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Original article

Antiproliferative effects of bioaccessible fractions of honeys from Sicilian black honeybee (*Apis mellifera ssp. sicula*) on human colorectal carcinoma cells

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Summary The aim of this study was to evaluate the antiproliferative activity of bioaccessible fractions (BFs) obtained by the internationally standardized INFOGEST static digestion method to Sicilian honeys of three distinct floral origins (Sulla, Thistle and Dill) and the Manuka honey (gold standard), and to compare their effects based on total polyphenol content (TPC). Differentiated CaCo-2 cells (intestinal-like) and non-differentiated CaCo-2 and HCT-116 colonic tumour-like cells were incubated for 24 h with BFs of honeys to test viability, apoptosis, mitochondrial membrane potential (MMP), ROS and cell cycle. TPC after digestion ranked in the following order: Dill > Thistle > Sulla > Manuka. No decrease in cell viability in differentiated CaCo-2 cells was observed, while a reduction to 25–85% (CaCo-2) and 20–80% (HCT-116) of viability was obtained. This descent in viability was caused by a cell cycle block with an increase in apoptosis through dissipation of MMP and raise in ROS levels, being Thistle and Dill the most effective honeys followed by Sulla and finally Manuka, in agreement with TPC after digestion.

Keywords Antiproliferative effects, colon cancer, honey, *in vitro* digestion, polyphenols.

Introduction

Honey contains over 200 compounds, consisting mainly of sugars and water, as well as enzymes, group B vitamins, minerals, phenolic compounds (flavonoids and phenolic acids), volatile compounds and pigments (Afrin *et al.*, 2020). Chemical and phytochemical composition of honeys depends not only on the source of the floral species but also on the geographical area and seasonal and environmental factors (Attanzio *et al.*, 2016; Mohammed *et al.*, 2020). Many *in vitro* and *in vivo* studies demonstrate its antibacterial, antifungal, anti-inflammatory and antidiabetic activities (Cianciosi *et al.*, 2018). In this context, honey is continuously explored to find natural dietary agents capable of controlling induction, and preventing or slowing progression of cancer with particular attention to the digestive tract, especially colon cancer (Waheed *et al.*, 2019; Afrin *et al.*, 2020). The main compounds in honey responsible for its anticancer activity are flavonoids (kaempferol, catechin and quercetin) and phenolic acids (caffeic acid and gallic acid) (Waheed *et al.*, 2019), whose anticancer effect has been linked to different mechanisms, such as inhibition of proliferation, arrest of the cell cycle, regulation of the oxidative stress and loss of mitochondrial membrane integrity (Waheed *et al.*, 2019; Afrin *et al.*, 2020).

Some researchers have investigated the effects of crude honey on colorectal cancer cell lines, as recently reviewed by Afrin *et al.* (2020). However, all researches investigating antiproliferative and apoptotic effects of honey on colon cancer cells have been carried out using crude honey samples, a condition that does not take into account the physiological changes that food components undergo during the digestive process and their bioaccessibility (De la Fuente *et al.*, 2020). The bioaccessibility of polyphenols involves several factors that strictly depend on the type of food matrix, the digestion process and the structure of the phytochemical. To our knowledge, only three studies in the literature have

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addressed the impact of gastrointestinal digestion on the fate of polyphenols in different kinds of honeys including Manuka (O'Sullivan *et al*., 2013; Cianciosi *et al*., 2020) and Bracatinga honeydew (Seraglio *et al*., 2017) honeys, showing a general decrease in total polyphenols after digestion. On the other hand, from a scarce number of studies only two of them evaluated whether the digestive process modifies the biological activities of honeys in different colon cancer cell lines, CaCo-2 (O'Sullivan *et al*., 2013) and HCT-116 (Cianciosi *et al*., 2020).

Recently, we have studied the functional properties of thirteen monofloral honeys produced by the Sicilian black honeybee (*Apis mellifera ssp. sicula*), highlighting their antiradical and antioxidant capacity (Attanzio *et al*., 2016). The aim of this work was to evaluate the antiproliferative activity of the bioaccessible fractions (BFs) obtained after simulated digestion of Sicilian honeys of three distinct floral origins (Sulla, Thistle and Dill) against two tumoral cell lines of human colorectal carcinoma (HCT-116 and CaCo-2). Manuka honey was used as gold standard owing to the known nutraceutical properties in the world. We also matched their effects based on total polyphenol content (TPC).

Materials and methods

All the sections, 2.1. Honey samples and reagents; 2.2. *In vitro* gastrointestinal digestion (INFOGEST static digestion); 2.3. Total polyphenol content (TPC) (Folin–Ciocalteu method – spectrophotometry); 2.4. Cell culture treatments; 2.5. Viability assay (MTT test – spectrophotometry); 2.6. Measurement of phosphatidylserine exposure (apoptosis) (Annexin V/PI – flow cytometry); 2.7. Measurement of mitochondrial transmembrane potential (MMP) ($DiOC_6$ – flow cytometry); 2.8. Measurement of intracellular reactive oxygen species (ROS) (DCFDA – flow cytometry); 2.9. Cell cycle analysis (PI/RNAse – flow cytometry); and 2.10. Statistical analysis, are described in the Appendix S1.

Results and discussion

Total polyphenol content (TPC) before and after digestion

The TPC of the different Sicilian honeys and Manuka honey before digestion is shown in Table 1. The TPC ranked from 59.7 to 244.8 mg GAE/100 g honey following the descending order: Dill > Manuka > Thistle > Sulla ($P < 0.05$). This exact order was reported in our previous study (Attanzio *et al*., 2016) pointing out that the TPC varies with the plant family, with the lowest value in honeys from *Leguminosae* (Sulla) and the highest in those from *Apiaceae* (Dill). Our results are comprised or slightly higher than those of other honeys such as Sardinian strawberry tree honeys (69–100 mg GAE/100 g; Afrin *et al*., 2017), Brazilian Bracatinga honeydew honeys (109.6–142.0 mg GAE/100 g; Seraglio *et al*., 2017) and Saudi Arabian acacia honeys (81.5–91.3 mg GAE/100 g; Mohammed *et al*., 2020), among others. In the case of Manuka honey, lower (127 mg GAE/100 g; Cianciosi *et al*., 2020) and higher (~290 mg GAE/100 g) (O'Sullivan *et al*., 2013) values of TPC have been reported indicating potential differences due to seasonal and environmental factors.

Table 1 Total phenolic content in honeys before and after simulated gastrointestinal digestion

Sample	Prior digestion	After digestion	% Decrease
Sulla	59.7 ± 1.2^{a}	55.3 ± 11.1^{a}	7.3
Thistle	114.5 ± 2.8^{b}	73.1 ± 8.0^{a}	36.2
Dill	244.8 ± 6.4^{c}	122.9 ± 19.7^{b}	49.8
Manuka	185.5 ± 20.7^{d}	12.8 ± 0.5^{c}	93.1

Results are expressed as mg gallic acid equivalents (GAE)/100 g honey. Values are the mean of three independent experiments ± SD. Different superscript letters (a-d) in the same column indicate statistically significant differences ($P < 0.05$).

The simulated gastrointestinal digestion evoked a diverse decrease in the values of TPC of the digested honeys that were comprised of 12.8 to 122.9 mg GAE/100 g (Table 1). The highest descent in TPC was found in Manuka honey (93.1%), whereas the lowest was present in Sulla honey (7.3%). This fact provides a different ranking after digestion with Dill honey still containing the highest TPC in the BFs of honeys followed by Thistle > Sulla > Manuka ($P < 0.05$). This means that phenolic compounds from Sicilian honeys show higher stability during simulated digestion than Manuka honey and have considerable antioxidant bioactive compounds able to exert potential beneficial effects in the gastrointestinal tract in concert with other bioactive compounds still present in the matrix (vitamins, organic acids and peptides) despite the chemical and structural modifications that have taken place during digestion. In fact, in agreement with our results a sharp decrease of 84% in TPC in Manuka honey (Cianciosi *et al*., 2020) has been described. In addition, ~14% decrease in TPC in Manuka honey has also been reported (O'Sullivan *et al*., 2013); although being a lower decrease than in the present study, Manuka was the only honey that suffered a reduction in TPC compared with other Irish honeys. On the other hand, similar decreases (25.8–59%) in TPC in Brazilian honeys (Seraglio *et al*., 2017) have been observed compared with the Sicilian honeys in our study (7.3–49.8%), highlighting the importance of considering the specific food matrix, as well as the floral and geographical origin.

The most plausible explanation for the decrease in total polyphenols during the digestion process is attributed to a rapid decrease in phenolic stability under the mild alkaline conditions achieved in the intestinal environment (Cilla *et al.*, 2011). In this sense, it has been reported that the influence of pH on polyphenols depends on the structure of the compound, being phenolic acids quite stable while multi-ring phenolic compounds such as flavonoids are more sensitive to the neutral or slightly basic pH found in the duodenum (Seraglio *et al.*, 2017; Cianciosi *et al.*, 2020). This fact can compromise the phenolic structure with transformations of unstable quinone intermediates or oxidised compounds (Friedman & Jurgens, 2000) that finally affect their antioxidant activity.

The more detailed analysis of main metabolites and phenolic profile before and after digestion would be interesting for future studies in the Sicilian honeys, although in this work, the main objective was to know the specific effect of digestion on the main antioxidants present in honey (determined generally as total polyphenols by the Folin–Ciocalteu assay) and then the impact of digested honey on antiproliferative action in a preclinical model of colon cancer, attributing the possible beneficial effect to the complex and specific mixture of phytochemicals present in the analysed samples.

Cell viability

Exposure of the HCT-116 and CaCo-2 cells at 24 and 48 h with a different range of dilutions of BF (1:5–1:20 v/v) (6.25–25 mg mL^{-1} honey) showed significantly ($P < 0.05$) higher growth inhibitory effects of Sicilian honeys in a concentration- and time-dependent manner in comparison with blank of digestion and Manuka honey (Fig. 1).

Cytotoxicity of the BFs on the HCT-116 and CaCo-2 cells followed the order Thistle > Dill > Sulla > Manuka in close agreement with their TPC after digestion. It is likely that the different cytotoxic effects of the three types of honey reflect their different composition. The polyphenolic content can vary widely depending on the habitat of the botanical species and the source of the flower of the nectar (Attanzio *et al.*, 2016). In fact, although the BF of Sulla honey has a lower polyphenolic content than the other two tested Sicilian honeys (Table 1), it still shows significant antiproliferative activity. Other components present in honey that can contribute to the antioxidant activity (though in minor proportion than phenolic compounds) such as organic acids, peptides, vitamins and carotenoids could also account for this antiproliferative effect (Afrin *et al.*, 2020; Cianciosi *et al.*, 2020).

A reduction to 5–85% (CaCo-2) and 3-80% (HCT-116) of viability was obtained after 24- to 48-h treatment with the assayed honeys at 6.25–25 mg mL^{-1} honey. In agreement, three Irish and Manuka digested honeys decreased to 15–90% of CaCo-2 cell viability after 24-h treatment at 0.5–3 mg mL^{-1} honey with IC_{50} below 2 mg mL^{-1} honey (O'sullivan *et al.*, 2013). These honeys displayed similar antiproliferative effect with lower concentrations of honey compared with our study, a fact attributable to the higher TPC in their digested honeys (>200 vs. 12.8–122.9 mg GAE/100 g honey in our study). Similarly, digested Manuka honey evoked a reduction in HCT-116 cell viability to 15–90% in the range of 0–26 mg mL^{-1} honey for 24, 48 and 72 h (IC_{50}: 28.4 mg mL^{-1} 24 h, 16.1 mg mL^{-1} 48 h and 14.3 mg mL^{-1} 72 h; Cianciosi *et al.*, 2020). In our study, Manuka honey only showed a decrease in HCT-116 cell viability with 12.5 and 25 mg mL^{-1} honey at 24 h (~65–80% viability) and 48 h (~50–75% viability). This slightly lower descent in viability could be attributed to the lower TPC after digestion at 12.8 vs. 20.3 mg GAE/100 g honey in the mentioned study.

Based on our results, the concentration of 12.5 mg mL^{-1} honey and 24-h treatment were considered optimal and were used for subsequent cellular assays since provided CaCo-2 and HCT-116 cell viability was between 20% and 85%, as previously described (Cianciosi *et al.*, 2020).

Further experiments were conducted on the viability of human intestinal normal-like differentiated CaCo-2 cells. After 24 h of treatment at 12.5 mg mL^{-1} honey, the BFs of honeys did not significantly affect cell viability, highlighting a potential cytotoxic effect of honeys on cancer cells but no or less toxicity to normal colon epithelial cells (Figure S1). Likewise, strawberry tree and Manuka honeys reduced viability of HCT-116 colon cancer cells and displayed no toxic effects in human dermal fibroblasts (non-cancer cells) (Afrin *et al.*, 2017; Afrin *et al.*, 2018a).

Cell death

To explore the cell death mechanism induced by the BFs of the honeys, HCT-116 and CaCo-2 cells were treated at 12.5 mg mL^{-1} honey for 24 h before double staining with Annexin V/PI. As shown in Fig. 2 for both cell lines, the percentage of cells in early apoptosis increased significantly ($P < 0.05$) compared with the blank of digestion from 9.9-fold (Manuka) to 30.1-fold (Thistle) and 5.6-fold (Manuka) to 17.5-fold (Thistle) for HCT-116 and CaCo-2 cells respectively. Interestingly, the BFs of Sicilian honeys show an increase in the percentage of cells in apoptosis greater than that of Manuka honey following the order: Thistle > Dill > Sulla > Manuka in both cell lines. These results are closely concordant with their TPC after digestion and the cell viability.

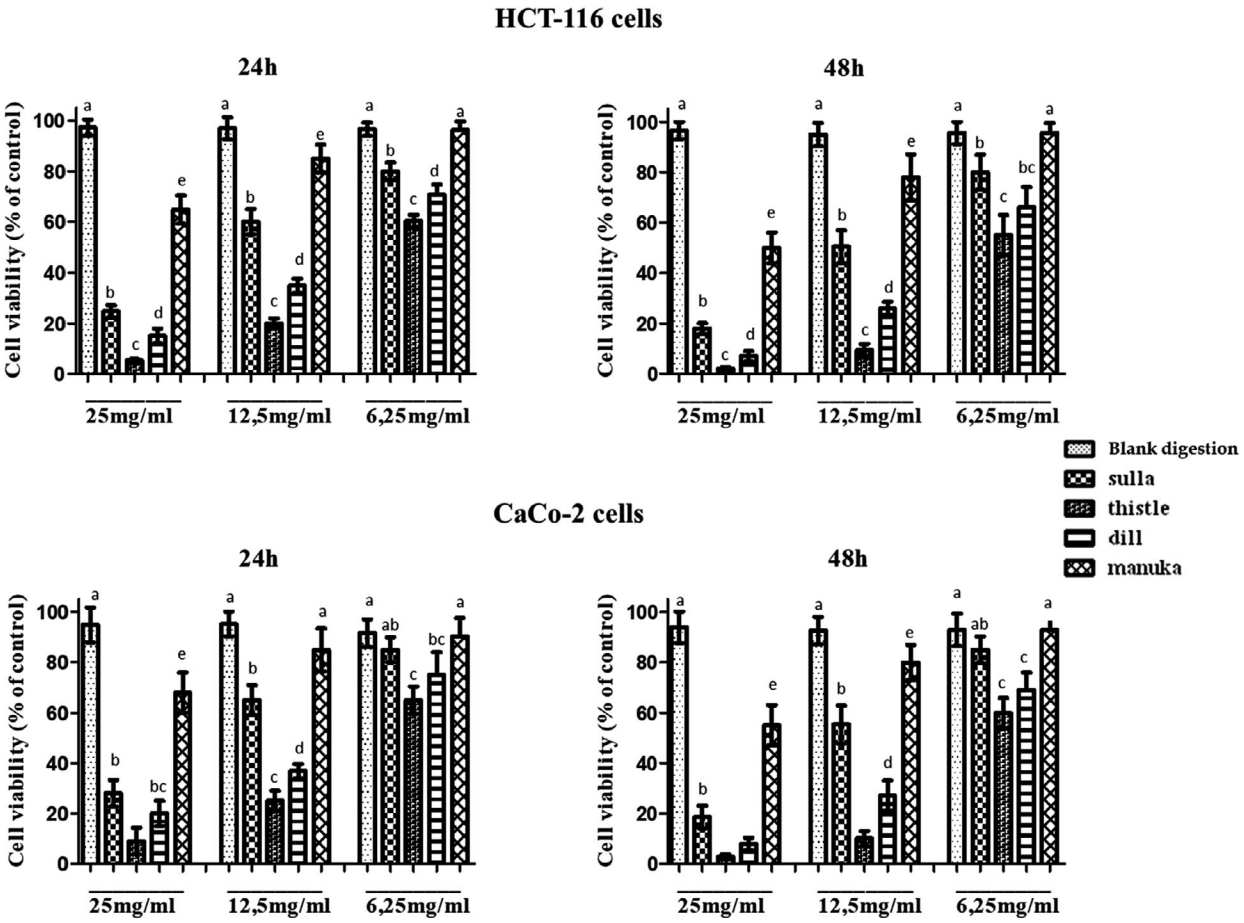

Figure 1 Effect of BFs of three samples of Sicilian black honeybee (Sulla, Thistle and Dill) and Manuka honey on the viability of HCT-116 and Caco-2 cells after 24- and 48-h treatment at 6.25, 12.5 and 25 mg mL^{-1} honey. Cell survival was measured by MTT assay in comparison with blank of digestion. Values are the mean ± SD of three separate experiments carried out in triplicate. Different superscript letters (a-e) in each bar for the same treatment indicate significant differences ($P < 0.05$).

Similarly, the pro-apoptotic effect of undigested Manuka honey has been described in HCT-116 colon cancer cells treated at 10–20 mg mL^{-1} honey for 48 h due to an increase in caspase-3, caspase-8 and caspase-9, p53 and cleaved-PARP (Afrin *et al.*, 2018a). Nevertheless, information on the effect of digested honeys on apoptosis is scarce with only one study to our knowledge. In accordance with our study, it was reported that digested Manuka honey increased apoptosis in HCT-116 cells vs. control by 1.67-fold and 2.16-fold at 16 and 24 mg mL^{-1} honey respectively after 48-h treatment (Cianciosi *et al.*, 2020).

Mitochondrial dysfunction

In many systems, the depolarisation of MMP reflects mitochondrial dysfunction with the loss of mitochondrial inner membrane potential, which is responsible for the release of some pro-apoptotic factors from the organelle and considered an early apoptotic event (Allegra *et al.*, 2020).

Flow cytometry measurements of the MMP showed that treatment of HCT-116 and CaCo-2 cells for 24h at 12.5 mg mL^{-1} honey resulted in an increase in mitochondrial dysfunction (1.8-fold to 7.6-fold and 3.7-fold to 9.8-fold in HCT-116 and CaCo-2 cells respectively) compared with blank of digestion following the order: Thistle > Dill > Sulla ≥ Manuka. In addition, the BF of the three Sicilian honeys displayed, in general, a greater negative effect on mitochondrial integrity than Manuka honey (Fig. 3). These results are in fashion with those of TPC after digestion, cell viability and apoptosis previously indicated.

Many bioactive compounds, including polyphenols and vitamins found in honey, have been found to affect mitochondrial functions (Afrin *et al.*, 2018a and

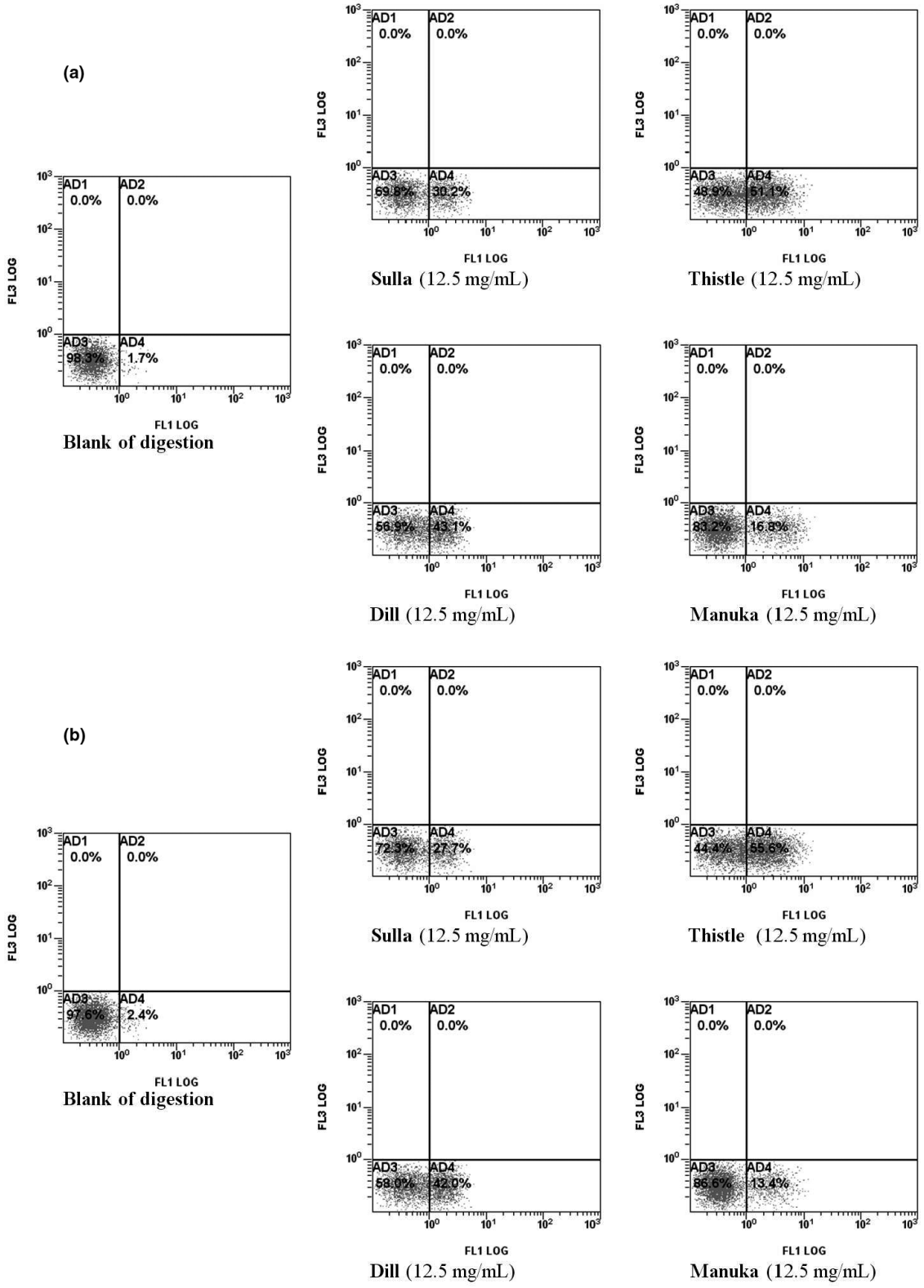

Figure 2 Flow cytometric analysis for the quantification of apoptosis by Annexin V/PI double staining, of three samples of Sicilian black honeybee (Sulla, Thistle and Dill) and Manuka honey on HCT-116 (a) and CaCo-2 (b) cells after 24-h treatment with BFs at 12.5 mg mL^{-1} honey. AD3, viable cells (Annexin V−/PI−); AD4, cells in early apoptosis (Annexin V+/PI); AD2, cells in late apoptosis (Annexin V+/PI+); AD1, necrotic cells (Annexin V−/PI+). Representative images of three experiments with comparable results.

Afrin *et al.*, 2018b). In this context, Tualang honey at 1–10% for 6-72h induced apoptosis in breast (MDA-MB-231 and MCF-7) and cervical (HeLa) cancer cells through an increase in caspase-3/7 and caspase-9 due to a decrease in MMP (Fauzi *et al.*, 2011). Likewise, it has been reported that Indian honey evoked apoptosis in HCT-15 and HT-29 colon cancer cells at 1–20% for 12–48 h via a decrease in MMP and non-protein thiols, with a concomitant decrease in Bcl-2 and rise in Bax, p53, caspase-3 and PARP cleavage pro-apoptotic events (Jaganathan & Mandal, 2010). However, as far as we are aware, no study has previously addressed the impact of digested honeys in MMP in cancer cells.

Reactive oxygen species (ROS)

Loss of mitochondrial integrity could be related to excessive production of ROS causing irreversible cell damage and induction to apoptosis (Allegra *et al.*, 2020). As shown in Fig. 4, BF of the three Sicilian honeys significantly ($P < 0.05$) increased the production of intracellular ROS (31.6-fold to 118.6-fold and 5.2-fold to 19.6-fold in HCT-116 and CaCo-2 cells respectively), when compared to blank of digestion, being the order: Thistle > Dill > Sulla. Furthermore, Manuka honey increased ROS levels less than Sicilian honeys, with about 15% in both colorectal carcinoma cell lines, in agreement with the TPC obtained after digestion and results on viability, apoptosis and MMP.

Previous studies with undigested honeys have reported an increase in ROS levels in different colon cancer cells as triggers of intracellular signalling cascades that can activate a sequence of pathways for programmed cell death as in our study. For instance, Manuka honey at 20 mg mL^{-1} for 48h and strawberry honey at 6 and 9 mg mL^{-1} for 72 h induced 22% and 20.5% rise in ROS levels respectively in HCT-116 colon cancer cells (Afrin *et al.*, 2017). In the same way, Afrin *et al.* (2018b) reported a dose-dependent increase in ROS production in HCT-116 cells treated with Manuka honey for 48 h, being 15 mg mL^{-1} the dose that evoked the highest effect (4.5-fold higher vs. control). Likewise, the only study that evaluates the effect of digested honey on the intracellular ROS levels (Cianciosi *et al.*, 2020) indicated that digested Manuka honey at 24 mg mL^{-1} for 48 h provided the greatest increase in ROS (4.3-fold compared with control cells) in HCT-116 colon cancer cells, in line with our own results.

Cell cycle distribution

Honeys and its bioactive compounds can inhibit the cell growth of cancer cells, inducing arrest in different cell cycle phases (G0/G1, S and G2/M) (Afrin *et al.*, 2020). The effect of treatment with BFs of Sicilian and Manuka honeys on the cell cycle distribution of CaCo-2 cells at 12.5 mg mL^{-1} honey for 24h is shown in Figure S2. All the Sicilian honeys produced significant ($P < 0.05$) variations in some cell cycle phases, with a coincident decrease in S phase (6.3%, 6.4% and 6.4% vs. 9.0% in blank of digestion for Sulla, Thistle and Dill respectively). In addition, Thistle honey also significantly ($P < 0.05$) arrested cells at G2/M phase (41.0% vs. 36.6% in blank of digestion), while Dill evoked a significant ($P < 0.05$) arrest in G1 phase (53.9% vs. 50.5% in blank of digestion) as well. On the contrary, Manuka honey did not significantly ($P > 0.05$) vary cell cycle in comparison with the blank of digestion, with only a trend to decrease G1 and rise G2/M phases. Taken together, this fact confirms and complements all the above-mentioned results, in agreement with TPC obtained after digestion, viability, apoptosis, MMP and ROS.

Some previous studies have reported that undigested honeys have the ability to arrest cell cycle in colon cancer cells in different phases. In this context, Indian commercial honeys at 1–20% for 12–48 h increased the accumulation in sub-G1 phase and arrested cell cycle in GO/G1 phase in colon cancer HCT-15 and HT-29 cells (Jaganathan & Mandal, 2010). Moreover, undigested Manuka honeys at 10, 15 and 20 mg mL^{-1} honey for 48 h have addressed a significant arrest in S phase with concomitant decreases in GO/G1 and G2/M phases in HCT-116 colon cancer cells. This arrest in S phase was linked with the upregulation of cyclin-dependent kinase (CDK) inhibitors p21Cip and p27Kip, and the down-regulation of CDK2, CDK4, cyclin D1, cyclin E and p-RB expression (Afrin *et al.*, 2018a). On the other hand, the only study with digested honey (Cianciosi *et al.*, 2020) reported that Manuka honey at 16 and 24 mg mL^{-1} honey for 48 h induced a sharp increase in sub-G1 phase (apoptotic marker) accompanied by a decrease in S and G0/G1 phases in HCT-116 cancer cells. Although the general outcome of our study is similar, with alterations in the progression of the cell cycle at specific phases, comparison with other works is difficult, possibly due to the different cell lines studied and differences in the bioactive compound profile present in the honeys analysed.

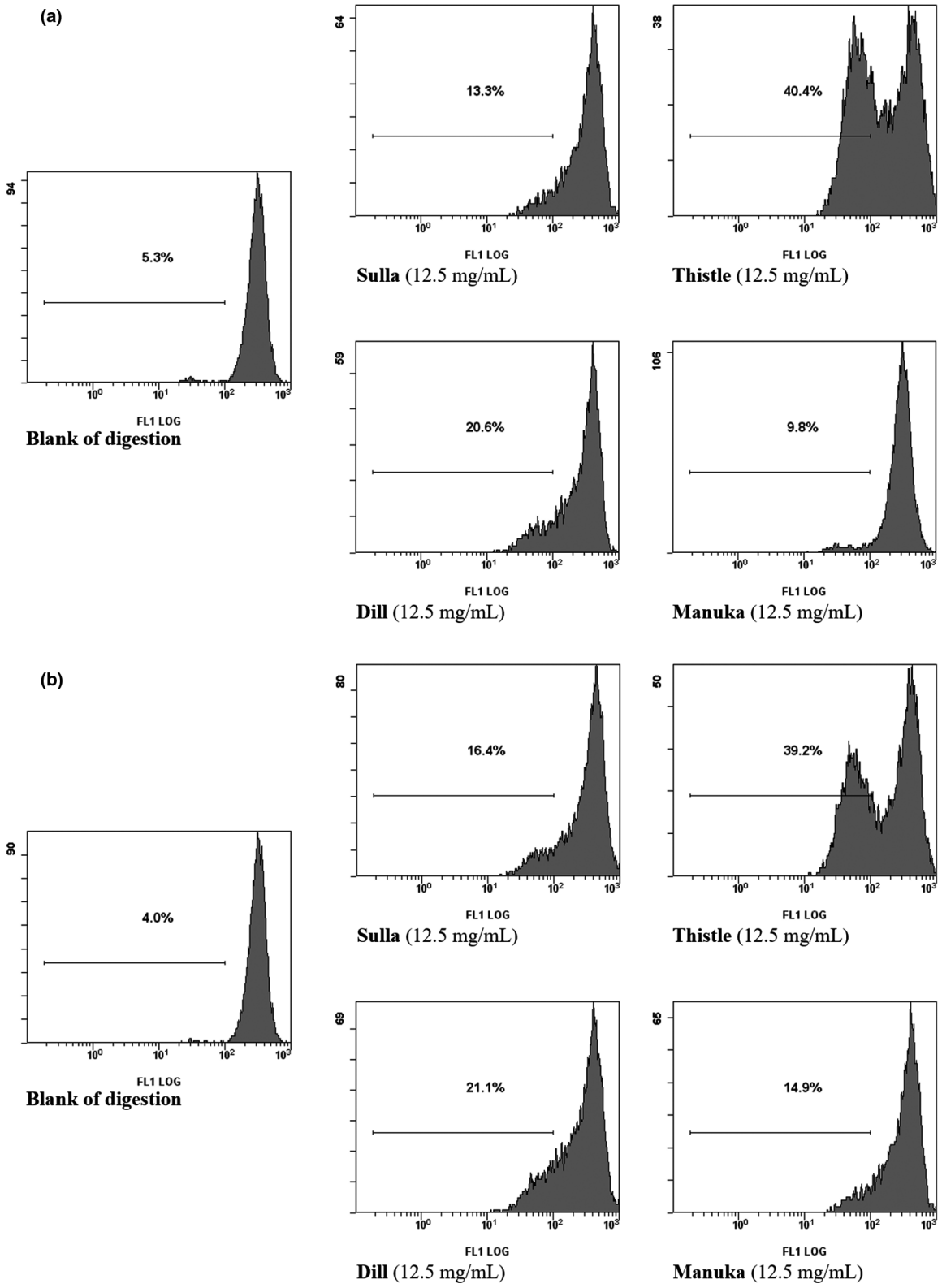

Figure 3 F Effects of three samples of Sicilian black honeybee (Sulla, Thistle and Dill) and Manuka honey on dissipation of mitochondrial transmembrane potential (MMP) in HCT-116 (a) and Caco-2 (b) cells after treatment with the BFs for 24 h at 12.5 mg mL^{-1} honey. Representative images of three experiments with comparable results.

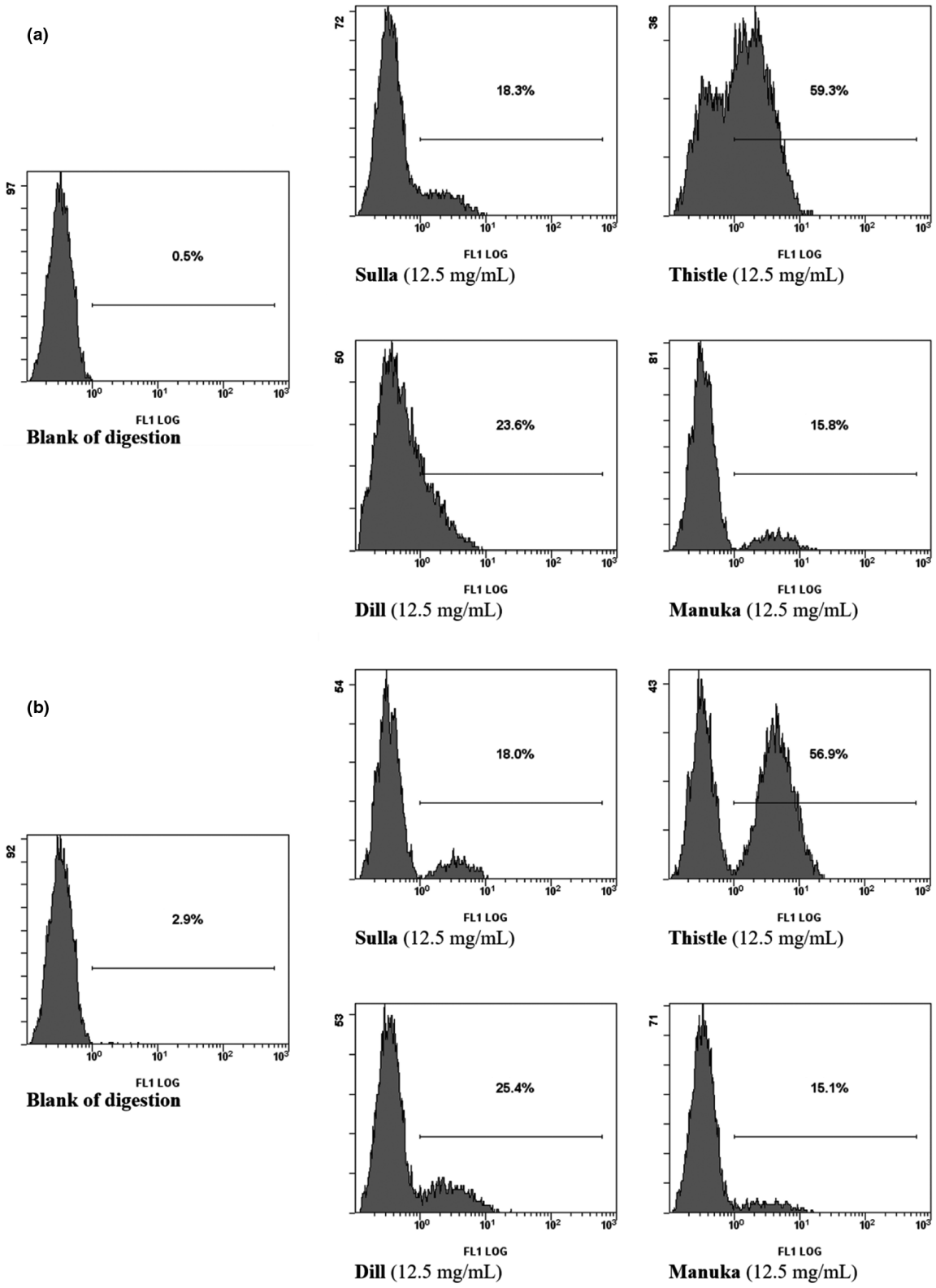

Figure 4 Effects of three samples of Sicilian black honeybee (Sulla, Thistle and Dill) and Manuka honey on the production of ROS in HCT-116 (a) and Caco-2 (b) cells after treatment with the BFs for 24 h at 12.5 mg mL^{-1} honey. Representative images of three experiments with comparable results.

Conclusions

The bioaccessible fractions from Sicilian honeys induce greater apoptosis and cell cycle arrest in colon cancer cells through an increase in ROS levels and mitochondrial membrane depolarisation than the gold standard Manuka honey, with no cytotoxic effect in non-malignant cells at similar concentrations. The higher the level of total polyphenols in BFs, the greater the antiproliferative effect. Despite the general considerable decrease in total polyphenols occurred after digestion, the specific combination of phytochemicals (phenolic compounds and others such as vitamins, organic acids and peptides) present in the bioaccessible fractions of Sicilian honeys still provides anticancer effects indicating the importance to check a whole matrix subjected to a simulated gastrointestinal digestion to be closer to the *in vivo* situation when applying preclinical studies. These results show promise in terms of chemoprevention in the functional food context for Sicilian honeys.

Ethical guidelines

Ethics approval was not required for this research.

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Conflicts of interest

The authors confirm that they have no conflict of interest with respect to the work described in this manuscript.

Author contribution

Antonio Cilla: Conceptualization (equal); Formal analysis (equal); Investigation (lead); Methodology (equal); Writing-original draft (lead); Writing-review & editing (lead). **Gabriel López-García:** Formal analysis (equal); Investigation (equal); Writing-original draft (supporting). **Reyes Barberá Sáez:** Methodology (equal); Writing-original draft (equal); Writing-review & editing (equal). **Anna Frazzitta:** Formal analysis (equal); Investigation (equal); Writing-original draft (supporting). **Ignazio Restivo:** Formal analysis (equal); Investigation (equal); Writing-original draft (supporting). **Luisa Tesoriere:** Conceptualization (equal); Writing-original draft (equal); Writing-review & editing (equal). **Alessandro Attanzio:** Conceptualization (lead); Formal analysis (equal); Investigation (equal); Methodology (equal); Writing-original draft (equal); Writing-review & editing (equal).

Peer review

The peer review history for this article is available at https://publons.com/publon/10.1111/ijfs.15169.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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This review summarises the findings from different experimental approaches (cell cultures, animal models and clinical studies), and provide an overview of the bioactive profile and bioavailability of the most commonly studied honey types, with special emphasis on the chemopreventive and therapeutic properties of honey and its major phenolic compounds in cancer.

In this study the effect of in vitro gastrointestinal digestion of Manuka honey was investigated, evaluating how the content of phenolic compounds and antioxidant activity changed, and studying whether this modification could affect the biological activities of honey in a human colon adenocarcinoma cell line (HCT-116).

This study provides for the first time information on the impact of in vitro gastrointestinal digestion on total antioxidant activity and total phenolic compounds in four different honeys and their effect on the antiproliferative activity in colon cancer Caco-2 cells.

This studies addresses the effect of simulated gastrointestinal digestion on the bioaccessibility of phenolic compounds, minerals and antioxidant capacity of bracatinga honeydew honeys.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Materials and methods.

Figure S1. Effect of BFs of three samples of Sicilian black honeybee (Sulla, Thistle and Dill) and Manuka honey on the viability of human intestinal normal-like differentiated Caco-2 cells after 24 h treatment at 12.5 mg mL^{-1} honey.

Figure S2. Effect of three samples of Sicilian black honeybee (Sulla, Thistle and Dill) and Manuka honey on cell cycle distribution of Caco-2 cells after treatment with the BFs for 24 h at 12.5 mg mL^{-1} honey.