Comparison of the Antioxidant Activity of Commercial Honeys, Before and After In-Vitro Digestion

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Honey is a rich source of antioxidant and anti-septic compounds including Maillard reaction products, vitamins, carotenoids and polyphenols. The objective of the present study was to determine the effect of digestion on the antioxidant activity of a range of honey samples including two economy brands (Tesco and Lidl), a premium Irish brand (Fainne Or Fine Foods) and a New Zealand Manuka honey. Samples were subjected to an in-vitro digestion which simulates the human gastric and intestinal digestion system. The antioxidant activity of the honey samples before and after digestion was determined by measuring total phenol content (TPC), 2,2-diphenyl-2-picrylhydrazyl hydrate assay (DPPH) radical scavenging and ferric reducing antioxidant potential (FRAP). The ability of the samples to protect against H2O2-induced DNA damage in the Caco-2 cell line was measured by the Comet assay. The Manuka honey had the highest TPC and the Tesco honey had the highest FRAP and DPPH scavenging activity. TPC was not altered following digestion however there was a significant decrease in the FRAP values for Manuka and Tesco honey and in the DPPH radical scavenging activity for all four of the samples. The Tesco and Manuka honeys demonstrated a significant protective effect against H2O2-induced DNA damage in Caco-2 cells, following digestion. The premium brand honey samples did not demonstrate significantly higher antioxidant activity in comparison with economy brand honey.

INTRODUCTION

Honey is a naturally sweet food produced by bees from the nectar of a large variety of plants. Aside from its culinary uses, honey has been employed since 350 BC, as a treatment for ailments such as wound infections, peptic ulcers, gastro-enteritis and eye problems [Molan, 1999]. The health benefits of honey can be attributed to its antimicrobial, antioxidant and anti-inflammatory activities. The antimicrobial activity of honey is due to its high osmolarity, low pH and the presence of glucose oxidase (GOx), methylglyoxal (MGO) and polyphenols [Mundo et al., 2004; Mandal & Mandal, 2011]. Compounds such as polyphenols, ascorbic acid, enzymes (catalase, glucose oxidase), organic acids (malic, citric acid), Maillard reaction products, amino acids, peptides and carotenoids contribute to the antioxidant [Gheldof et al., 2002] and anti-inflammatory [Kassim et al., 2010] effects of honey.

The physico-chemical properties and antioxidant capacity of honey depend largely on the floral source of the nectar as well as seasonal and environmental conditions [Al-Mamary et al., 2002]. Studies have found a direct correlation between honey colour, its phenolic content and antioxidant activity [Alvarez-Suarez et al., 2010; Dezmirean et al., 2012]. Monofloral honeys, such as Manuka, are produced from nectar obtained primarily from one plant source. The majority of honeys sold commercially are a blended product and contain a mix of honeys obtained from different floral sources and places of origin. Economy brand honeys, in particular, often contain a blend of honeys sourced from many different countries.

Studies investigating the stability of antioxidants during digestion have found that digestive enzymes and pH changes can result in the degradation of certain antioxidant compounds. Polyphenols in Concord grape juice were found to be stable during gastric digestion but were significantly reduced following duodenal digestion [Stalmach et al., 2012]. However, the phenolic content of vegetable juices was shown to be increased following digestion due to the release of antioxidant compounds from the food matrix [Wootton-Beard et al., 2011].

Phenolic extracts prepared from Cuban monofloral honey have been shown to protect against AAPH-induced membrane lipid peroxidation and reduction in antioxidant enzyme activity in human erythrocytes [Alvarez-Suarez et al., 2012]. A multifloral honey (1% w/v) also protected against oxidant induced damage in EA.hy926, endothelial cells [Beretta et al., 2007]. Makpol et al. [2012] found that honey derived from nectar of the Gelam tree significantly protected against gamma-radiation induced DNA damage in human diploid fibroblasts and proposed that honey could act as a radioprotectant for patients undergoing radiotherapy treatment.

The aim of the present study was firstly to measure the antioxidant activity of four honeys, a New Zealand Manuka (Comvita UMF ® 5 + Manuka honey), a 100% pure Irish honey and two economy brands (Tesco and Lidl). The antioxidant activity was determined both before and after an in-vitro digestion procedure by the Folin-Ciocalteu assay, the 2,2-di-
To measure DPPH radical scavenging, 100 µL honey was diluted in 900 µL methanol (MeOH) and 500 µL of this mix was added to 3.5 mL of DPPH (0.06 mmol in methanol). Samples were incubated in the dark for 60 min. Samples were centrifuged at 4100 rpm for 10 min, and the absorbance of the supernatant was measured at 515 nm. Data were presented as % radical scavenging relative to blank consisting of 3.5 mL DPPH and 0.5 mL methanol.

Cell culture
Human colon adenocarcinoma Caco-2 cells were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with Foetal Bovine Serum (FBS) (10% v/v) and non-essential amino acids (1% v/v). Cells were incubated in an atmosphere of CO₂-air (5:95) at 37°C and were maintained in the absence of antibiotics. For experiments, Caco-2 cells were plated at a density of 1×10⁴ cells/mL. Honey samples were sterile filtered through a 0.22 µm filter (Millipore, Cork, Ireland) before addition to cells.

Statistical analysis
Results for all measurements (antioxidant activities, cell viabilities and DNA damage) are presented as mean values of three independent experiments± SE. Statistical analysis was by one-way ANOVA or repeated measures ANOVA followed by Dunnett’s test or Tukey’s test (Prism 4.0, GraphPad Inc, CA, USA). The level of statistical significance was determined as P<0.05.

RESULTS AND DISCUSSION
Antioxidant activity
The total phenol content of honey can vary widely depending on floral source and geographical origin. Manuka honey had the highest TPC (Figure 1A) of the four honey samples. Sangsrichan & Wanson [2008] reported TPC values for Thai honeys between 10 and 14.4 GAEq/100 g. The total phenol content of Polish honeys of different floral origin ranged from 17.57–189.52 GAEq/100 g [Wilczyńska, 2010] and the total phenol content of Cuban honeys ranged from 21.39–59.5 GAEq/100 g [Alvarez-Suarez et al., 2010]. Tesco honey had

**MATERIALS AND METHODS**

**Materials**
Honeys were purchased from a local supermarket (Cork, Ireland). A single jar for each honey was used throughout the study. Human colon adenocarcinoma Caco-2 cells were purchased from the European Collection of Animal Cell cultures (Salisbury, UK). Foetal bovine serum (FBS) was purchased from Invitrogen (Paisley, Scotland). Cell culture plastics were purchased from Cruinn Diagnostics (Greiner Bio-One, Frickenhauen, Germany). All other cell culture chemicals and reagents were purchased from Sigma Chemical Co. (Dublin, Ireland).

**In vitro digestion**
The simulated digestion was conducted under amber light to prevent photo-decomposition of the antioxidants present in the honey samples. Honey samples were weighed accurately (0.5 g) and dissolved in 10 mL HBSS. The in-vitro digestion procedure was performed according to the method described in Daly et al. [2010]. The pH of the samples was adjusted to 2 using 1 mol/L HCl, porcine pepsin was added to a final concentration of 0.4 µg/mL sample and the samples were incubated in a shaking water bath (95 rpm; 37°C) for 1 hr. The pH was then increased to 5.3 using 0.9 mol/L NaHCO₃ and the bile salts: glycodeoxycholate, taurodeoxycholate and taurocholate were added to a final concentration of 0.8 mmol/L, 0.45 mmol/L and 0.75 mmol/L, respectively. Pancreatin (0.08 g/mL) was added and the pH was increased to 7.4 using 0.1 mol/L NaOH. Samples were incubated for 2 hr in the shaking water bath (95 rpm; 37°C). Following digestion the samples were centrifuged (53,000 rpm, 95 mins), the aqueous fraction was isolated and filtered (0.22 µm) and the samples were stored at -80°C under nitrogen.

**Antioxidant activity**
The antioxidant activity of the honey samples was determined before and after digestion by TPC, FRAP and DPPH, as previously described in O’Sullivan et al. [2011]. Briefly, for the TPC assay, the honey samples were incubated with Folin-Ciocalteau reagent for 5 mins and the absorbance was measured at 765 nm (WPA Lightwave S2000). Gallic acid was used to prepare a calibration curve and TPC of the honey samples was expressed as mg gallic acid equivalents (GAEq)/g.

The FRAP assay quantified the antioxidant potential of the samples by measuring the reduction of ferric iron (Fe³⁺) to ferrous iron (Fe²⁺). FRAP reagent [2 mL; 0.01 mol TPTZ (2,4,6-tripryridyl-s-triazine) in 0.04 mol HCl, 0.02 mol FeCl₃, 6H₂O and 0.3 mol acetate buffer] was added to 200 µL of each sample diluted in 800 µL distilled H₂O. A calibration curve was constructed using FeSO₄, 7H₂O. Samples were incubated for 30 min and the absorbance was measured at 593 nm (WPA Lightwave S2000). Data were expressed as mmol Fe²⁺/mg honey.
the highest FRAP activity (Figure 1B) followed by Manuka, Lidl and Irish honeys. Similarly, Tesco honey had the highest DPPH radical scavenging activity and there was good correlation ($R^2=0.96$) between DPPH and FRAP data for all honey samples. There was no evident relationship between TPC and the FRAP and DPPH data obtained for the samples. Overall, the antioxidant activity of the premium brand honey samples (Manuka and Irish) was not greater than that of the economy honey samples. The Manuka honey used in the present study was UMF 5+. The UMF (unique Manuka factor) which is measured on a scale of 5+ to 25+, is a measure of a honeys antibacterial strength and also an indirect measure of polyphenol content. Honeys with a therapeutic value are generally between UMF 16–18, therefore it is possible that higher strength Manuka honeys than that assessed in the present study may have a higher antioxidant capacity.

**Antioxidant activity of honey following in-vitro digestion**

Polyphenols and other antioxidants are susceptible to degradation during digestion due to the effects of pH and enzymes and in the present study, the antioxidant activity of the honey samples was determined following an in-vitro digestion. There was no significant change in TPC of honey samples following digestion (Figure 1A) however, DPPH was significantly ($P<0.05$) decreased in all honey samples (Figure 1C) and FRAP was significantly decreased in Tesco and Manuka honey (Figure 1B). The effect of an in-vitro digestion on antioxidant capacity varies depending on a number of factors including the food matrix, a decrease in antioxidant activity has been observed for fruit juices [Cilla et al., 2009] and herbal teas [Gião et al., 2012]. However, Chohan et al. [2012] found a significant increase in the TPC and radical scavenging activity of cooked herbs following digestion. Parker et al. [2010] investigated the effect of an in-vitro digestion on the antioxidant capacity of various combinations of compounds representative of the antioxidants present in honey including a sugar solution, rutin, $p$-coumaric acid, abscisic acid and ascorbic acid and found that ascorbic acid was the greatest contributor to antioxidant activity followed by $p$-coumaric acid and the sugar solution. Overall there was little change in the antioxidant capacity of the various combinations following digestion as measured by oxygen radical absorbance capacity (ORAC).

**DNA protective effects of honey**

The cytotoxicity of each of the honey samples at concentrations ranging from 2.5 to 7.5 mg/mL (undigested samples) and from 1 to 3 mg/mL (digested samples) were assessed in Caco-2 cells using the MTT assay (Figure 2A & 2B, respectively) and a concentration of 2.5 mg/mL was selected...
for the comet assay as the samples did not display any cytotoxic effects at this concentration. The addition of 50 μmol/L H₂O₂ to Caco-2 cells increased DNA damage from a control level of 4.8% tail DNA in untreated cells to 62% tail DNA. Pre-incubation (24 hr) with digested Manuka and digested Tesco honey significantly (P<0.05) reduced DNA damage to approximately 47% and 48.5% tail DNA, respectively (Figure 3). Buckwheat honey and Tualang honey have been shown to reduce hydroxyl radical and UVB induced DNA damage, respectively [Zhou et al., 2012; Ahmad et al., 2012] and Serem & Bester [2012] found that a number of honeys protected against 2,2’-Azobis(2-amidinopropane) dihydrochloride (AAPH)-induced plasmid DNA damage from Caco-2 and SC-1 cell lines. Although the TPC and antioxidant activity of our honey samples were similar before and after digestion, none of the undigested honey samples protected against DNA damage induced by H₂O₂. Tavares et al. [2012] also found that blackberry (Rubus sp.) polyphenols protected neuroblastoma cells against H₂O₂-induced damage after, but not before, in vitro digestion and suggested that the enhanced protection was related to alterations in the polyphenolic composition which occurred during the digestion.

**SUMMARY AND CONCLUSIONS**

The premium brand honeys did not demonstrate superior antioxidant activity in comparison to the less expensive honeys. DPPH radical scavenging activity was reduced following in-vitro digestion of honey but TPC was not significantly altered. Future work will attempt to identify the honey component which is responsible for its antioxidant activity. Manuka and Tesco honey protected against H₂O₂ induced DNA damage in Caco-2 cells after, but not before, in vitro digestion.

**REFERENCES**


