Antioxidant and anti-inflammatory activities, bioaccessibility, transmembrane transport of major phenolics from selected floral honeys using Caco-2 BBe1 cell model

Yan Zhu[a], Ronghua Liu[a], Lili Mats[a], Honghui Zhu[a], Tauseef Khan[b,c,d],
John Sievenpiper[b,c,d,e,f], Dan Ramdath[a] and Rong Tsao[a*]

[a]Guelph Research and Development Centre, Agriculture and Agri-Food Canada, 93 Stone Road West, Guelph, Ontario N1G 5C9, Canada
[b]Department of Nutritional Sciences, Temerty Faculty of Medicine, University of Toronto, Toronto, Canada
[c]Toronto 3D Knowledge Synthesis and Clinical Trials Unit, Clinical Nutrition, St Michael's Hospital, Toronto, Ontario, Canada.
[d]Clinical Nutrition and Risk Factor Modification Centre, St Michael's Hospital, Toronto, Ontario, Canada
[e]Division of Endocrinology and Metabolism, St. Michael's Hospital, Toronto, Ontario, Canada
[f]Li Ka Shing Knowledge Institute, St Michael's Hospital, Toronto, Ontario, Canada.
[*]**Corresponding author:** Rong Tsao, Guelph Research and Development Centre, Agriculture and Agri-Food Canada, 93 Stone Road West, Guelph, Ontario N1G 5C9, Canada. E-mail: rong.cao@agr.gc.ca

Abstract

In the present study, we assessed the antioxidant activity of the phenolic extracts and major phenolic compounds of alfalfa, buckwheat, clover and orange honeys collected in North America using chemical-based and cell-based antioxidant assays (CAA). Cell culture models using Caco-2 BBe1 were established to evaluate the effect of honey phenolics on antioxidant enzyme activities and secretion of interleukin 8 (IL-8). Moreover, bioaccessibility, transmembrane transport and cellular uptake of honey phenolics were also studied. Based on the same quantity of the honey studied, phenolic extract of buckwheat honey showed the highest FRAP, DPPH, ORAC and CAA values, and strongest activity in restoring activities of antioxidant enzymes (GR, SOD and CAT) and in reducing TNF-α-induced IL-8 secretion. Our results showed that compared to the major phenolic component of honey of plant origin, minor phenolics or combination of different phenolic compounds, particularly those derived from propolis, and their phase II metabolites may play contribute more to the overall antioxidant and anti-inflammatory effects. Future research will focus on bioavailability of honey phenolics and their metabolites, and the molecular mechanism of the antioxidant, anti-inflammatory activities.

Keywords: Honey; Phenolics; antioxidant; Anti-inflammatory; Bioaccessibility; Cellular uptake; Transmembrane transport; Metabolism.

1. Introduction

Honey has been appreciated by humans as a natural sweetener since antiquity, but in recent years, it has been found to have additional health benefits beyond being merely a sweetener and energy source (Battino et al., 2021; Zammit Young and Blundell, 2023).

In the meantime, in addition to sugars, its main component, other nutrients and bioactive components have been identified (Machado De-Melo et al., 2018; Zhu et al., 2024). The potential health benefits of honey have been mainly associated with the antioxidant and anti-inflammatory activities, which has been attributed to mainly the phenolic content of honey (Sultana et al., 2022; Tan-

leque-Alberto et al., 2020; Tel-Çayan et al., 2023; Yu et al., 2023; Zammit Young and Blundell, 2023). Majority of the studies focus on the phenolic extracts and examine the antioxidant and anti-inflammatory activities *in vitro* (Giordano et al., 2018; Ruiz-Ruiz et al., 2017; Tanleque-Alberto et al., 2020). However, the phenolic composition varies significantly among different floral honeys, and in fact, phenolic profile, along with other unique honey components have been used as chemical markers to identify botanic origin, geographic origin, and environmental origins (Wang et al., 2022; Zhu et al., 2024). (Tomás-Barberán et al., 2001). Other factors, such as extraction and analytical methods may also add complexity to the composition of honey phenolics, consequently their bioactivities. There is no consented extraction method on honey phenolics. In some studies, honey phenolics were simply extracted by methanol or acetonitrile/10%NaCl partitioning and used for compositional analysis (Ruiz-Ruiz et al., 2017; Yu et al., 2023), and in others adsorbent such as XAD-2 resin was used, following liquid-liquid partitioning between ethyl acetate and water (Sun et al., 2020). Solid state extraction (SPE) was found to be a simple and valid extraction and semi-purification method for honey phenolics (Bertoncelj et al., 2011; Tanleque-Alberto et al., 2020), therefore, it was adopted in our recent study (Zhu et al., 2024). We recently reported that up to 29 phenolic compounds, mostly phenolic acids and flavonoids, were found in selected North American floral honeys (Zhu et al., 2024). Among them, pinobanksin-5-methyl ether, pinobanksin and pinocembrin are propolis-derived flavonoids which are rarely reported in honey (Tomás-Barberán et al., 2001).

Majority of the studies use *in vitro* chemical-based assays e.g., ferric reducing antioxidant power (FRAP), 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and oxygen radical absorption capacity (ORAC), to assess the antioxidant activity of honey phenolics (Poulsen-Silva et al., 2023; Sun et al., 2020; Tanleque-Alberto et al., 2020; Tel-Çayan et al., 2023), and cell culture models for anti-inflammatory activities (Ruiz-Ruiz et al., 2017; Silva et al., 2021; Sun et al., 2020); however, in most of these studies, contributions of honey phenolics to the antioxidant and anti-inflammatory activities were made by correlating those activities with total phenolic content (TPC) or concentrations of individual phenolics by statistic analyses, (Shen et al., 2019; Tanleque-Alberto et al., 2020). These activities have not been verified by measuring the activities of individual phenolic compounds of honey using the same *in vitro* assays.

Bioaccessibility and bioavailability are important properties for phenolics to exert their in vivo bioactivities. Bioaccessibility of honey phenolics is rarely studied. One report showed that *in vitro* gastric digestion did not significantly affect the TPC, thus it concluded that phenolics of honey had high bioaccessibility (O'Sullivan et al., 2013); however, its effect on individual honey phenolics was not evaluated. The same study also showed that that *in vitro* gastric digestion had mixed effects on FRAP, DPPH activities. While bioavailability of phenolics need to be assessed *in vivo*, intestinal epithelial cells such as Caco-2 cells have been used as a model to evaluate potential bioavailability of these bioactives, by assessing cellular uptake and transmembrane transport of phenolics across the Caco-2 monolayer (Chen et al., 2021; Zhang et al., 2020).

In the present study, we assessed the antioxidant activity of the phenolic extracts and major phenolic compounds of these honeys using cell-based antioxidant assay (CAA) in addition to the chemical-based assays. Caco-2 BBe1 cells was used to measure the effects of honey phenolics on antioxidant enzyme activities and secretion of interleukin 8 (IL-8). Moreover, bioaccessibility, transmembrane transport and cellular uptake of *p*-hydroxybenzoic

acid, caffeic acid, *p*-coumaric acid, isoferulic acid, pinobanksin-5-methyl ether, pinobanksin, kaempferol and pinocembrin, the major phenolic compounds identified in four North American alfalfa, buckwheat, clover and orange honeys (Zhu et al., 2024). Bioaccessibility of honey phenolics was also assessed. The objective of this study is to better understand the roles of individual honey phenolics in the antioxidant and anti-inflammatory activities, and provide initial insight into their bioaccessibility and bioavailability.

2. Material and methods

2.1. Samples, chemicals

Honey samples were provided by the US National Honey Board. Four floral honey varieties including alfalfa, buckwheat, clover, and orange were collected and produced between 2020 and 2022. All samples were stored at −20 °C before analysis.

Phenolic standards, including *p*-hydroxybenzoic acid (PHBA), caffeic acid (CA), *p*-coumaric acid (PCA), isoferulic acid (IFA), pinobanksin-5-methyl ether (P5ME), pinobanksin (PBK), kaempferol (KAE), pinocembrin (PCB), and other solvents and reagents including acetonitrile, formic acid, dimethyl sulfoxide (DMSO), L-ascorbic acid, ferric chloride, 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH), (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) were obtained from Sigma–Aldrich (Oakville, ON, Canada). 2,4,6-Tri(2-pyridyl)-1,3,5-triazine (TPTZ) and 2,2-Diphenyl-1-picrylhydrazyl (DPPH) were obtained from Fisher Scientific (Nepean, ON, Canada). LC-MS grade methanol was obtained from Fisher Scientific (Ottawa, ON, Canada). Distilled and deionized water was purified in-house using a Milli-Q system (Bedford, MA, USA).

2.2. Extraction of phenolic compounds in honey

A total of 300 g of each honey sample was weighed and diluted with 1% aqueous formic acid (v/v) to 30% (w/v). One liter of this acidified honey solution was then purified using 3 Strata-X 33 polymeric SPE cartridges (2 g, Phenomenex, Torrance, CA, USA), the absorbed phenolic compounds were eluted with 20 mL 1% (v/v) formic acid in methanol per cartridge. The eluates were combined and dried by a vacuum concentrator (Savant Speedvac SPD1030, ThermoFisher Scientific, Mississauga, ON, Canada). This yielded 259.4, 190.9, 312.8 and 429.9 mg of dried extract from clover, alfalfa, orange and buckwheat honey, or 0.09%, 0.06%, 0.10% and 0.14% by weight, respectively. Stock solutions (2 mL) of honey phenolic extracts were prepared by dissolving the dried extract in DMSO at a concentration of 150 g honey equivalent extract per mL (HE/mL), and stored in −20 °C freezer before use. HE concentrations were used in this study to compare the strength different bioactivities of different honeys.

2.3. Antioxidant activities assays

2.3.1. Ferric-reducing antioxidant power (FRAP) assay

FRAP was measured following a previously reported procedure (Li et al., 2012) with slight modifications. Briefly, 10 µL of diluted extract or phenolic standards or L-ascorbic acid was allowed to react with 300 µL of ferric-TPTZ reagent in wells of a 96-well microplate for 2 h at room temperature. The absorbance was read

at 593 nm using the visible–UV microplate reader (PowerWave XS2, Bio-Tek Instruments, Inc., Winooski, VT, USA). FRAP value was expressed as µmol L-ascorbic acid equivalent per gram sample (µmol AAE/g).

2.3.2. DPPH assay

Measurement of DPPH antioxidant activity also followed Li et al. (Li et al., 2012) with slight modifications. Briefly, 25 µL of diluted extract or phenolic standards of Trolox was mixed with 200 µL of 350 µM DPPH in methanol in a 96-well plate. The mixtures were reacted for 6 h in darkness at room temperature. The absorbance was measured at 517 nm using the microplate reader as stated above. The DPPH antioxidant activity was expressed as µmol of Trolox equivalents per gram sample (µmol TE/g).

2.3.3. Oxygen radical absorption capacity (ORAC) assay

The ORAC assay also followed the procedure by Li et al. (Li et al., 2012). Briefly, 25 µL of blank, Trolox or diluted extracts or phenolic standards were mixed with 200 µL fluorescein solution (0.0868 µM) and incubated for 30 min at 37 °C before injection of 25 µL AAPH (153 mM) in a black 96-well microplate. The fluorescence (excitation: 485 nm, emission: 528 nm) was measured every minute for 120 min until the signal reached zero in a Bio-Tek Fluorescence Spectrophotometer equipped with an automatic thermostatic holder (FLX 800, Bio-Tek Instruments, Inc., Winooski, VT, USA). A calibration curve was constructed by plotting the calculated differences of area under the fluorescein decay curve between the blank and a series of Trolox solutions. The results were expressed as µmol Trolox equivalent per gram sample (µmol TE/g).

2.4. Cell culture of Caco-2 BBe1

The Caco-2 BBe1 human intestinal cell line (American Type Culture Collection, Rockville, MD, USA) was grown in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Burlington, ON, Canada) with 10% foetal bovine serum (FBS; Hyclone Co., Logan, UT, USA) and 1X Antibiotic-Antimycotic (100 units/mL of penicillin, 100 µg/mL of streptomycin, and 250 ng/mL of Gibco Amphotericin B; Gibco). The cells were incubated at 37 °C in 5% CO_2 and the medium was replaced every 3 days. Cell passages of 60–80 were used in all monolayers for this experiment. Caco-2 BBe1 cells were cultured in T-75 culture flasks at 2×10^5 cells/mL and grown for 5–7 days to reach 80–90% confluency. Cells were sub-cultured to appropriate culture plates depending on the experiment to be performed.

2.5. Cell viability assay (WST-1 assay)

Caco-2 BBe1 cells were incubated with honey extracts at concentrations of 0.25, 0.5, 1.0, and 2.0 g HE/mL, as well as 1 mM of phenolic standards (PHBC, CA, PCA, IFA, P5ME, PBK, KAE, and PCB) for 6 h at 37 °C in 5% CO_2, followed by adding water-soluble tetrazolium (WST-1) at a final concentration of 250 µM and incubating for 2 h. The absorbance was measured at 450 nm using a UV/vis microplate reader mentioned above. Untreated cells were used as control. The cell viability was reported as the ratio of optical density of samples to control.

2.6. Cellular antioxidant activity (CAA) assay

CAA was evaluated using a published method with slight modifications (Li et al., 2014). Briefly, Caco-2 BBe1 cells were grown in a 96-well black/clear flat bottom plate until formation of monolayer. Cells were simultaneously treated with 50 µL of DCFH-DA (100 µM) and 50 µL of FBS-free DMEM (as control), honey extracts (0.1, 0.2, 0.5, and 1.0 g HE/mL), or phenolic standards (1 mM) for 1 h at 37 °C in 5% CO_2. After washing twice with 200 µL of HBSS, media were removed and replaced with 100 µL of 0.6 mM AAPH. Background samples were prepared by replacing media with 100 µL of HBSS. The intensity of fluorescence was measured using a fluorescence spectrophotometer mentioned above at an excitation wavelength of 485 nm and an emission wavelength of 528 nm for 2 h. The results were calculated according to the Equation (1):

$$CAA\ unit\ (\%) = \left(1 - \frac{\int SA - \int BA}{\int CA}\right) \times 100$$

where $\int SA$, $\int BA$, and $\int CA$ are integrated area under the sample, background, and control fluorescence versus time curve, respectively.

2.7. Interleukin-8 (IL-8) immunoassay

Caco-2 BBe1 cells were grown in a 48-well plate until reaching a 80–90% confluence. Cells were treated with 200 µL of DMEM with 5% FBS (as control) or honey extracts (0.25, 0.5, and 1.0 g HE/mL) for 2 h at 37 °C in 5% CO_2. Oxidative stress was induced by adding 2 ng/mL of TNF-α to both control and honey extracts samples (TNF-α +). A parallel groups of samples were treated with HBSS (TNF-α -) to identify the baseline of IL-8 release. All samples were incubated for 4 h and the supernatant was collected and stored in −80 °C before analysis.

Released IL-8 was determined by using a human IL-8 enzyme-linked immunosorbent assay (ELISA) kit following the manufacturer's instructions (eBioscience, Inc., San Diego, CA, USA). Mouse anti-human IL-8 antibodies were coated in 100 µL of coating buffer in a 96-well plate incubated overnight at 4 °C. The wells were washed 3 times with 300 µL of wash buffer and then blocked with 200 µL of blocking buffer for 1 h. The plate was washed 3 times between each of following steps. The 100 µL of sample and IL-8 standards were added into the wells and incubated for 2 h at room temperature. A 100 µL of secondary anti-human IL-8 antibody was added into the well for 1 h incubation, followed by 30 min incubation with 100 µL avidin-horseradish peroxidase conjugate (Av-HRP). A 100 µL of 3,3′,5,5′-tetramethylbenzidine (TMB) for colour development. This reaction was stopped by adding 100 µL of 1M H_3PO_4. The absorbance was measured at 450 nm using a UV/vis microplate reader mentioned above. The concentration (pg/mL) of IL-8 was extrapolated from the standard calibration curve.

2.8. Intracellular antioxidant enzyme activities

Glutathione reductase (GR), glutathione peroxidase (GPx), superoxide dismutase (SOD), and catalase (CAT) activities were measured using colorimetric assay kits (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions. Briefly, Caco-2 Bbe1 cells in a 6-well plate were pre-incubated with or without 1.0 g HE/mL extracts for 30 min, and then H_2O_2 was di-

rectly added into the wells to reach a final concentration of 1 mM and incubated for 6 h at 37 °C in 5% CO_2. The cells were washed twice with HBSS and then 0.5 mL of cold buffer (100 mM potassium phosphate buffer containing 1 mM EDTA, pH 7.5) was added into the wells. The cells were collected into 2 mL bead mill tubes with five 2.8 mm ceramic beads and were lysed for 20 sec at s speed of 5 m/s using a bead mill homogenizer (OMNI, Kennesaw, GA, USA). The mixture was centrifuged at 5,000×g for 10 min at 4 °C and the supernatant was collected for the assays of enzyme activities. The activity of each enzyme was calculated in milliunits per mg of protein (mU/mg). The results were reported as percent of the negative control.

2.9. In vitro digestion

Bioaccessibility of honey extracts was evaluated by a simulated gastrointestinal digestion described in our previous study (Zhang et al., 2017) with slight modifications. Briefly, 50 μL of each honey extract stock solution was mixed with 1.5 mL of artificial saliva and made up to 8 mL by adding HBSS. The mixtures were incubated for 15 min at 37 °C in a water bath shaker at 200 rev/min, and then adjusted to pH 1.5 using 6 M HCl. Porcine pepsin was added to reach a final concentration of 1.3 mg/mL. The mixture was continuously incubated for 1.5 h in a water bath shaker at 200 rev/min, and then added KH_2PO_4 (2 mL, 0.5 M), pancreatin (final concentration: 0.175 mg/mL) and procine bile salt (final concentration: 1.1 mg/mL). The pH of this solution was then adjusted to 8.0 using 10 M NaOH and incubated at 37 °C in a water bath shaker for another 2 h. The digestion process was terminated by adding phenylmethylsulfonyl fluoride (PMSF) at a final concentration of 1 mM. The digested mixture was acidified with 1% formic acid (for analysis of phenolic acids) or without (for analysis of flavonoids) before centrifuging at 2,000 × g for 10 min. The supernatant was cleaned by an OASIS HLB cartridge (150 mg, Waters, Milford, MA, USA). The elute was stored at −80 °C before LC-MS analysis.

2.10. Cell transport assay

Transport experiments were carried out by following a previously described procedure (Zhang et al., 2017) with slight modifications. Briefly, Caco-2 BBe1 cells were seeded onto polyethylene terephthalate (PET) membrane permeable support inserts (10.5 mm, 0.4 μm pore size, Corning Inc., Corning, NY, USA) at a density of 2 × 10^5 cells/mL. The culture medium (DMEM with 10% FBS and 1× Antibiotic-Antimycotic) was changed every second day until the cells became confluent monolayer. Only monolayers displaying trans-epithelial electrical resistance (TEER) values greater than 1,000 Ω·cm^2 was used in the experiment. TEER values were measured using a Millicell-ERS Volt-Ohm Meter (Millipore, Bedford, MA, USA). The medium in both wells and inserts was washed twice with HBSS, and added with FBS-free DMEM and 1.0 g HE/mL honey extract in FBS-free DMEM, respectively. Cells were treated with honey extracts at 1.0 g HE/mL. After 6 h incubation at 37 °C in 5% CO_2, the solution from apical and basal compartments were collected, acidified by 1% formic acid, and cleaned using Strata-X polymeric cartridges (30 mg, Phenomenex, Torrance, CA, USA). The cells with cold buffer were collected into 2 mL bead mill tubes with five 2.8 mm ceramic beads and were lysed for 20 sec at s speed of 5 m/s using a bead mill homogenizer (OMNI, Kennesaw, GA, USA). The mixture was centrifuged at 5,000 × g for 10 min at 4 °C and the supernatant was collected, acidified, and

cleaned using the procedure mentioned above.

2.11. Analysis of phenolic compounds by HPLC and LC-MS/MS

Phenolic compounds were analyzed using an Agilent 1260 series HPLC system consisting of an autosampler, a degasser, a quaternary pump, a thermostatted column compartment and a diodearray detector. LC-MS/MS analysis was performed using a Thermo® Scientific Q-Exactive™ Orbitrap mass spectrometer coupled to a Vanquish™ Flex Binary UPLC System with a diode array detector (DAD) (Waltham, MA, USA). A Kinetex XB-C18 100A column (100 x 4.6 mm, 2.6 μm, Phenomenex Inc., Torrance, CA, USA) was used. The binary mobile phase consisted of solvent A (99.9% H_2O/0.1% formic acid) and solvent B (94.9% methanol/5% acetonitrile/0.1% formic acid). The phenolic compounds were analyzed by using negative ionization mode. The solvent gradient was: 0–5 min, 0% to 12% B; 5–15 min, 12% to 23% B; 15–30 min, 23% to 50% B; 30 - 40 min, 50% to 80% B; 40–42 min, 80% to 100% B; 42–45 min, 100% B; 45–46 min, 100% to 0% B; 46–52 min, 0% B. The column temperature was set at 40 °C, the flow rate was 0.700 mL/min, and the injection volume was 2 μL. UV peaks were monitored at 280 nm, 320 nm, 360 nm and 520 nm. The spray voltages for both negative and positive modes were set at 4.5 kV. Mass spectrometry data were collected using DDMS2 method (TopN = 10, NCE = 30, intensity threshold was set at 1.0e5 counts) for compound identification, and with Full-MS mode for quantification. Data were visualized and analysed using Thermo FreeStyle™ 1.7PS2 software. Quantification was achieved using standard curves generated from individual compounds in serial dilutions (0.005–10 mg/L; r^2 > 0.995).

2.12. Statistical analysis

Results were expressed as mean ± standard deviation of then mean of at least triplicated measurements unless otherwise specified. A one-way analysis of variance (ANOVA) followed by Tukey's Honest Significant Difference (HSD) test was performed using Sigma-Plot 15.0 (Palo Alto, CA, USA) to analyze the difference of mean values of antioxidant activities, cell viability, CAA unit, released IL-8 concentration, and antioxidant enzyme activities. Significant differences were considered at p < 0.05.

3. Results and discussion

3.1. In vitro antioxidant activities

The SPE extracted phenolics of honeys were assessed for their antioxidant activities using FRAP, DPPH and ORAC assays. As shown in Table 1, among the four honeys, buckwheat honey showed the highest antioxidant activity in all three *in vitro* methods, followed by orange, clover and alfalfa honeys in FRAP and DPPH values, and clover, alfalfa and orange in ORAC value. The difference in antioxidant activity depended on the assays used; however, the results corresponded closely to the total phenolic content (TPC) of the same honeys that we reported recently (Zhu et al., 2024). While the phenolic profiles varied significantly, and unique compounds were identified as chemical markers for the four floral honeys (Zhu et al., 2024), the TPC of honeys of the present study were similar except for buckwheat honey which was three times higher than the rest, i.e., 379, 415, 425 and 1,269 μg GAE/g of alfalfa, orange,

Table 1. Antioxidant activities of honey extracts and their major phenolic compounds

Honey extracts	Mean ± SD (n = 3)		
	FRAP (μmol AAE/g honey)	DPPH (μmol TE/g honey)	ORAC (μmol TE/g honey)
Alfalfa	0.709 ± 0.012[d]	0.365 ± 0.007[c]	1.651 ± 0.052[c]
Buckwheat	1.781 ± 0.018[a]	0.918 ± 0.009[a]	3.377 ± 0.112[a]
Clover	0.809 ± 0.001[c]	0.401 ± 0.028[c]	2.500 ± 0.030[b]
Orange	0.954 ± 0.042[b]	0.583 ± 0.013[b]	1.484 ± 0.122[c]

Phenolic compounds (PCs)	Mean ± SD (n = 3)		
	FRAP (μmol AAE/μmol PC)	DPPH (μmol TE/μmol PC)	ORAC (μmol TE/μmol PC)
p-Hydroxybenzoic acid	<0.050	<0.125	7.274 ± 0.232[b]
Caffeic acid	3.382 ± 0.075[a]	1.049 ± 0.039[a]	7.356 ± 0.688[b]
p-Coumaric acid	0.480 ± 0.043[d]	0.269 ± 0.060[d]	5.609 ± 0.192[c]
Isoferulic acid	1.560 ± 0.063[c]	0.395 ± 0.032[c]	6.339 ± 0.363[bc]
Pinobanksin-5-methyl ether	<0.050	<0.125	4.070 ± 0.117[d]
Pinobanksin	<0.050	<0.125	3.257 ± 0.493[d]
Kaempferol	2.643 ± 0.027[b]	0.868 ± 0.033[b]	11.303 ± 0.226[a]
Pinocembrin	<0.050	<0.125	3.549 ± 0.359[d]

Means followed by a common letter within the same column (honey extracts or phenolic compounds) are not significantly different by the Tukey's HSD test at the 5% level of significance.

clover and buckwheat honey, respectively (Zhu et al., 2024). Buckwheat honeys also had the highest TPC and antioxidant among the 39 different honey batches in another study (Shen et al., 2019).

While it is evident that collectively phenolic compounds are the principal antioxidants of honeys as demonstrated in the present as well as other studies, to better understand the role of individual honey phenolics in the total antioxidant activity, we examined the antioxidant activity of major phenolic compounds common to all four selected honeys, i.e., PHBA, CA, PCA, IFA, P5ME, PBK, KAE AND PCB (Zhu et al., 2024), using the same *in vitro* assays. As shown in Table 1, cinnamic acids, particularly caffeic and isoferulic acid, and kaempferol had higher FRAP, DPPH and ORAC values. *p*-Hydroxybenzoic acid which had the highest concentration of all honeys, especially in buckwheat honey (Zhu et al., 2024), showed very little antioxidant activities in all three assays. P5ME and PBK (flavanonols), and PCM, a flavanone, the three flavonoids unique to honey also had relatively lower *in vitro* antioxidant activity (Table 1). In the meantime, PHBA was 12.5 μg/g in buckwheat honey, 2–11 fold higher than the other three honeys (Zhu et al., 2024), yet it had the weakest FRAP and DPPH activities, and moderate ORAC values among the compounds tested; conversely, the highest FRAP and DPPH values were found in CA which was only 1.1 μg/g in buckwheat honey, but 6.9 μg/g in Alfalfa honey (Table 1). These suggest that phenolic compounds with high concentrations in honey may not be the major contributors to the total antioxidant activities, and vice versa, compounds at low concentrations may contribute more to the *in vitro* antioxidant activities. Our result suggests that these *in vitro* results need to be verified *in vivo* or by other more biologically relevant method. It also necessitates further studies focusing on the roles of other polyphenols found in relatively lower concentrations in honeys (Zhu et al., 2024). Antioxidant activities have been reported for other honey phenolics using similar *in vitro* assays (Ruiz-Ruiz et al., 2017; Sun et al., 2020; Tanleque-Alberto et al., 2020), and our result from the DPPH assay (0.365–0.918 μmol TE/g honey) was in agreement of that of Mozambique honeys (Tanleque-Alberto et al., 2020); however, most studies derive their conclusions by associating the total antioxidant activity of honey with TPC or concentrations of individual phenolics, rather than verifying the antioxidant activity of individual compounds using the same *in vitro* assays (Shen et al., 2019; Tanleque-Alberto et al., 2020). Since the most prevailing phenolics did not show

3.2. Cell-based antioxidant activity (CAA)

Caco-2 BBe1 cells were used to evaluate the CAA of the phenolic extracts of honeys. Extracts of honey showed no toxicity to the cells at and below 1.0 g HE/mL (Figure S1), therefore the following CAA assays were conducted at 0.1, 0.2, 0.5 and 1.0 g HE/mL. Major honey phenolic compounds PHBC, CA, PCA, IFA, P5ME, PBK, KAE, and PCB also had no significant effect on cell viability at 1mM (Figure S1). The results showed a clear dose-dependent CAA for all honeys. CAA of alfalfa, buckwheat and clover honeys were generally similar, but that of orange was significantly lower (Figure 1). However, at 0.1 g HE/mL, extract of buckwheat honey had significantly higher CAA compared to other honeys at the same concentration, suggesting the former is a stronger antioxidant at lower concentration. This is in agreement with other studies which also showed that buckwheat honeys had the highest CAA among different honeys (Shen et al., 2019). The lower CAA of orange honey might be due to the significantly lower total concentration of all detected phenolic compounds compared to other three honeys of this study. The sum of all detected phenolic compounds was 11 μg/g of orange honey, compared to 40, 40 and 44 μg/g of alfalfa, clover and buckwheat honeys (Zhu et al., 2024). Phenolic extracts of different honeys showed significant differences in FRAP, DPPH and ORAC values, and that of buckwheat was substantially
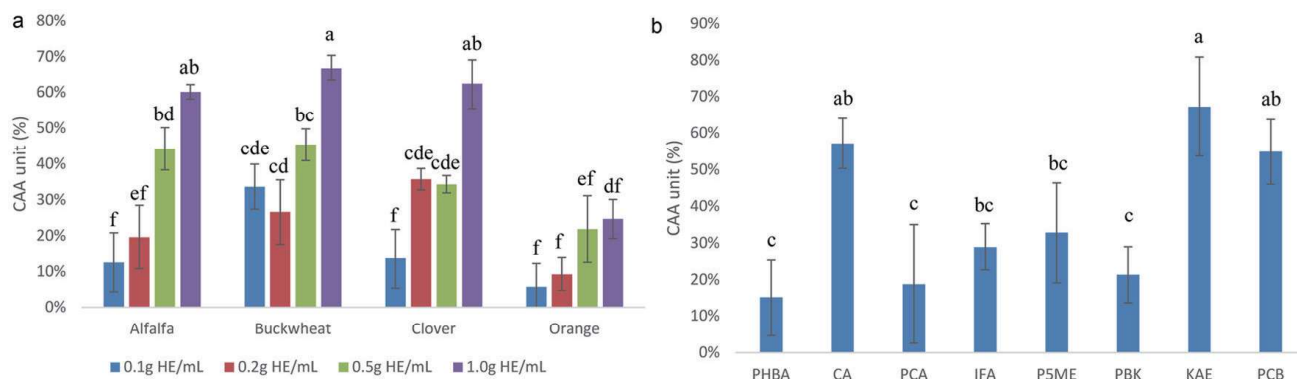
Figure 1. CAA of Caco-2 BBe1 cell treated with 0.1, 0.2, 0.5 and 1.0 g HE/mL of the phenolic extracts of alfalfa, buckwheat, clover, and orange honeys (a), and 1 mM of major phenolic compounds in honeys (b). Value are expressed as CAA Unit (%) and presented as mean ± SD, n = 3. Shared letters indicate no significant difference (p < 0.05).

higher (Table 1), but this was not true for CAA. Considering there might be compounds other than phenolics in honey extracts that may react to reducing agents or free radicals in chemical based assays, giving overestimated values, CAA is a result of activity in live cells after phenolic compounds are transported across the cell membrane, therefore, only those enter the cytosol of the cells will exert antioxidant activity. CAA is also more relevant to *in vivo* activity than chemical based antioxidant assays (Furger, 2021). In the meantime, nearly all phenolics detected in honeys of this study were aglycones which are most likely absorbed into the cytosol via passive transport (Zhang et al., 2020). Honeys containing higher concentrations of phenolic compounds will therefore have higher CAA, and those with lower concentrations, lower CAA. This explains the significantly lower CAA of orange honey than the other three honeys (Figure 1a). CAA of the major honey phenolics were also obtained, among them caffeic acid, kaempferol and pinocembrin had the highest CAA and were significantly higher than other phenolics at the concentration tested (Figure 1b). P5ME also showed similar CAA to other major honey phenolics of this study. It is worth noting that pinocembrin and P5ME, particularly the former possesses CAA, despite its very FRAP, DPPH and ORAC activities. Exactly how these major phenolics of honey contribute to the overall antioxidant activity of honey needs future investigation. Shen et al. suggested that honey phenolic acids might have synergistic CAA activity (Shen et al., 2019).

3.3. Phenolic extracts of honeys on antioxidant enzymes

Phenolic extracts of honeys at 1.0 g HE/mL were also evaluated for their effect on endogenous antioxidant enzymes, i.e., GR, GPx, SOD and CAT, in a Caco-2 BBe1/H_2O_2 cell model. In general, exposure to H_2O_2 significantly reduced the activity of all enzymes over the normal control; however, treatment by phenolic extracts lowered the enzyme activities. Only extract of buckwheat honey significantly restored the H_2O_2-induced reduction of GR, SOD and CAT (Figure 2). These results indicate that honey phenolics can also provide protection against reactive oxygen species such as H_2O_2. The stronger activity of buckwheat honey is highly likely due to its higher TPC and sum of all phenolic compounds (Zhu et al., 2024). Effect of honey phenolics on antioxidant enzymes was studied on Manuka honey, not the phenolic extract, in a different cell model (Gasparrini et al., 2018), which showed that the increased antioxidant enzyme activities were through enhanced

NF-E2-related factor 2 (Nrf2) expression. Although the authors hinted that phenolics may be responsible for the activity. Our results suggest that honey phenolics contribute to the antioxidant defense by enhancing the activity of the endogenous antioxidant enzymes, but the exact mechanism, especially how collectively or individually these phenolics modulate the Nrf2/antioxidant responsive element (Nrf2/ARE) pathway which regulates the antioxidant enzymes needs to be confirmed (Furger, 2021).

3.4. Phenolic extracts of honeys on IL-8 secretion

There is sufficient *in vitro* and *in vivo* evidence that honey, mainly due to its phenolic content, possesses anti-inflammatory activity (Ranneh et al., 2021; Silva et al., 2021). While extensive experiment was not conducted in the present study, the phenolic extracts of honeys at 0.25, 0.5 and 1.0 g HE/mL were examined for their effect on the release of the proinflammatory cytokine IL-8 in a Caco-2 BBe1/TNF-α cell model. The anti-inflammatory effect of honey phenolic extracts as expressed in IL-8 inhibition appeared to be dose-dependent. All honeys showed significant inhibition of TNF-α-induced IL-8 at 1.0 g HE/mL, but phenolic extract of buckwheat showed significant inhibition at 0.5 1.0 g HE/mL, suggesting its stronger anti-inflammatory effect compared to other honeys (Figure 3). This is in agreement with the above CAA results, which showed that buckwheat honey also had stronger antioxidant activity, especially at lower concentration (Figure 1a). Phenolics of honey have been found to contribute to the anti-inflammatory effects in a different cell model (Yu et al., 2023); however, roles of individual honey phenolics in anti-inflammatory activity and the underlying mechanisms need to be further studied.

3.5. Bioaccessibility of honey phenolics

It is understood that the *in vivo* activity of any food bioactives depends on its bioaccessibility and bioavailability. The bioaccessibility of honey phenolics of the present study was evaluated using a simulated upper gut digestion system, and overall, all phenolics except kaempferol showed good stability after *in vitro* digestion (Table 2). The bioaccessibility of the three propolis-derived flavonoids pinobanksin, pinocembrin and P4ME was between 79–95%. Very limited number of studies have assessed the bioaccessibility of honey phenolics, but results of the present study generally
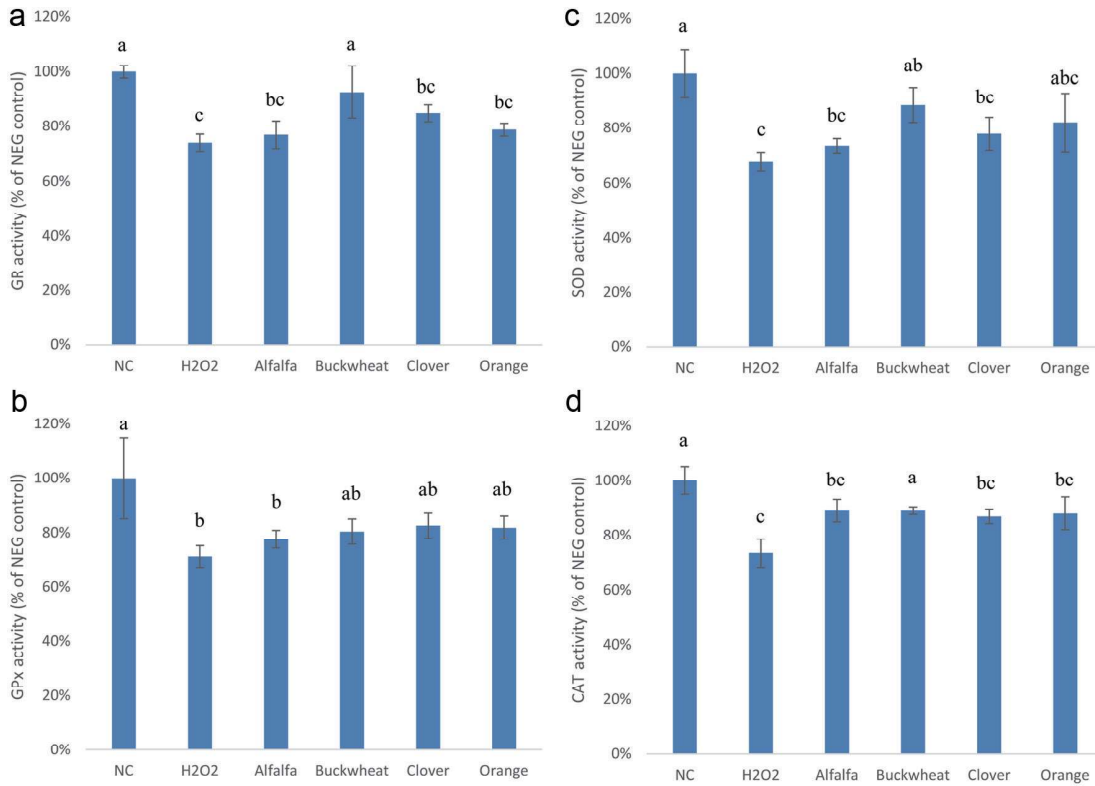
Figure 2. Effect of the phenolic extracts of alfalfa, buckwheat, clover, and orange honeys on endogenous antioxidant enzyme activities (a: GR; b:GPx; c: SOD; d: CAT) in H_2O_2-stimulated Caco-2 BBe1 cells. The negative control (NC) represents untreated cells. Positive control (H_2O_2) represents cells treated with H_2O_2 only. Values are presented as mean ± SD, n = 3. Shared letters indicate no significant difference (p < 0.05).
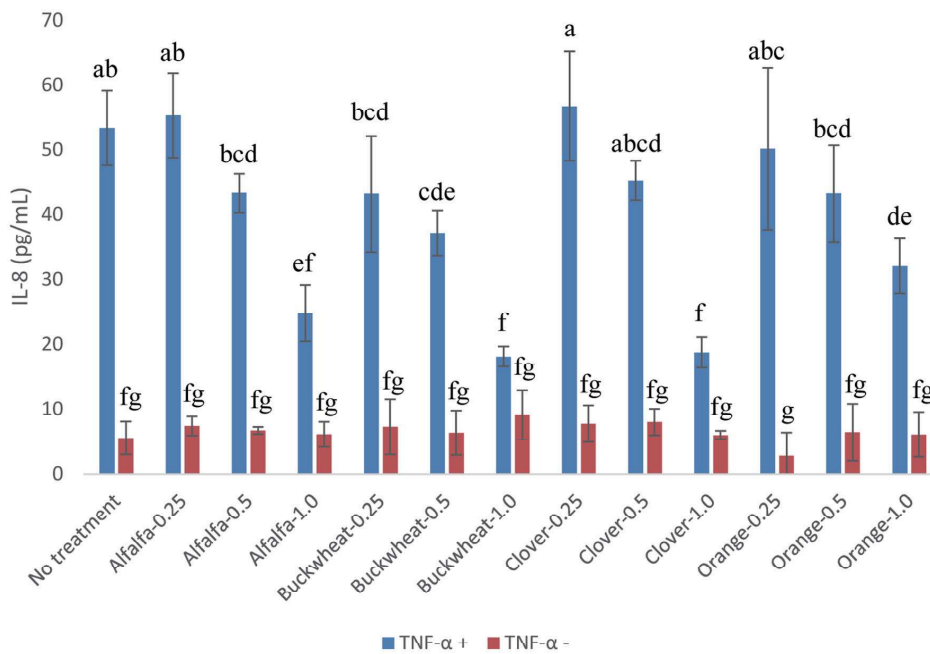

Figure 3. Released IL-8 of Caco-2 BBe1 cell treated with 2 ng/mL of TNF-α in combination with 0.25, 0.5 and 1.0 g HE/mL of the phenolic extracts of alfalfa, buckwheat, clover, and orange honeys. Value are expressed as IL-8 concentration and presented as mean ± SD, n = 4. Shared letters indicate no significant difference (p < 0.05).

Table 2. Bioaccessibility of major phenolic compounds in honeys through *in vitro* digestion

	Relative bioaccessibility (%), Mean ± SD, n = 3			
	Alfalfa	**Buckwheat**	**Clover**	**Orange**
p-Hydroxybenzoic acid	111.3% ± 2.3%*	96.7% ± 4.5%	106.5% ± 3.9%	97.2% ± 1.7%
Caffeic acid	79.9% ± 2.3%*	86.8% ± 4.0%*	89.5% ± 7.9%	78.4% ± 1.5%*
p-Coumaric acid	93.2% ± 3.7%	92.1% ± 4.4%	95.5% ± 5.9%	91.6% ± 1.2%*
Isoferulic acid	103.1% ± 3.1%	84.5% ± 17.9%	111.8% ± 15.0%	89.5% ± 17.7%
P5ME	94.8% ± 0.5%*	94.4% ± 1.9%*	100.2% ± 5.0%	89.9% ± 6.0%
Pinobanksin	88.0% ± 0.8%*	83.3% ± 2.9%*	96.4% ± 3.3%	78.9% ± 1.4%*
Kaempferol	40.5% ± 2.7%*	48.5% ± 2.4%*	49.6% ± 4.1%*	40.9% ± 4.1%*
Pinocembrin	87.5% ± 2.4%*	84.4% ± 1.1%*	95.8% ± 4.6%	80.6% ± 0.9%*

Means followed by a star symbol (*) indicate a significantly difference between before and after *in vitro* digestion by the Tukey's HSD test at the 5% level of significance. P5ME, Pinobanksin-5-methyl ether

agree with those of similar studies. For example, *in vitro* gastric digestion did not affect the TPC of honey thus phenolics of honey were considered to have high bioaccessibility, although the effect on FRAP, DPPH activity was mixed (O'Sullivan et al., 2013). In another study, not only TPC, FRAP and DPPH values were not significantly affected by *in vitro* gastric and duodenal phase digestions, but individual phenolic compounds, including the major honey phenolic of the present study, such as PBK, KAE, CA, also had high bioaccessibility (Seraglio et al., 2017). One study, however, showed mixed effect of *in vitro* digestion on the antioxidant activities and anti-inflammatory activity of honey phenolics (Alevia et al., 2021). Because of the high bioaccessibility of the honey phenolics, *in vitro* antioxidant and anti-inflammatory activities of digested honey extracts were not tested in the present study.

3.6. Transmembrane transport of major honey phenolics

To assess the potential bioavailability of the major honey phenolics, extracts of the four honeys were applied to the apical side of the Caco-2 BBe1 cell monolayer. Concentrations and percent distribution of major honey phenolics in the apical and basolateral compartments and the monolayer cells were analyzed after 6 h incubation using LC-MS (Figure 4). In general these phenolic compounds showed different stability in cell medium and varied ability to cross the Caco-2 BBe1 cell monolayer. PHBA and PCA were most stable in the apical medium regardless of its honey origin, with 98.4–109.5% and 93.6–95.7% remaining, 2.5–3.0% taken up by the cells, and 6.2–7.3% and 3.6–4.2% transported to the basolateral side, after 6 h, respectively. Stability and rate of transmembrane transport of IFA varied significantly depending on the origin of honey. IFA of alfalfa, buckwheat and clover honey was relatively stable in the apical medium (75.4–105.7%) (Figure 4a–c), but only IFA from the alfalfa honey was permeated to the basolateral medium at 13.1% (Figure 4a). No IFA was detected in cells for all treatments. For the monolayer treated phenolics of orange honey, IFA was not detected after 6 h (Figure 4d). Contrary to these phenolic acids, KAE, the major flavonol aglycone of honeys, was not stable in the cell medium, and showed minimum cellular uptake and transmembrane transport. Phenolics unique to honey, i.e. P5ME, PBK and PCB, showed higher potential bioavailability than other compounds, as seen in higher percentage of these flavanonol/flavanone polyphenols permeated to the basolateral side after 6 h incubation. Among them, P5ME had the highest

transmembrane transport rate, at 22.0, 23.9, 25.3 and 20.1% for alfalfa, buckwheat, clover and orange honeys, respectively, and ca. 4.1–4.6% of P5ME was taken up by the cells. These numbers suggest that P5ME was stable in cells and cell medium under the experimental conditions. PBK and PCB were less stable, especially the latter, with ca. 9.0–19.1% and 9.0–21.4% transported across the Caco-2 BBe1 cell monolayer, and 4.1–4.9% and 0–6.9% taken up by the cells, respectively (Figure 4). These data clearly demonstrate that honey phenolics are generally stable, and can be absorbed by the Caco-2 BBe1 cells and permeated through the monolayer, particularly the honey specific, propolis-derived flavonoids P5ME, PBK and PCB.

Absorption of phenolic compounds both in transmembrane transport and cellular uptake depends on the structural feature when other conditions are the same. Percent flavonoids transported from the apical to basolateral compartment of the Caco-2 monolayer was found to be higher than phenolic acids (Hithamani et al., 2017). In another study, quercetin 3-glucoside and glucuronide had very low to no permeability in both apical-basolateral and basolateral- apical directions, but quercetin and cinnamic acids of hibiscus extract were transported across the monolayer from apical to basolateral; however, only quercetin aglycone was detected in the cell, suggesting the aglycone reaches its molecular targets in a more effective way (Borrás-Linares et al., 2015). The authors also suggested the existence of a transport efflux mechanism of these compounds (Borrás-Linares et al., 2015), but such mechanism does not appear to involve transporter proteins (Rastogi and Jana, 2016). The mechanism of absorption of flavonoids has been revealed in our recent study, which showed that absorption of flavonoids depended not only on the structural features, but also the number and type of the sugar moieties attached to the flavonoid backbone (Zhang et al., 2020). We further discovered that two intestinal hexose transporters, SGLT1 and GLUT2, were key players in the active transport of flavonoid glycosides, particularly diglycosides, but not of aglycones which may be by passive diffusion (Zhang et al., 2020).

In the present study, while PCB was relatively unstable, it had the highest permeability rate (Figure 4). Moreover, further examination using LC-MS of the phenolic profile of the basolateral compartment showed that PCB was transformed into two phase II metabolites, PCB glucuronides peaks M1 (Rt = 28.5 min) and M2 (Rt = 28.7 min), possibly conjugated at 7- and 5- positions, respectively (Figures 5 and 6). These two metabolites were detected only in the basolateral side of Caco-2 BBe1 cell membrane treated with
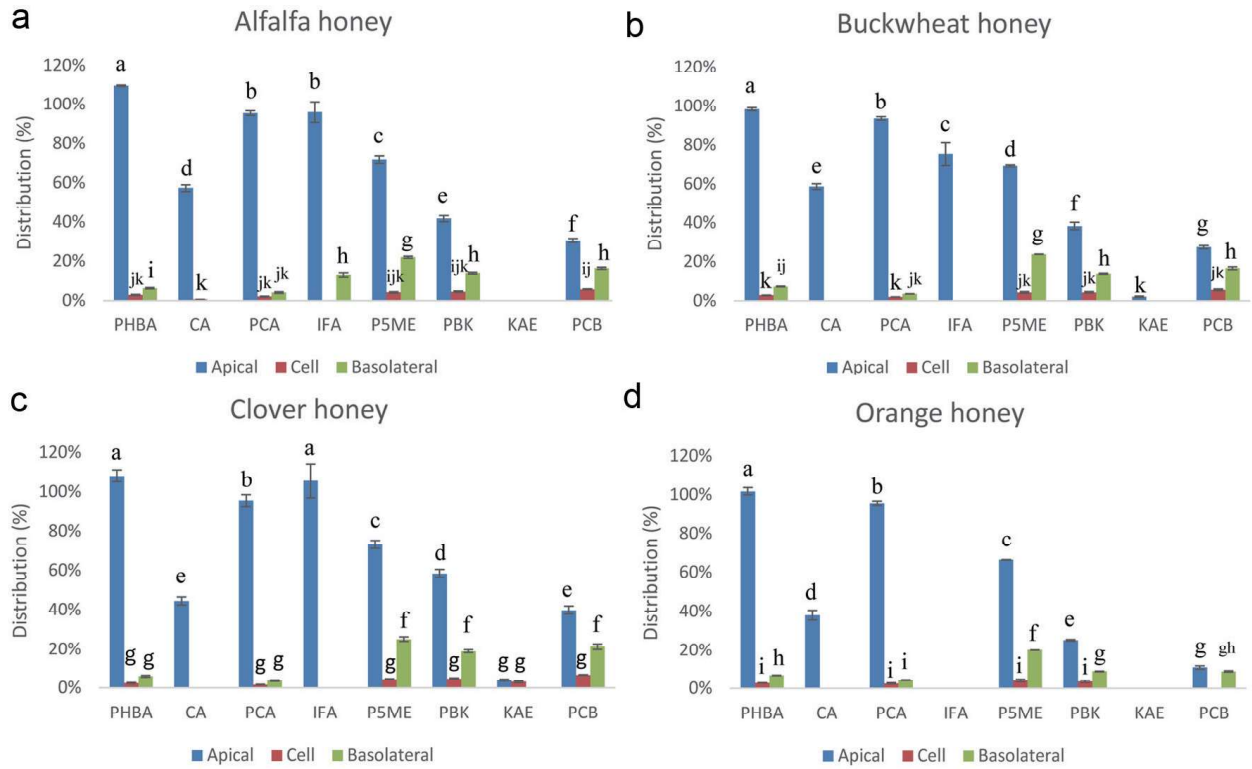
Figure 4. Apical, basal, and Caco-2 BBe1 cell uptake fractions of major phenolic compounds in the extract from alfalfa (a), buckwheat (b), clover (c), and orange honeys (d) after 6 h incubation. The percentage was determined by the ratio of the concentration of each compartment to the original extract (1.0 g HE/mL). Values are presented as mean ± SD, n = 3. Shared letters indicate no significant difference (p < 0.05).

pure PCB and the alfalfa, buckwheat and clover honey phenolic extracts, not the orange honey extract, possibly due to the relatively very low PCB concentration in the orange honey (Figure 5) (Zhu et al., 2024). The two PCB glucuronides were not detected in the apical side and the cell (Figure 7), suggesting it was conjugated in the cell and immediately effluxed into the basolateral medium. The tentative assignment of the two glucuronosyl positions was based on the eluding order of two similar isomers, apigenin 7-O-glucoside and apigenin 5-O-glucoside separated on a C18 column similar to the one used in the present study (Beszterda and Frański, 2021). Further study using other instrumental analysis can help identify the exact position of the two PCB glucuronide isomers of the present study. No such metabolites were detected for other honey phenolics (Figure 6).
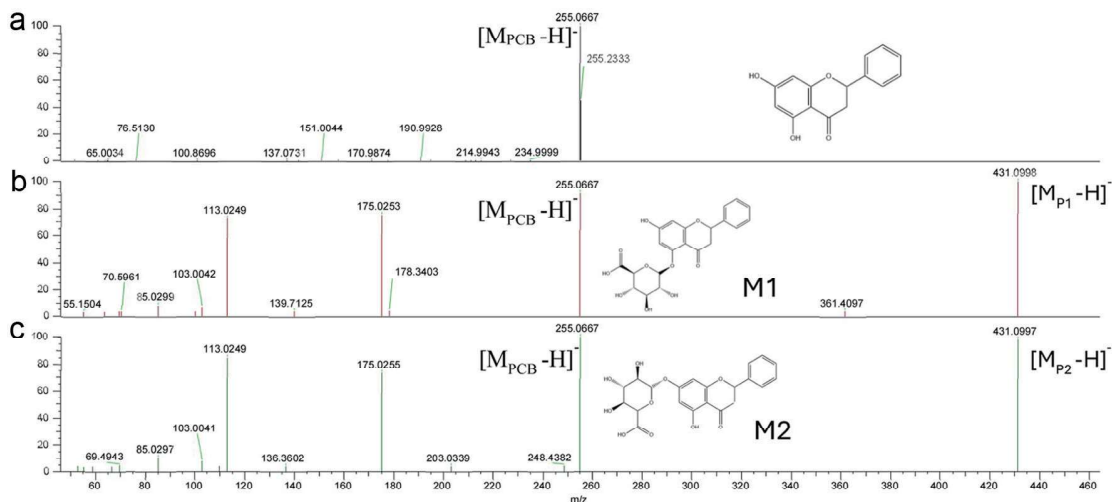

Figure 5. MS/MS spectra of PCB (a) and its metabolites (P1, b; P2, c) extracted from basolateral side of Caco-2 BBe1 cells after 6 h incubation.
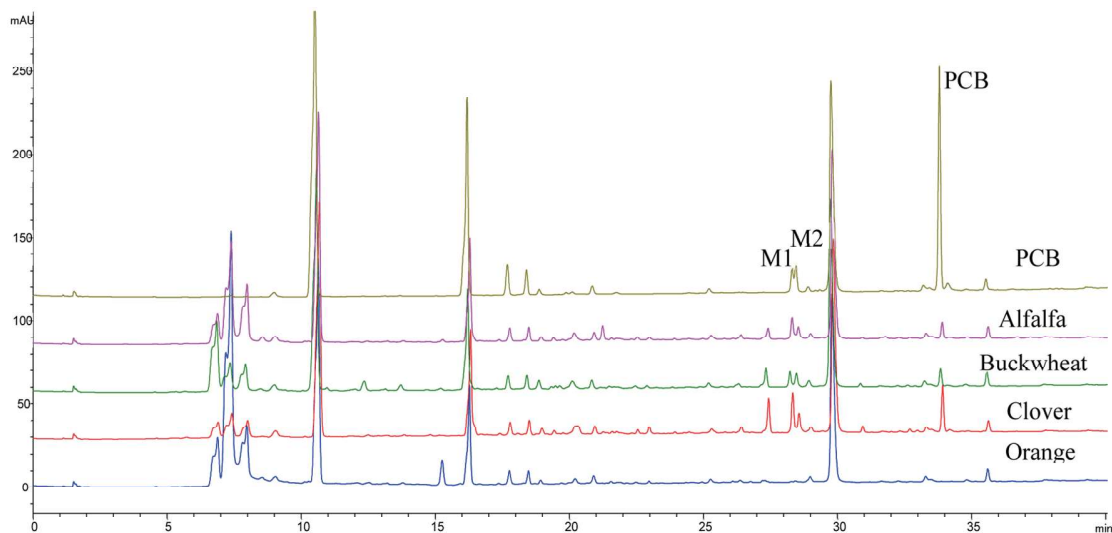
Figure 6. HPLC chromatograms of phenolic compounds extracted from basolateral side of Caco-2 BBe1 cells after 6 h incubation with 100 μM PCB, 1 g HE/mL alfalfa, buckwheat, clover, and orange honey extracts.

Flavonoids such as flavan-3-ols and their phase II metabolites were found to be relatively stable in the cell medium (Rodriguez-Mateos et al., 2014). For epicatechin, a flavan-3-ol, incubation in the Caco-2 monolayer system produced mainly phase II metabolites, including 3'- and 4'-methyl epicatechin sulphates and glucuronides at 5- and 7- positions of the A ring, and overwhelming majority of epicatechin and its metabolites were found in the apical compartment. Only 3'-methyl epicatechin 7-glucuronide and 3'-methyl epicatechin 7-sulphate were also detected in the basolateral side and in cells (Rodriguez-Mateos et al., 2014); however, epicatechin glucuronides were not found in other studies (Redan et al., 2017; Sanchez-Bridge et al., 2015). This may be due to the method and sensitivity of the analytical methods used, cell culture conditions, concentrations and length of treatment times (Redan et al., 2017).

Cellular uptake and transport mechanism and metabolism of

honey phenolic compounds have not been well studied. Propolis-derived hydroxy cinnamic acid esters and a set of aglycone flavonoid compounds, mainly chrysin, galangin, PCB and PBK were found to be transformed into phase II metabolites in the form of glucuronides and sulphates in plasma after oral ingestion, but dominantly as 7-O-glucuronides (Bloor and Mitchell, 2021). No PCB 5-, but only PCB 7-O-glucurodide was detected as a phase II metabolite of PCB in humans (Bloor and Mitchell, 2021). Despite the difference, the Caco-2 BBe1 cell monolayer model used in the present study is helpful in understanding the cellular uptake and transmembrane transport mechanism and detection of potential phase II metabolites. Certain co-existing polyphenols could significantly increase the transmembrane efflux of epicatechin, thus potentially enhances the bioavailability (Sanchez-Bridge et al., 2015), therefore, adsorption of honey phenolics may also differ
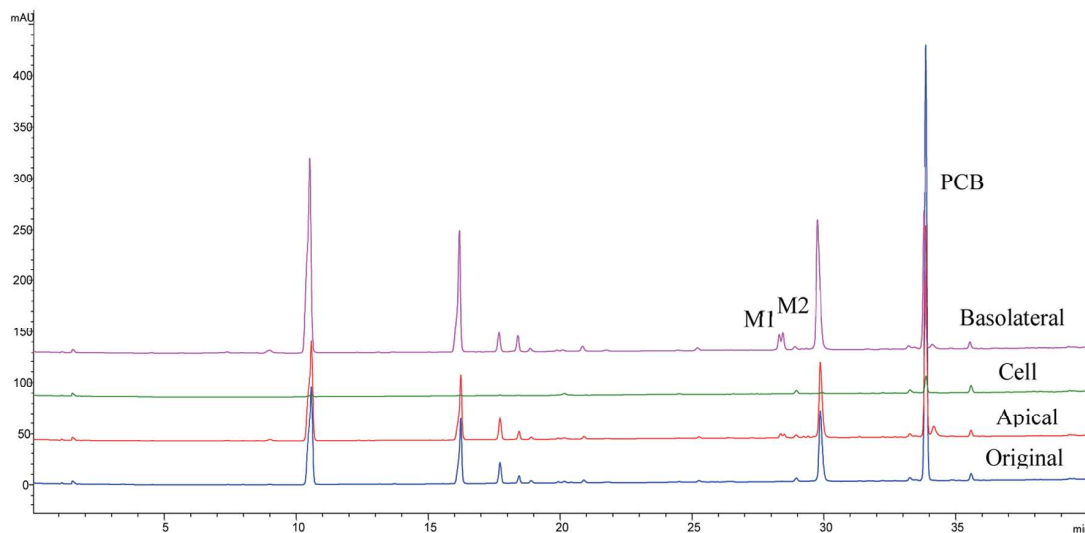

Figure 7. HPLC chromatograms of PCB and its metabolites P1 and P2 extracted from basolateral, apical, cells of Caco-2 BBe1 cells after 6 h incubation with 100 μM PCB.

between using pure compounds versus extracts.

In summary, four honeys collected in North America were extracted and assessed for their antioxidant and anti-inflammatory activities using chemical-based and cell culture based models. Buckwheat showed the highest FRAP, DPPH and ORAC values. This corresponds to the TPC of buckwheat. Eight major phenolic compounds of the studied honeys also showed varied in vitro antioxidant activities, among them, CA and KAE and IFA were most strongest; however, they are not the most prevalent phenolics of honey. Honey phenolics with higher concentrations such as PHBA did not show higher antioxidant activity in chemical-based assays, suggesting the other factors than major phenolic compounds contribute to the overall antioxidant potential of honeys. The higher antioxidant activity of buckwheat honey was also found in CAA. CAA is considered a more biologically relevant marker because compounds must be transported across the live cell membrane to exert antioxidant activity. The higher antioxidant potential of buckwheat honey was also demonstrated in the different antioxidant enzymes in Caco-2 BBe1 cells. Only the phenolic extract of buckwheat honey showed significant activity in restoring H_2O_2-induced reduction of GR, SOD and CAT, key defense enzymes against oxidative stress. Similar effect was found for phenolics of buckwheat honey in reducing TNF-α-induced IL-8 secretion, a proinflammatory biomarker in Caco-2 BBe1 cell. It is not clear on what phenolic compound or combinations of them in buckwheat might have caused the significantly higher antioxidant and anti-inflammatory activities; however, results from the cellular uptake and transmembrane transport of the phenolic compounds of the different honeys may provide some insight. The three propolis-derived flavonoids P5ME, PBK and PCB, showed similar and higher ability than other phenolics to permeate through Caco-2 BBe1 cell monolayer, particularly P5ME which had the highest transmembrane transport cellular take up rate. These three unique phenolics were similar in all honeys studied except Orange honey, suggesting they may present higher potential of *in vivo* activity. While only PCB glucuronides were found as phase II metabolites in the basolateral medium of the Caco-2 BBe1 cell monolayer, the role of metabolites are not clear. Further research is needed to focus on the metabolism of honey phenolics and their role in antioxidant and anti-inflammatory activities. Another factor that might affect the potential *in vivo* activity may be the bioaccessibility of honey phenolics. While most phenolics were stable during in vitro digestion, KAE was not. It also was not stable in cell culture medium. Results from the present study demonstrate that honey phenolics are the underlying contributor to the antioxidant and anti-inflammatory activities; however, at individual phenolic compound level, and when bioaccessibility, potential bioavailability and metabolism are taken into consideration, more research is needed. In particular, bioavailability of honey phenolics and their metabolites, and the molecular mechanism of the antioxidant, anti-inflammatory activities should be investigated.

Acknowledgments

Supplementary material

Suppl 1. Cell viability of honey phenolic extracts and individual phenolics. Caco-2 BBe1 cell were treated with 0.25, 0.5, 1.0 and 2.0 g HE/mL of the phenolic extracts of alfalfa, buckwheat, clover, and orange honeys (A); and major phenolic acids (). Value are expressed as relative viability (%) and presented as mean ± SD, n = 4. *p*-hydroxybenzoic acid (PHBA), caffeic acid (CA), *p*-coumaric acid (PCA), isoferulic acid (IFA), pinobanksin-5-methyl ether (P5ME), pinobanksin (PBK), kaempferol (KAE), pinocembrin (PCB).

References

Alevia, M., Rasines, S., Cantero, L., Sancho, M.T., Fernández-Muiño, M.A., and Osés, S.M. (2021). Chemical Extraction and Gastrointestinal Digestion of Honey: Influence on Its Antioxidant, Antimicrobial and Anti-Inflammatory Activities. Foods 10(6): 1412.

Battino, M., Giampieri, F., Cianciosi, D., Ansary, J., Chen, X., Zhang, D., Gil, E., and Forbes-Hernández, T. (2021). The roles of strawberry and honey phytochemicals on human health: A possible clue on the molecular mechanisms involved in the prevention of oxidative stress and inflammation. Phytomedicine 86: 153170.

Bertoncelj, J., Polak, T., Kropf, U., Korošec, M., and Golob, T. (2011). LC-DAD-ESI/MS analysis of flavonoids and abscisic acid with chemometric approach for the classification of Slovenian honey. Food Chem. 127(1): 296–302.

Beszterda, M., and Frański, R. (2021). Electrospray ionisation mass spectrometric behaviour of flavonoid 5-O-glucosides and their positional isomers detected in the extracts from the bark of *Prunus cerasus* L. and *Prunus avium* L. Phytochem. Anal. 32(3): 433–439.

Bloor, S.J., and Mitchell, K.A. (2021). Metabolic products of European-type propolis. Synthesis and analysis of glucuronides and sulfates. J. Ethnopharmacol. 274: 114035.

Borrás-Linares, I., Herranz-López, M., Barrajón-Catalán, E., Arráez-Román, D., González-Álvarez, I., Bermejo, M., Fernández Gutiérrez, A., Micol, V., and Segura-Carretero, A. (2015). Permeability Study of Polyphenols Derived from a Phenolic-Enriched Hibiscus sabdariffa Extract by UHPLC-ESI-UHR-Qq-TOF-MS. Int. J. Mol. Sci. 16(8): 18396–411.

Chen, Y., Zhang, H., Fan, W., Mats, L., Liu, R., Deng, Z., and Tsao, R. (2021). Anti-Inflammatory Effect and Cellular Transport Mechanism of Phenolics from Common Bean (Phaseolus vulga L.) Milk and Yogurts in Caco-2 Mono- and Caco-2/EA.hy926 Co-Culture Models. J. Agric. Food Chem. 69(5): 1513–1523.

Furger, C. (2021). Live Cell Assays for the Assessment of Antioxidant Activities of Plant Extracts. Antioxidants (Basel) 10(6): 944.

Gasparrini, M., Afrin, S., Forbes-Hernández, T.Y., Cianciosi, D., Reboredo-Rodriguez, P., Amici, A., Battino, M., and Giampieri, F. (2018). Protective effects of Manuka honey on LPS-treated RAW 264.7 macrophages. Part 2: Control of oxidative stress induced damage, increase of antioxidant enzyme activities and attenuation of inflammation. Food Chem. Toxicol. 120: 578–587.

Giordano, A., Retamal, M., Leyton, F., Martínez, P., Bridi, R., Velásquez, P., and Montenegro, G. (2018). Bioactive polyphenols and antioxidant capacity of Azara petiolaris and Azara integrifolia Honeys. CyTA - J. Food 16(1): 484–489.

Hithamani, G., Kizhakayil, D., and Srinivasan, K. (2017). Uptake of phenolic compounds from plant foods in human intestinal Caco-2 cells. J. BioSci. 42(4): 603–611.

Li, H., Deng, Z., Liu, R., Loewen, S., and Tsao, R. (2014). Bioaccessibility, in vitro antioxidant activities and in vivo anti-inflammatory activities of a purple tomato (*Solanum lycopersicum L*.). Food Chem. 159: 353–360.

Li, H., Deng, Z.Y., Zhu, H., Hu, C., Liu, R., Young, J.C., and Tsao, R. (2012). Highly pigmented vegetables: Anthocyanin compositions and their role in antioxidant activities. Food Res. Int. 46: 250–259.

Machado De-Melo, A.A., Almeida-Muradian, L.B.D., Sancho, M.T., and Pascual-Maté, A. (2018). Composition and properties of Apis mellifera honey: A review. J. Apic. Res. 57(1): 5–37.

O'Sullivan, A.M., O'Callaghan, Y.C., O'Connor, T.P., and O'Brien, N.M. (2013). Comparison of the Antioxidant Activity of Commercial Hon-

eys, Before and After In-Vitro Digestion. Pol. J. Food Nutr. Sci. 63(3): 167–171.

Poulsen-Silva, E., Gordillo-Fuenzalida, F., Velásquez, P., Llancalahuen, F.M., Carvajal, R., Cabaña-Brunod, M., and Otero, M.C. (2023). Antimicrobial, Antioxidant, and Anti-Inflammatory Properties of Monofloral Honeys from Chile. Antioxidants 12(9): 1785.

Ranneh, Y., Akim, A.M., Hamid, H.A., Khazaai, H., Fadel, A., Zakaria, Z.A., Albujja, M., and Bakar, M.F.A. (2021). Honey and its nutritional and anti-inflammatory value. BMC Complementary Med. Ther. 21(1): 30.

Rastogi, H., and Jana, S. (2016). Evaluation of physicochemical properties and intestinal permeability of six dietary polyphenols in human intestinal colon adenocarcinoma Caco-2 cells. Eur. J. Drug Metab. Pharmacokinet. 41(1): 33–43.

Redan, B.W., Chegeni, M., and Ferruzzi, M.G. (2017). Differentiated Caco-2 cell monolayers exhibit adaptation in the transport and metabolism of flavan-3-ols with chronic exposure to both isolated flavan-3-ols and enriched extracts. Food Funct. 8(1): 111–121.

Rodriguez-Mateos, A., Toro-Funes, N., Cifuentes-Gomez, T., Cortese-Krott, M., Heiss, C., and Spencer, J.P.E. (2014). Uptake and metabolism of (–)-epicatechin in endothelial cells. Arch. Biochem. Biophys. 559: 17–23.

Ruiz-Ruiz, J.C., Matus-Basto, A.J., Acereto-Escoffié, P., and Segura-Campos, M.R. (2017). Antioxidant and anti-inflammatory activities of phenolic compounds isolated from Melipona beecheii honey. Food Agric. Immunol. 28(6): 1424–1437.

Sanchez-Bridge, B., Lévèques, A., Li, H., Bertschy, E., Patin, A., and Actis-Goretta, L. (2015). Modulation of (–)-Epicatechin Metabolism by Coadministration with Other Polyphenols in Caco-2 Cell Model. Drug Metab. Dispos. 43(1): 9.

Seraglio, S.K.T., Valese, A.C., Daguer, H., Bergamo, G., Azevedo, M.S., Nehring, P., Gonzaga, L.V., Fett, R., and Costa, A.C.O. (2017). Effect of in vitro gastrointestinal digestion on the bioaccessibility of phenolic compounds, minerals, and antioxidant capacity of Mimosa scabrella Bentham honeydew honeys. Food Res. Int. 99: 670–678.

Shen, S., Wang, J., Chen, X., Liu, T., Zhuo, Q., and Zhang, S.Q. (2019). Evaluation of cellular antioxidant components of honeys using UPLC-MS/MS and HPLC-FLD based on the quantitative composition-activity relationship. Food Chem. 293: 169–177.

Silva, B., Biluca, F.C., Gonzaga, L.V., Fett, R., Dalmarco, E.M., Caon, T., and Costa, A.C.O. (2021). In vitro anti-inflammatory properties of honey flavonoids: A review. Food Res. Int. 141: 110086.

Sultana, S., Foster, K., Lim, L.Y., Hammer, K., and Locher, C. (2022). A Review of the Phytochemistry and Bioactivity of Clover Honeys (Trifolium spp.). Foods 11(13): 1901.

Sun, L.-P., Shi, F.-F., Zhang, W.-W., Zhang, Z.-H., and Wang, K. (2020). Antioxidant and Anti-Inflammatory Activities of Safflower (Carthamus tinctorius L.) Honey Extract. Foods 9(8): 1039.

Tanleque-Alberto, F., Juan-Borrás, M., and Escriche, I. (2020). Antioxidant characteristics of honey from Mozambique based on specific flavonoids and phenolic acid compounds. J. Food Compos. Anal. 86: 103377.

Tel-Çayan, G., Çiftçi, B.H., Taş-Küçükaydın, M., Temel, Y., Çayan, F., Küçükaydın, S., and Duru, M.E. (2023). Citrus Honeys from Three Different Regions of Turkey: HPLC-DAD Profiling and in Vitro Enzyme Inhibition, Antioxidant, Anti-Inflammatory and Antimicrobial Properties with Chemometric Study. Chem. Biodiversity 20(9): e202300990.

Tomás-Barberán, F.A., Martos, I., Ferreres, F., Radovic, B.S., and Anklam, E. (2001). HPLC flavonoid profiles as markers for the botanical origin of European unifloral honeys. J. Sci. Food Agric. 81: 485–496.

Wang, X., Chen, Y., Hu, Y., Zhou, J., Chen, L., and Lu, X. (2022). Systematic Review of the Characteristic Markers in Honey of Various Botanical, Geographic, and Entomological Origins. ACS Food Sci. Technol. 2: 206–220.

Yu, W., Sun, F., Xu, R., Cui, M., Liu, Y., Xie, Q., Guo, L., Kong, C., Li, X., Guo, X., and Luo, L. (2023). Chemical composition and anti-inflammatory activities of Castanopsis honey. Food Funct. 14(1): 250–261.

Zammit Young, G.W., and Blundell, R. (2023). A review on the phytochemical composition and health applications of honey. Heliyon 9(2): e12507.

Zhang, H., Hassan, Y.I., Liu, R., Mats, L., Yang, C., Liu, C., and Tsao, R. (2020). Molecular Mechanisms Underlying the Absorption of Aglycone and Glycosidic Flavonoids in a Caco-2 BBe1 Cell Model. ACS Omega 5(19): 10782–10793.

Zhang, H., Hassan, Y.I., Renaud, J., Liu, R., Yang, C., Sun, Y., and Tsao, R. (2017). Bioaccessibility, bioavailability, and anti-inflammatory effects of anthocyanins from purple root vegetables using mono- and co-culture cell models. Mol. Nutr. Food Res. 61(10): 1600928.

Zhu, Y., Liu, R., Mats, L., Zhu, H., Roasa, J., Khan, T., Ahmed, A., Brummer, Y., Cui, S., Sievenpiper, J., Ramdath, D.D., and Tsao, R. (2024). A comprehensive characterization of phenolics, amino acids and other minor bioactives of selected honeys and identification of botanical origin markers. J. Food Bioact. 25: 25–41.