noted that the antioxidant activities of honey samples increased after digestion compared to undigested samples, attributing this to the release of bioactive compounds induced by enzymatic degradation. In another study, FRAP antioxidant activity was found to peak during the gastric digestion phase before decreasing during intestinal digestion, whereas DPPH antioxidant activity exhibited the opposite trend. This observation was linked to the stabilization provided by low pH and structural changes, such as the ionization of phenolic compounds, which enhance their electron-donating capacity (Seraglio et al., 2017).

3.4. Individual phenolic compounds of honey

The results of the identified phenolic compounds and their changes during in vitro digestion are summarized in Table 2. Among the identified phenolic compounds, the amounts of gallic acid, methyl-3,4,5-trihydroxybenzoate, and syringic acid in H1 and H2 samples; syringic acid and 3,4-dimethoxycinnamic acid in H3 sample; gallic acid and vanillic acid in H4 sample; and methyl-3,4,5-trihydroxybenzoate and syringic acid in H5 and H6 samples were found to be higher. However, other investigated compounds such as rosmarinic acid, chrysin, quercetin, kaempferol, luteolin, and naringenin were not detected in the honey samples. In addition, there were no detected phenolic compounds in oral, gastric, and intestinal stage control samples. Kıvrak and Kıvrak (2017) have highlighted that different phenolic compounds dominate in different honey types, with these variations largely attributed to the plant flora and production season. For instance, ferulic acid is predominant in rhododendron honey, syringic acid in euphorbia, lavender, pine, and carob honey (Kıvrak & Kıvrak, 2017), ellagic acid in black cumin honey (Kolayli et al., 2023), and gallic acid in chestnut honey (Taş-Küçükaydın et al., 2023). Besides, various studies have reported phenolic acid concentrations within the following ranges: 0.02–6.62 mg/100 g for gallic acid, 0.68–4.64 mg/100 g for vanillic acid, 0.01–34.48 mg/100 g for syringic acid, 0.04–1.83 mg/100 g for *p*-coumaric acid, 0.11–0.17 mg/100 g for ferulic acid, and 0.02–7.73 mg/100 g for caffeic acid (Cheung et al., 2019; Gośliński et al., 2021; Kıvrak & Kıvrak, 2017; Seraglio et al., 2017).

Although botanical origins significantly influence the types and amounts of phenolic compounds in honey, Matkovits et al. (2023) emphasized that a standardized phenolic compound profile could not be established even for honey of the same botanical origin. They further highlighted that factors such as geographical origin and extraction methodologies could also contribute to these variations. In another study on lavender honey, which investigated 24 different phenolic compounds, it was reported that gallic acid, *p*-hydroxybenzoic acid, *p*-coumaric acid, caffeic acid phenethyl ester, chrysin, and pinocembrin were identified. However, some phenolic compounds previously detected in lavender honey were not identified in this study. This divergence was attributed to variations in monofloral ratios, which could alter the phenolic composition of honey (Kolayli et al., 2024).

The digestion stages and differences among the honey samples had a statistically significant effect on the phenolic compound profiles ($p < 0.01$). Although the changes varied depending on the sample, the amounts of some compounds either increased or decreased as digestion progressed. The concentrations of syringic, trans-cinnamic, caffeic, ferulic, *p*-coumaric, and vanillic acids were higher during gastric digestion, whereas methyl-3,4,5-trihydroxybenzoate was predominantly higher during intestinal digestion, except in samples H5 and H6. Specifically, the amounts of gallic acid and methyl-3,4,5-trihydroxybenzoate in the H1 sample; 3,4-dimethoxycinnamic acid in the H2 sample; methyl-3,4,5-trihydroxybenzoate and trans-cinnamic acid in the H3 sample; and methyl-3,4,5-trihydroxybenzoate, trans-cinnamic acid, *p*-coumaric acid, and 3,4-dimethoxycinnamic acid in the H4 sample were all higher compared to their respective undigested counterparts. Cianciosi, Forbes-Hernández, Afrin, et al. (2020) reported that the 3,4-dihydroxybenzoic acid content in digested Manuka honey remained unchanged compared to the undigested sample, while the salicylic acid content was higher, and the syringic acid, *p*-coumaric acid, ferulic acid, and pinocembrin contents were significantly lower. They also noted that phenolic acids demonstrate greater stability than flavonoids during digestion. The findings of this study align with these observations, as the amounts of syringic acid, *p*-coumaric acid, and ferulic acid decreased as digestion progressed. Besides, Seraglio et al. (2017)

Table 2
Changes in phenolic composition (mg/100 g) of honey samples during in vitro digestion.

Sample	H1												H2											
Digestion phase	Undigested			Oral			Gastric			Intestinal			Undigested			Oral			Gastric			Intestinal		
Gallic acid	2.39^{b}	±	0.03	0.97^{c}	±	0.03	1.11^{c}	±	0.03	3.76^{a}	±	0.11	2.30^{a}	±	0.00	0.00^{b}	±	0.00	0.00^{b}	±	0.00	0.00^{b}	±	0.00
Methyl-3,4,5-trihydroxybenzoate	1.68^{b}	±	0.09	0.00^{c}	±	0.00	0.00^{c}	±	0.00	2.30^{a}	±	0.04	2.48^{a}	±	0.20	0.00^{c}	±	0.00	0.00^{c}	±	0.00	1.07^{b}	±	0.00
Syringic acid	0.97^{c}	±	0.05	1.07^{b}	±	0.00	2.25^{a}	±	0.01	0.00^{d}	±	0.00	0.89^{a}	±	0.00	0.35^{b}	±	0.02	0.90^{a}	±	0.05	0.00^{c}	±	0.00
Trans-cinnamic acid	0.87^{a}	±	0.03	0.51^{c}	±	0.01	0.69^{b}	±	0.02	0.00^{d}	±	0.00	nd			nd			nd			nd		
Caffeic acid	0.65^{b}	±	0.01	0.25^{c}	±	0.02	1.00^{a}	±	0.02	0.00^{d}	±	0.00	0.59^{a}	±	0.07	0.05^{b}	±	0.01	0.62^{a}	±	0.02	0.00^{b}	±	0.00
p-coumaric acid	0.51^{b}	±	0.04	0.36^{c}	±	0.02	0.91^{a}	±	0.02	0.00^{d}	±	0.00	0.54^{a}	±	0.06	0.15^{c}	±	0.01	0.55^{a}	±	0.01	0.38^{b}	±	0.01
3,4-dimethoxycinnamic acid	nd			nd			nd			nd			0.00^{d}	±	0.00	0.15^{c}	±	0.01	0.39^{a}	±	0.02	0.29^{b}	±	0.01
Sample	**H3**												**H4**											
Digestion phase	**Undigested**			**Oral**			**Gastric**			**Intestinal**			**Undigested**			**Oral**			**Gastric**			**Intestinal**		
Gallic acid	nd			nd			nd			nd			1.48^{a}	±	0.25	0.66^{b}	±	0.02	1.73^{a}	±	0.04	0.00^{c}	±	0.00
Methyl-3,4,5-trihydroxybenzoate	0.00^{b}	±	0.00	0.00^{b}	±	0.00	0.00^{b}	±	0.00	1.24^{a}	±	0.04	0.00^{b}	±	0.00	0.00^{b}	±	0.00	0.00^{b}	±	0.00	0.67^{a}	±	0.01
Vanillic acid	nd			nd			nd			nd			2.25^{a}	±	0.24	0.95^{b}	±	0.01	2.52^{a}	±	0.02	0.00^{c}	±	0.00
Syringic acid	1.64^{b}	±	0.03	1.32^{c}	±	0.03	2.57^{a}	±	0.08	1.27^{c}	±	0.01	nd			nd			nd			nd		
Trans-cinnamic acid	0.61^{c}	±	0.01	0.55^{c}	±	0.00	1.20^{a}	±	0.03	0.86^{b}	±	0.02	0.00^{d}	±	0.00	0.30^{c}	±	0.00	1.00^{a}	±	0.01	0.82^{b}	±	0.02
Caffeic acid	nd			nd			nd			nd			0.00^{b}	±	0.00	0.00^{b}	±	0.00	0.43^{a}	±	0.01	0.00^{b}	±	0.00
p-coumaric acid	0.64^{b}	±	0.02	0.33^{d}	±	0.01	0.92^{a}	±	0.01	0.60^{c}	±	0.01	0.00^{c}	±	0.00	0.10^{b}	±	0.00	0.44^{a}	±	0.04	0.46^{a}	±	0.03
Ferulic acid	nd			nd			nd			nd			0.00^{b}	±	0.00	0.00^{b}	±	0.00	0.02^{a}	±	0.01	0.00^{b}	±	0.00
3,4-dimethoxycinnamic acid	1.16^{a}	±	0.06	0.00^{b}	±	0.00	0.00^{b}	±	0.00	0.00^{b}	±	0.00	0.00^{d}	±	0.00	0.07^{c}	±	0.00	0.15^{b}	±	0.00	0.58^{a}	±	0.01
Sample	**H5**												**H6**											
Digestion phase	**Undigested**			**Oral**			**Gastric**			**Intestinal**			**Undigested**			**Oral**			**Gastric**			**Intestinal**		
Methyl-3,4,5-trihydroxybenzoate	3.63^{a}	±	0.81	0.60^{b}	±	0.00	1.41^{b}	±	0.01	0.68^{b}	±	0.01	6.85^{a}	±	0.02	3.43^{b}	±	0.00	6.91^{a}	±	0.52	0.82^{c}	±	0.02
Syringic acid	3.47^{a}	±	0.10	0.32^{c}	±	0.01	2.23^{b}	±	0.05	0.00^{d}	±	0.00	4.08^{a}	±	0.19	1.28^{c}	±	0.02	2.80^{b}	±	0.04	0.97^{c}	±	0.03
Trans-cinnamic acid	1.13^{a}	±	0.02	0.42^{c}	±	0.01	1.15^{a}	±	0.01	0.69^{b}	±	0.01	0.94^{b}	±	0.05	0.44^{d}	±	0.01	1.30^{a}	±	0.01	0.75^{c}	±	0.00
Caffeic acid	2.09^{a}	±	0.06	0.18^{c}	±	0.01	1.34^{b}	±	0.07	0.04^{c}	±	0.01	0.64^{b}	±	0.01	0.00^{c}	±	0.00	0.73^{a}	±	0.00	0.00^{c}	±	0.00
p-coumaric acid	0.81^{a}	±	0.02	0.18^{d}	±	0.00	0.60^{b}	±	0.01	0.45^{c}	±	0.01	1.23^{a}	±	0.05	0.00^{c}	±	0.00	1.21^{a}	±	0.03	0.59^{b}	±	0.00
Ferulic acid	0.74^{a}	±	0.06	0.00^{c}	±	0.00	0.44^{b}	±	0.00	0.00^{c}	±	0.00	0.77^{a}	±	0.01	0.13^{c}	±	0.00	0.62^{b}	±	0.01	0.00^{d}	±	0.00
3,4-dimethoxycinnamic acid	0.60^{a}	±	0.01	0.14^{c}	±	0.01	0.52^{b}	±	0.01	0.00^{d}	±	0.00	nd			nd			nd			nd		

nd: not detected.

found that the phenolic compound levels in a mixture of three honeydew honey samples generally increased during the intestinal digestion stage. However, caffeic acid content was higher during the gastric digestion stage in two samples, consistent with the results of this study. Furthermore, 3,4-dihydroxybenzoic acid and salicylic acid contents were reported to be higher in digested samples than in undigested ones in all honey samples. It was emphasized that the amounts and compositional changes of phenolic compounds in honey during digestion stages may affect depending on various factors, including ionic equilibrium, pH levels, enzymatic activity, the sample matrix, and interactions between phenolic compounds and macromolecules such as carbohydrates, proteins, and minerals within the sample (Seraglio et al., 2021).

3.5. Cytotoxic activity of honey

The biological activity of honey is primarily linked to its phenolic compounds and their concentration (Seraglio et al., 2021). Based on this, the cytotoxic activity of honey samples was evaluated using the digested H3 and H6 samples, which exhibited higher phenolic and flavonoid contents after digestion. The cytotoxic effects of the digested honey samples on various cancer and healthy cell lines are presented in Fig. 3A, while the concentration effects on cell viability levels are shown in Fig. 4. The order of cytotoxic effects, based on the average IC_{50} values of the honey samples, was as follows: A549, Hep-G2, H1299, Bj, Caco-2, and A431 cells. Interestingly, low concentrations of digested honey samples (e.g., 0.025 and 0.050 mg/mL) resulted in increased growth of cancer cells. This stimulatory effect persisted up to a concentration of 0.20 mg/mL in A431 cells. Consequently, it was concluded that low concentrations of honey did not inhibit cancer cell proliferation, and higher concentrations (> 0.20 mg/mL) were more effective in suppressing the growth of these cancer cell types. Notably, the cytotoxic effect of sample H6 was greater than that of sample H3, except for H1299 cells.

While the anticancer activity of honey has been extensively studied in various cancer cell lines, research examining the impact of digestion-induced modifications on this activity remains limited. To date, only a few studies have reported the anticancer effects of digested honey, particularly in colorectal cancer cell lines such as HCT-116 and CaCo-2 (Cianciosi, Forbes-Hernández, Afrin, et al., 2020; Cilla et al., 2022; O Sullivan et al., 2013). Digested honey samples, applied at concentrations ranging from 6.25 to 25 mg/mL to Caco-2 and HCT-116 colon cancer cells, resulted in a reduction in cell viability by 25–85 % and 20–80 %, respectively. This decrease in viability was linked to an increase in reactive oxygen species and a dissipation of mitochondrial transmembrane potential. It was suggested that these changes may contribute to increased apoptosis and disruption of the cell cycle mechanism. Additionally, the varying antiproliferative effects of the honey samples on these tumor cells were attributed to differences in the total phenolic content of the samples after in vitro digestion (Cilla et al., 2022). In another study, the IC_{50} values of digested Manuka honey after 24, 48, and 72 h incubation with HCT-116 colorectal cancer cells were reported to be 28.36, 16.11, and 14.32 mg/mL, respectively. It was also noted in the reported study that while the phenolic and flavonoid content, as well as the antioxidant activity of the honey, decreased after digestion, higher cytotoxic effects were observed in the digested samples despite their lower phenolic content (Cianciosi, Forbes-Hernández, Afrin, et al., 2020). This reported observation by Cianciosi et al. (2020) was attributed to the generation of metabolites that exhibited increased antiproliferative activity during in vitro digestion. A similar finding was noted by O Sullivan et al. (2013), who found that the cytotoxic effect of digested honey samples on Caco-2 cells was observed at concentrations between 1.0 and 3.0 mg/mL, while undigested honey samples showed cytotoxic effects at concentrations between 2.5 and 7.5 mg/mL. The study emphasized that, although the total phenolic content and antioxidant activity of the honey samples remained the same before and after digestion, alterations in the polyphenolic composition during digestion might have contributed to the variations in the effects of the samples on Caco-2 cells (O Sullivan et al., 2013).

4. Conclusion

In this work, we evaluated the antimicrobial and anti-inflammatory properties of selected mono- and multifloral honeys while monitoring dynamic changes in their phenolic and flavonoid profiles and antioxidant activity during in vitro gastrointestinal digestion. Additionally, we investigated the effects of the digested honey on various cancer cell lines (lung, epidermal, colon, and liver) as well as healthy fibroblast cells.

The digestion stages and differences among the honey samples had significant variation in the amount and composition of phenolic compounds and their biological activities. The phenolic and flavonoid bioaccessibilities increased during in vitro digestion, while antioxidant activities showed dissimilar trends for each sample. The increase in phenolic content following digestion suggests that honey could deliver even more potent bioactive compounds upon ingestion than previously understood. This has important implications for the development of honey-based nutraceuticals and functional foods, offering promising avenues for improving public health outcomes. Besides, digested honey samples showed notable inhibitory effects on a range of cancer cell lines, with particularly strong effects on lung (A549, H1299) and liver (Hep-G2) cancer cells. These findings suggest that the digestion process not only enhances the bioaccessibility of honey's bioactive compounds but also amplifies its potential therapeutic applications, especially in cancer treatment. As our understanding of its bioactive compounds continues to grow with further investigations into other biologically active compounds in honey—such as methylglyoxal, hydrogen peroxide, amino acids, and organic acids, honey presents an exciting and increasingly valuable component in functional food systems.

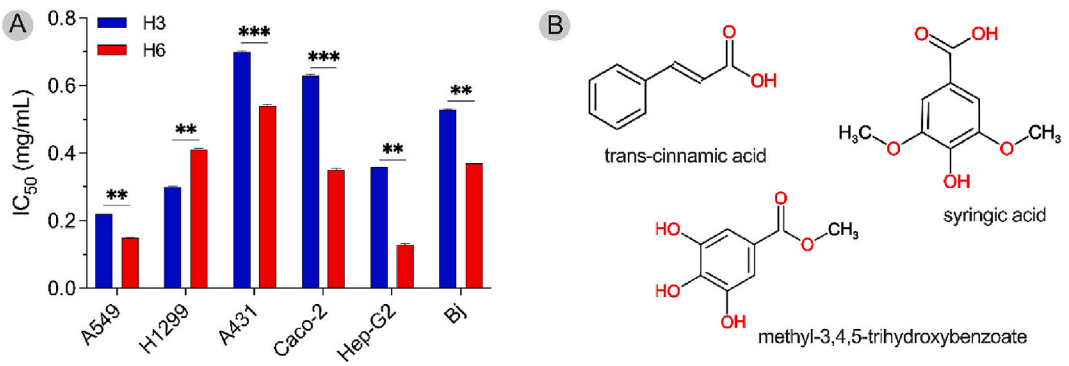

Fig. 3. (A) the IC_{50} values evaluated by two-tailed t-test of digested honey samples (H3 and H6) for some cancer (A549-lung, H1299-lung, A431-epidermal, Caco-2-colon, Hep-G2-liver) and healthy (Bj-fibroblast) cell lines, and (B) some phenolic compounds identified in these samples by HPLC-DAD.

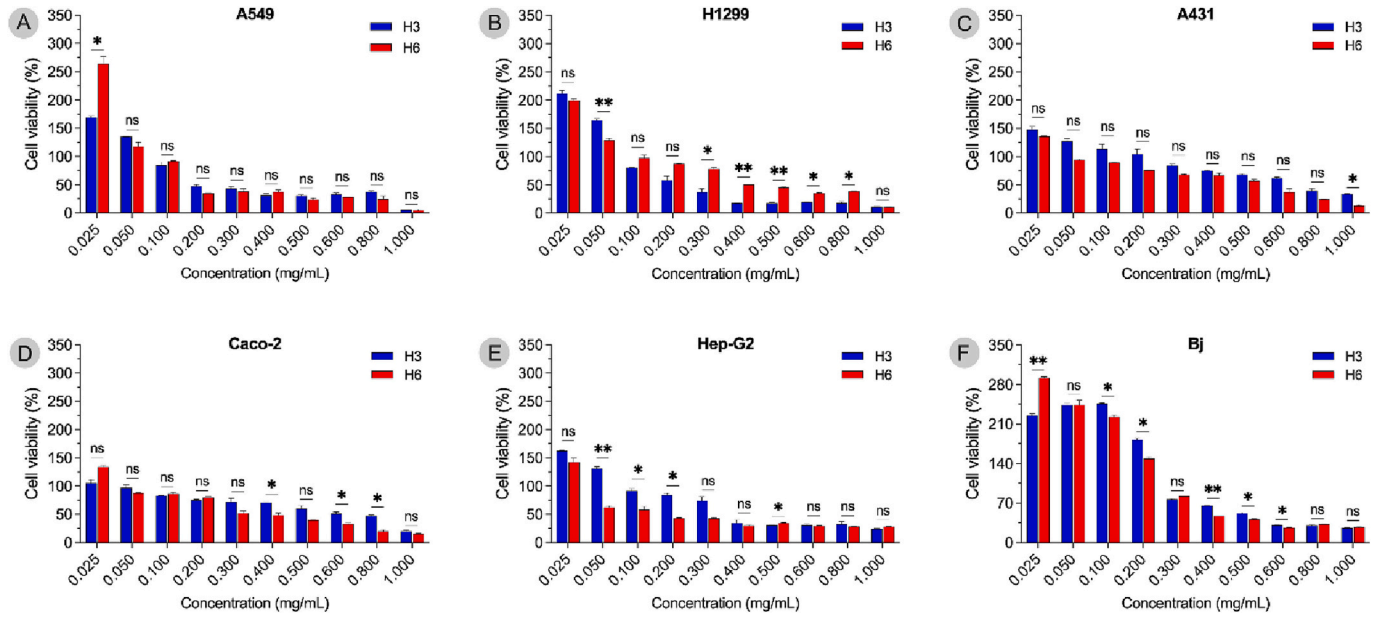

Fig. 4. The concentration effects of digested honey samples (H3 and H6) on cell viability levels of some cancer and healthy cell lines: (A) A549-lung, (B) H1299-lung, (C) A431-epidermal, (D) Caco-2-colon (E) Hep-G2-liver cancer cell lines, and (F) Bj-fibroblast cells.

CRediT authorship contribution statement

Ceren Mutlu: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Zeynep Demir:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Volkan Aylanc:** Writing – review & editing, Visualization, Formal analysis. **Aysun Özkan:** Writing – review & editing, Methodology, Investigation. **Mustafa Erbaş:** Writing – review & editing, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Ethical approval

Ethics approval is not required for this research.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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