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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 antiseptic 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 Oir 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 H_2O_2-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 H_2O_2-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, gastroenteritis 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-

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phenyl-2-picrylhydrazyl hydrate (DPPH) scavenging assay and the ferric reducing antioxidant potential (FRAP) assay. The ability of the undigested and digested honeys to protect against oxidant induced DNA damage in human adenocarcinoma, Caco-2 cells was determined by the Comet assay.

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, Frickenhausen, 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 $NaHCO_3$ 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 (Fe^{3+}) to ferrous iron (Fe^{2+}). FRAP reagent [2 mL; 0.01 mol TPTZ (2,4,6-tripyridyl-s-triazine) in 0.04 mol HCl, 0.02 mol $FeCl_3.6H_2O$ and 0.3 mol acetate buffer] was added to 200 μL of each sample diluted in 800 μL distilled H_2O. A calibration curve was constructed using $FeSO_4 7H_2O$. Samples were incubated for 30 min and the absorbance was measured at 593 nm (WPA Lightwave S2000). Data were expressed as mmol Fe^{2+}/mg honey.

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 CO_2: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 1×10^5 cells/mL. Honey samples were sterile filtered through a 0.22 μm filter (Millipore, Cork, Ireland) before addition to cells.

Cell viability

Caco-2 cells were supplemented with increasing concentrations (1–7.5 mg/mL) of honey samples for 24 hrs in 96-well plates with a final volume of 100 μL per well. Cell viability was assessed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide) I proliferation kit (Roche Diagnostics, UK) as described in O'Sullivan *et al*. [2011]. Sub-toxic concentrations of samples were determined and a concentration of 2.5 mg/mL honey and honey digestates was selected for the Comet assay.

Determination of DNA damage (Comet assay)

Caco-2 cells were exposed to each of the honey samples for 24 hrs. The cells were subsequently exposed to 50 μmol H_2O_2 for 30 mins at 37°C in FBS-free media. DNA damage was measured using the Comet assay as previously outlined in O'Sullivan *et al*. [2011].

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

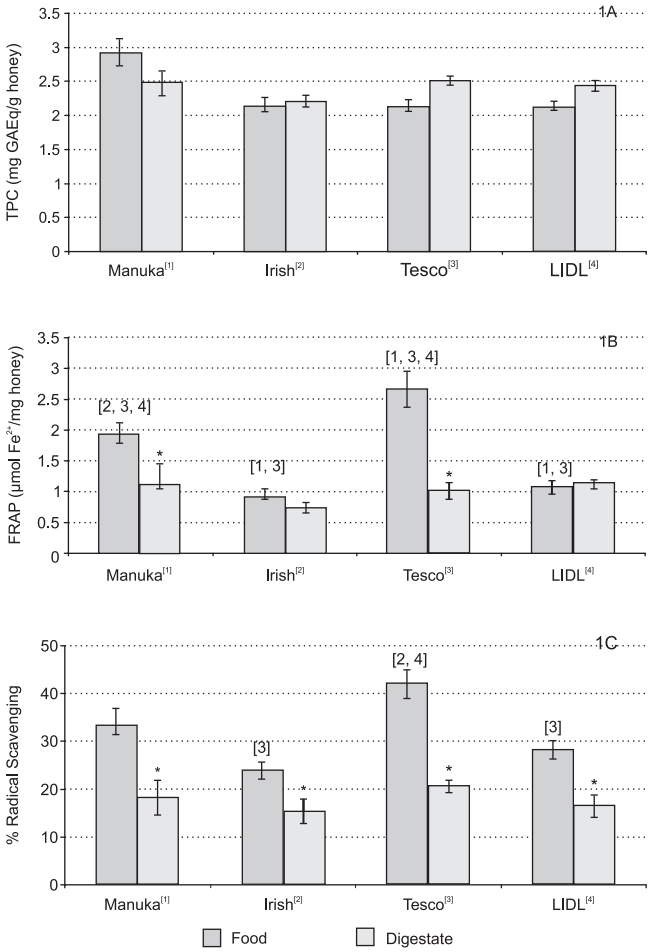

FIGURE 1A, 1B & 1C. Antioxidant activity of honey before and after digestion was determined by measuring total phenol content (TPC) [1A], ferric reducing antioxidant power (FRAP) [1B] and DPPH radical scavenging activity [1C]. Data represent the mean ± SE, * $P<0.05$ indicates significant difference between food and corresponding digestate, ANOVA followed by Dunnetts. Superscript numbers indicate a significant difference between sample and the sample corresponding to the superscript number, $P<0.05$, ANOVA followed by Tukey's test.

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

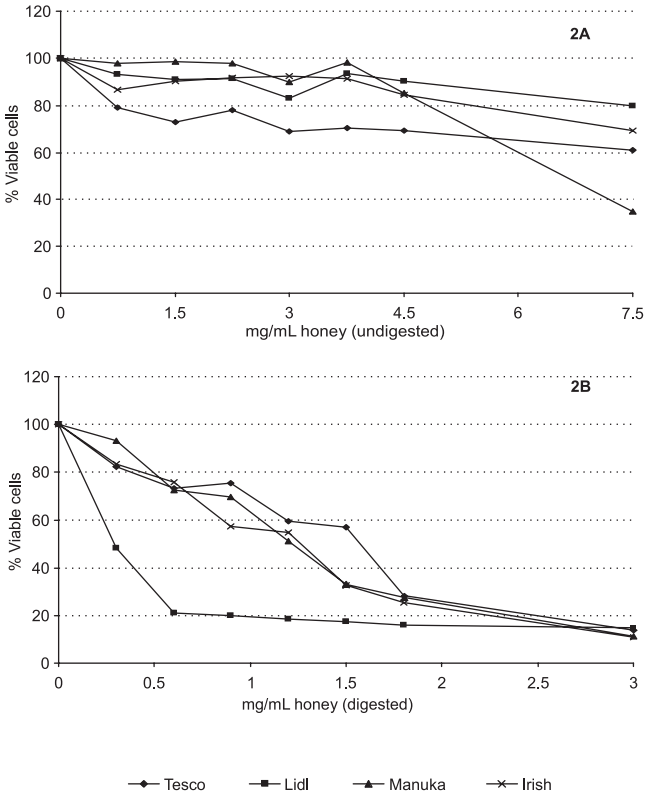

FIGURE 2A & 2B. Viability of Caco-2 cells, as determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide) assay, following a 24 hr incubation with undigested honey (2A) or digested honey (2B). Data are expressed as a percentage of the untreated, control cells and represent the mean of three independent experiments.

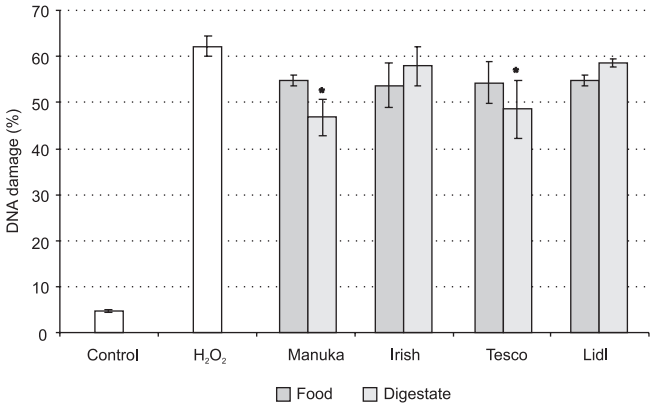

FIGURE 3. DNA damage in Caco-2 cells incubated with honey and digested honey samples (2.5 mg/mL) for 24 hr prior to a 30 min exposure to 50 mmol H_2O_2. DNA damage was determined by the Comet assay and expressed as % Tail DNA. Data represent the mean ± SE of three individual experiments. * $P<0.05$, ANOVA followed by Dunnett's test.

for the comet assay as the samples did not display any cytotoxic effects at this concentration. The addition of 50 μmol/L H_2O_2 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 H_2O_2. Tavares *et al*. [2012] also found that blackberry (*Rubus* sp.) polyphenols protected neuroblastoma cells against H_2O_2-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 H_2O_2 induced DNA damage in Caco-2 cells after, but not before, *in-vitro* digestion.

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