Original Research Article

Total Phenols Content and Antioxidant Power of Manuka Honey Is Related to 24hr Cytotoxicity Towards MCF-7 Breast Cancer Cells

ABSTRACT

Aims: To investigate the relations between total polyphenols content, antioxidant power and Manuka honey cytotoxicity towards MCF-7 cells.
Study design: *In vitro* study.
Place and Duration of Study: Department of Chemistry, University of Crete in partnership with the School of Biomedical Sciences, Ulster University, 09/ 2014 – 09/ 2015.
Methodology: Manuka honey (UMF 5+,10+, 15+ and 18+) were examined for total phenols content using the Folin-Ciocalteu method with results expressed as mg-gallic acid equivalents per kg honey (mg-GAE/kg). Antioxidant power was evaluated using the Ferric Reducing Antioxidant Power "FRAP" method and expressed as mg-GAE/kg. Honey cytotoxicity was examined with MCF-7 breast cancer cells cultured with RPMI 1640 supplemented with charcoals stripped serum and viability was monitored using the MTT assay.
Results: The total phenols content for Manuka honey ranged from 1367±152 mg-GAE/kg for UMF5+ honey to 2358 ±79 mg-GAE/ kg for UMF18+ honey. The antioxidant power for Manuka honey ranged from 170±22 mg-GAE/kg for UMF5+ honey rising to 266±21 mg-GAE/kg for UMF18+ honey. Manuka honey showed dose-dependent cytotoxicity towards MCF-7 cells after 24 hrs treatment. The concentration of honey which produces 50% inhibitory activity (IC_{50}) ranged from 4.7% (w/v) for UMF5+ honey to 2.2% (w/v) for UMF18+ honey. The cytotoxicity of Manuka honey was highly correlated with, values for the total phenols content (R^2=0.99) and antioxidant power (R^2=0.95) of Manuka
Conclusion: Manuka honey is cytotoxic to MCF-7 breast cancer cells *in vitro* and the effects are correlated with the total phenols content and antioxidant power.

Keywords: Manuka honey, MCF-7, antioxidant power, anticancer action, polyphenols

1. INTRODUCTION

There is renewed interest in honey owing to its antioxidant and anti-inflammatory potential, emerging role as functional food [1], possible use against drug resistant bacterial [2], and applications for cancer therapy [3]. Honey polyphenols produce antioxidant and anti-inflammatory action by scavenging reactive nitrogen and oxygen species [4]. Polyphenols also down-regulate cycloxygenase-2 and inducible nitric oxide synthase [5] and may hinder cell mutation by inhibiting cytochrome P450 family and inducing phase II detoxification enzymes [6]. The mechanisms proposed for honey anticancer activity include, induction of cell apoptosis via caspase-8/9 dependent pathways, cell cycle blockage at the G_0/G_1 phase, regulation of Tumor-Necrosis Factor (TNF) family proteins or anti-estrogenic activity [3,4].

Breast cancer is the most important gender-specific cancer in women with 1.7 million cases in 2012 [7]. Current research into the effect of honey on breast cancer cells is limited. Three studies focused on Tualang honey [8, 9, 10], one considered Manuka honey [11] whilst two studies examined the effect of honey extracts [12, 13]. Thyme and pine fir honey extracts showed no inhibition of MCF-7, but instead showed antiestrogen activity [12, 13]. Tualang honey was cytotoxic to the MCF-7 and MDA-MB-31 cells and protective with normal breast epithelial cells. Tualang honey and tamoxifen combinations produced synergistic interactions [8, 9, 10]. There was significant cytotoxicity when MCF-7 cells were exposed to honey with "Unique Manuka Factor" (UMF) rating 10+ but no other UMF ratings were examined [11].

Manuka honey exhibits non-peroxide antibacterial activity attributed to polyphenols and methylglyoxal. Indeed, the levels of polyphenols, methylglyoxal or methyl-syringate [14] are considered quality

markers for Manuka honey, indicative of geographic origin and harvesting season [15]. Polyphenols identified in Manuka honey include phenolic acids, gallic acid [1 & 3] methyl-syringate or leptosperin [14] and phenylacetic acid. The main flavonoids in Manuka were found to be chrysin, galangin, pinocembrin and pinobanskin are from Manuka honey [16]. We reported a strong correlation between total phenols content and ferric reducing antioxidant power (FRAP) and honey UMF rating 5+, 10+, 15+ and 18+ [17]. Manuka honey also inhibited MDA-DB-231 cells (unpublished results). However, the possible association of between total phenols content, antioxidant power and Manuka honey cytotoxicity has not been explored. The aims of this study were, to investigate whether Manuka honey total phenols content or antioxidant power are related to the cytotoxicity expressed towards MCF-7 breast cancer cells.

2. MATERIAL AND METHODS / EXPERIMENTAL DETAILS / METHODOLOGY

2.1. Manuka honey samples and reagents

Manuka honey samples rated "Unique Manuka Factor" (UMF) 5+, 10+, 15+, 18+ were purchased from Comvita Ltd (UK). Thyme honey (30%) was purchased from a Cretan honey producer and was used as control for total phenols assay and antioxidant power assay. The MCF-7 cells were a generous offer of the Cancer Biology Lab, Department of Medicine, University of Crete. RPMI 1640 L-glutamine, sodium bicarbonate, Charcoal stripped-Foetal Bovine Serum (CSFBS), 2,4,6-Tris(2-pyridyl)-s-triazine (≥99.0%) (TPTZ), gallic Acid (97.5-102.5%), sodium carbonate (≥99.5% purity) and Folin-Denis reagent were all purchased from Sigma Aldrich Germany. Other laboratory reagents unless otherwise stated were from Sigma Aldrich (UK), Fisher Scientific UK or GE Healthcare (UK).

2.2. Cell culture conditions

MCF-7 cells were cultured with RPMI640 (+L-glutamine) supplemented with 10% CSFBS and 1% penicillin-streptomycin solution. Confluent cells (70%) were treated with trypsin-EDTA 0.25% solution for detachment.

2.3. Folin-Ciocalteu assay for total phenols (total phenols content)

The total phenols content for honey was determined using the Folin-Ciocalteu method described by Singleton et al. [18] with minor modifications for microplate analysis [19, 20]. Briefly, test samples (50µL) were added to Eppendorf tubes, with 100µL Folin-Denis reagent and 850µL of sodium carbonate (3.5% w/v) solution. The samples were vortexed briefly and incubated for 20min at 37-40℃. The reacted samples (800µL) were transferred to cuvettes and absorbance was read at 760nm using a Shimadzu UV-2700 UV-VIS spectrophotometer. Base-line measurements were carried out using de-ionized water and blank values were deducted from all measurements. Calibrations were produced using gallic acid 3mM (0-1000µM). Manuka samples (1:10 w/v diluted) were analysed as above and values for total phenols content were expressed as mg-GAE /kg of Manuka honey. All analyses were performed in triplicate and repeated on two independent days (n=6) datasets.

2.4. Determination of antioxidant power

Antioxidant power was measured using the ferric reducing antioxidant power (FRAP) assay as described by Benzie and Strain [21] and adapted for microplate analysis [17]. Briefly, 75µL of test sample were added to Eppendorf tubes followed by 1425µL of FRAP solution. The mixture was vortexed briefly and incubated in 37℃ water bath for 30℃min. Samples (200uL) were transferred to 96-wells microplate and absorbance was read at 593nm in the Synergy HT, Bio-TEK microplate reader. Base line calibration was carried out using deionized water. Blank values were deducted from all measurements. The FRAP analysis was calibrated using GA (0-500µM) and Thyme honey from Crete was adopted as a "non-UMF" honey sample. The antioxidant power for samples was expressed as mg-gallic acid equivalent antioxidant power (GAEAC) per kilogram of honey. All analyses were performed in triplicate and repeated on two independent days.

2.6. Cytotoxicity and MTT assay

MCF-7 cells that were cultured in sterile T-75 flasks at 37℃ and 3.5% CO_2 atmosphere until 70% confluence, trypsinized and counted using a Neubauer chamber. Sterile 96-well micro-plates were loaded with 10^4/well and cells were allowed to attach for 24 hrs. Manuka honey samples were diluted with culture media (10%, 8.5%, 5%, 3.33%, 2.5%, 2% and control (0%), filter sterilized (0.2µm) and applied to the plated cells. After 24h honey and media were removed from microplates, cells were washed 2 xs with cold PBS and 20µL of MTT solution/well was added. Three hours after MTT application DMSO 100µL was added to each well to dissolve the blue formazan crystals and optical density (OD) was measured at 570nm two hours later using a Synergy HT, Bio-TEK microplate

reader. OD measurements were corrected for "assay" blanks. Results are presented as mean values of eight samples of two different days/datasets.

2.6. Statistical Analysis

Correlations between Manuka honey components and UMF strength, MCF-7 percentage cell viability and antioxidant power were calculated using MS-office excel 2010 (R^2 value). All measurements were carried out in triplicates except the cell viability assay which were done in eight repeats. Mean values and standard deviations (S.D.) are used in Tables and means and standard error of mean (S.E.M.) in figures. Group means were analysed for statistically significant differences using one-way ANOVA while followed by Tukey's HSD, or Dunnett's-T3 multiple comparisons *post-hoc* tests to locate statistically significant differences between pairs of means. Prior to one-way ANOVA data were tested for normality with the Kolmogorov-Smirnov test and for homogeneity of variances with the Levine's test. Where normality was violated replacement of the extreme values (>2 S.D. from the mean or in one case of an outlier very close to 2 S.D. from the mean (total 8 cases out of 256 in MTT assay) with the mean value was effected. Where variables had unequal variances the Dunnett's-T3 *post-hoc* test was used for the separation of means replacing Tukey's test for homogenous variances. Statistical significance was noted with *p*-value less than .05. All analyses were performed using IBM SPSS Statistics v.22 for Windows, Chicago, IL, USA.

3. RESULTS AND DISCUSSION

3.1. Total phenols and antioxidant power of honey samples

From Table (1), Manuka honey samples showed a total phenols content range of 1367-2357 mg-GAE /kg honey. A one-way ANOVA test showed the total phenols content for all honeys were significantly different (P = 0.05). Thyme honey had a lower mean total phenol content value compared to Manuka honeys. Samples rated UMF5+ had almost double the total phenols content than thyme honey, and UMF18+ had ~3.5 folds higher total phenols content. The total phenols content for honey was strongly correlated with UMF rating (thyme was assigned with 0 value in UMF strength) for honey samples (R^2= 0.9765). Upon exclusion of thyme honey, the correlation between total phenols content and UMF rating increased (R^2=0.9908).

Table 1. Total phenols content and antioxidant power for Manuka honey (GAEAC mg/kg honey) determined by the Folin Ciocalteu and FRAP assays

Honey type	Total Phenols Content Mg-GAE/ kg (n=6)	Antioxidant Power mg-GAE / kg (n=6)
Thyme	692±65	58.8±8
UMF 5+	1367±152	170±22
UMF 10+	1747 ±52	206±25
UMF 15+	2042 ±49	248±8
UMF 18+	2358±79	266±21

**Notes. Values within 2nd and 3rd column are significantly different from each other (P=0.05). Assay precision was 5.9% (Total phenols) and 9.9% (Antioxidant power) respectively*

The antioxidant power of honey samples determined by the FRAP assay is listed in Table (1). A one-way ANOVA test showed that the values for antioxidant power were significantly different for all honeys (P = 0.05). The antioxidant power of Manuka honey UMF5+ was nearly 3-fold higher compared to the value for thyme honey, whilst UMF 18+ Manuka had a 4.4-fold higher antioxidant power compared to thyme honey. There was a positive correlation between antioxidant power and UMF ratings for honey (R^2= 0.9252), which improved when Manuka samples were regarded alone (R^2= 0.9978). Analysis of linear regression showed that the total phenols content and antioxidant power were highly correlated (R^2= 0.977) (*P*=0.001) and when thyme honey was excluded the change of the regression coefficient was minor (R^2= 0.980) (*P*=0.01).

3.2. Cell viability changes due to honey

Preliminary cytotoxicity tests for honey where performed using treatment durations of 24h and 48h. One-way ANOVA showed there existed a statistically significant difference between the honey treatments and media-only cell culture control (F (4,35) =32.809, *P*=.000, eta squared=0.789) and Dunnett's T3 *post-hoc* showed that all cytotoxicity values differed statistically significantly from the control (5+,10+,15+ *P* =.001, 18+ *P* =.003) while between-UMF group comparisons revealed no

significant difference for honey at 8.5% dilution. Using a 48h treatment, one-way ANOVA found mean values of Manuka honeys and control groups differed significantly (F (4,35) =228.831, P=.000, eta squared=0.963). *Post-hoc* analysis revealed that all Manuka sample produced a statistically significantly change in cell viability compared with the media-only control (all *P*-values=.000). There were also statistically significant differences between some comparisons of the means (UMF5+ vs. UMF10+ (*P*-value=.012), UMF10+ vs. UMF18+ (*P*-value=.039)). A time interval of 24h was chosen to further investigate cytotoxicity towards MCF-7 cells using a range 2-10% (w/v) of honey

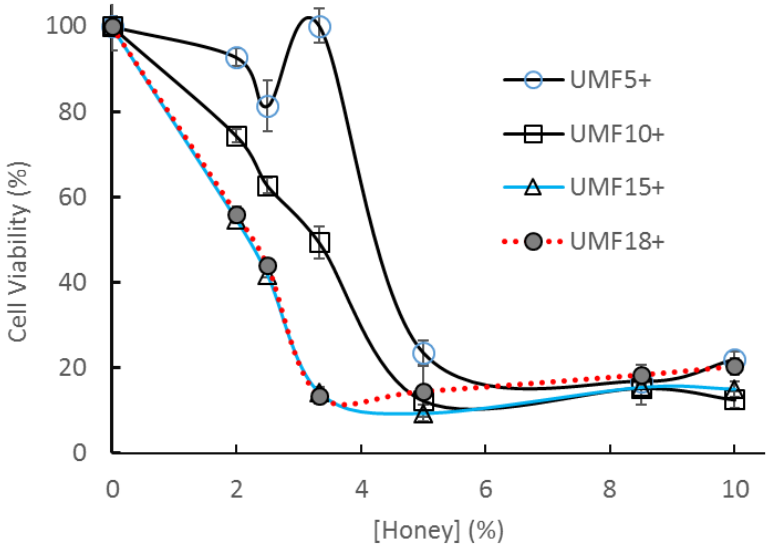

Fig. 1. Effect of Manuka honey treatment on breast cancer MCF-7 cell viability
Cell were culture with RPMI 1640 with 10% Charcoal stripped FBS, 1%penstrep and assay using the MTT assay

Figure 1 shows changes of MCF-7 cell viability following 24h treatment with UMF 5+, UMF10+, UMF15+ and UMF18+ Manuka honey. Generally, MCF-7 viability declined at honey concentration of 2-10%. The half-maximal inhibitory concentrations (IC_{50}), determined using log linear excel dose-response curves, are shown in Fig. 2. For honey rated UMF 15+ and 18+ the IC50 values were virtually identical (2.1-2.2% honey).

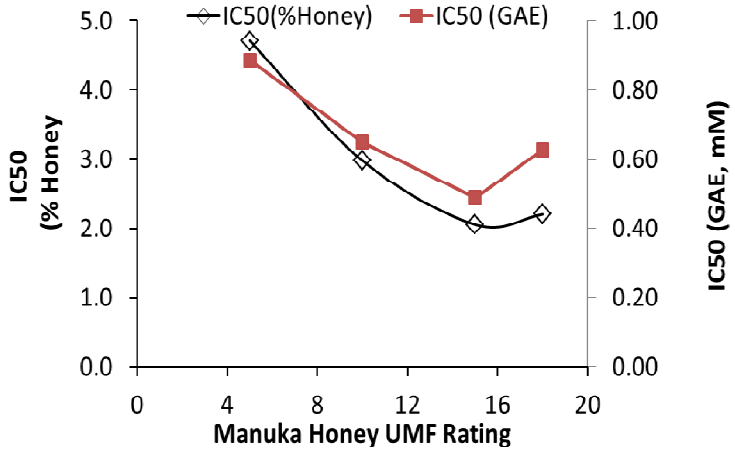

Fig. 2. Effect of Manuka honey UMF rating on the inhibitory concentration (IC50) for breast cancer MCF-7 cells

In Fig. (1) there was a significant difference in all group comparisons and a *post-hoc* analysis showed that all cell viability values decreased in comparison with the control except UMF5+ at 3.33%. For

UMF5+ to UMF15+ (Figure 2) there was a high degree of correlation between the IC50 value and UMF rating for honeys. Increasing UMF rating produced declining values for IC50. There was a correlation between IC50 values for honey and the total phenols content (R^2 = _0.9895) and also between IC50 and the antioxidant (FRAP) measurement (R^2= 0.9525; Fig. 3).

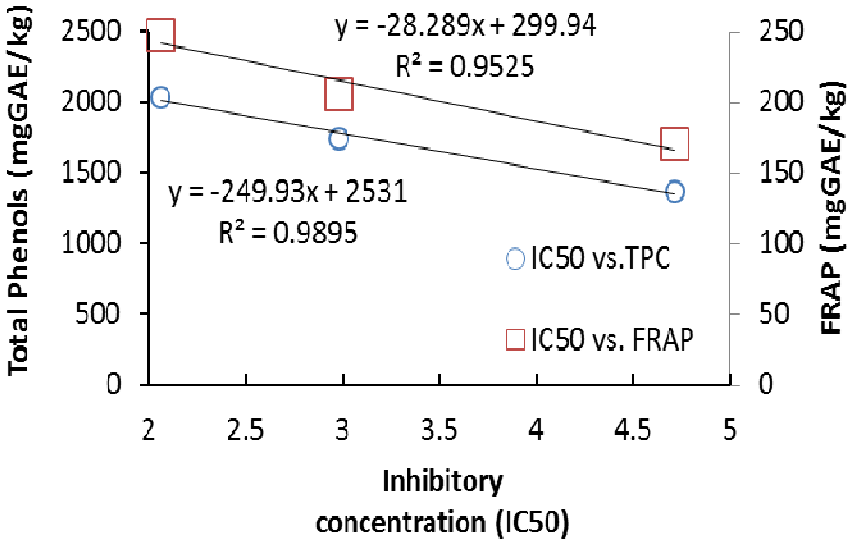

Fig. 3. Relating MC7-7 cell inhibitory concentration (IC50) and honey characteristics.
Total phenols content (TPC) and antioxidant power (FRAP) for Manuka honey samples, UMF5+, UMF10+ and UMF15+

4. DISCUSSION

4.1. Total phenols content

The total phenolic content of Manuka honey is an indicator of its antioxidant power [17]. In this investigation there was an increase in the total phenols content for the Manuka honey series UMF5+ < UMF10+< UMF15 < UMF18+ (Table 1). The total phenols values reported in this article (Table 1) are similar to reports for Manuka honey originating from the Northland region (903-2706mg/kg) of New Zealand [22]. The total phenols content for UMF5+ Manuka honey was 2-fold to 10-fold higher than values reported for other honeys in recent times (Table 2).

The total phenol content for honeys described in the literature were typically 500 mg-GAE/kg or lower [22-37]. A few honeys contain nearly 1000-mg GAE/kg including some from Argentina, Brazil, Italy and Burkina Faso (Table 2). Most honeys were from the honey bee (*Apis mellifera*). Interestingly, Kelulut honey from stingless bees (*Trigona spp*) possessed a higher total phenols content (791-1058 mgGAE/kg) compared to, values (510.4-589.2 mgGAE/kg) for Gelam, Borneo, Tualang and pineapple honey produced by Apis [23]. Overall, it seems that Manuka honey belongs to a rare grouping “super honey” types that contain at least 2000-mg GAE/kg. A few less well-known honeys from Sudan and Ethiopia were reported to have total phenols content similar or higher than Manuka honey but this data needs collaborating from other investigators (Table 2).

Table 2: Total phenols content for select honeys from the literature and this study

Honey	Total phenols mg-GAE/kg)*	Reference
Manuka honey	372-576	[17]
Manuka honey	1367-2358	This study
Manuka honey	903-2706	[22]
Malaysian honey (Kelulut honey)	791-1058	[23]
Turkish pine honey	156	[24]
Sourwood, Longan honeys	564-580	[25]
Cuban honey (v)	214-596	[26]
Saudi Arabia (v)	111-503	[27]
Ethiopian honey (v)	3300-6100	[28]
Sudanese Honeys (v)	794- 2327	[29]
Brazilian honey (v)	685-1085	[30]
Tualang honey, Malaysia	840	[31]
Argentina (v)	400-1930	[32]
Moroccan, citrus, thyme	164-924	[33]
Mexico (v)	510-1340	[34]
Italian (v)	605-2760	[35]
Obudu, Nigeria	1060-1300	[36]
Burkina Faso (v)	356-1148	[37]

**Total phenols content values are rounded up to nearest milligram, (v) several honeys were analysed*

4.2. Antioxidant power

Antioxidant power is one measure of the bioactivity from honey and other food [1, 3, 4]. Honey is derived from nectar and could potentially contain all classes of plant polyphenols, notably the phenolic acids being either hydroxy-benzoic acids (gallic, protocafeteric, syringic, and vanillic acids) or hydroxycinnamic acids (p-coumaric, ferulic, sinapic and caffeic acids). The flavonoids are also represented, notably flavan-3-ols (catechins, gallocatechin, epicatechin) and flavanols (kaemferol, quercetin, myrecetin) [36]. Specific polyphenols identified in Manuka honey include, gallic acid, methyl-syringate or leptosperin [1,3,14]. Flavonoids from Manuka honey (11 mg/kg) were principally pinobanksin, pinocembrin, luteolin and chrysin [16, 39]. Polyphenolic compounds are thought to contribute to the high antioxidant power of honey as measured by the FRAP assay [16].

Manuka honey showed increasing antioxidant power along with UMF rating; the order of decreasing antioxidant power was, UMF5+<UMf10+<UMF15+<UMF18+ (Table 1). The differences in antioxidant power were statistically significant and~ 4xs higher than values reported when the same samples were analysed earlier [17]. Interestingly, there were no differences in the antioxidant power for UMF15+ and UMF18+ Manuka honey samples though values of total phenols content were significantly different. Such results indicate either that polyphenols are not the only compounds contributing to the antioxidant power of Manuka honey samples, or that the FRAP and Folin assays for antioxidant power possess differences in sensitivity. The general correlation between total antioxidant power (FRAP) and total phenols content for honey has been reported previously [26] but other honey constituents (glucose oxidase, catalase, organic acids, amino acids and more) may contribute to the antioxidant power [1,3,4].

4.4. Anticancer activity of Manuka honey with increasing UMF rating

Despite modern scientific breakthroughs and discoveries, cancer mortality rates remain high [11] Chemotherapy, radiotherapy and surgery, all result in undesirable adverse health effects. The interest for alternative treatments has turned the focus to honey's anti-cancer potential. Investigations showed that Tualang honey was cytotoxic towards MCF-7 cells, and protective towards the MCF-10A non-cancerous cell line [8, 9, 10]. In previous studies, the MCF-7 cell lines was considered a good model for early stage hormone-sensitive cancer [8-11].

The results from the current study agree with those reported from a previous investigation which showed that treating MCF-7 cells with UMF10+ Manuka honey produced a dose dependent decline in

cell viability [11] though the IC50 was >5% and 4% for 24hrs or 72 hrs exposure. By comparison, the IC50 for Manuka honey UMF10+ was 3% after 24 hrs in the present study. We found also that IC50 decreased with increasing UMF rating from UMF5+, UMF10+, to UMF15+ (Figure 2). In addition, results in Figure 3 showed that MCF-7 inhibition is strongly correlated with the total phenols content an antioxidant power for Manuka samples.

5. CONCLUSION

Manuka honey rated UMF 5+, 10+, 15+, and 18+ show total phenols content higher than most other honey. There was also a strong correlation between the total phenols content, antioxidant power, and UMF rating for honey samples. The current study demonstrates also that the cytotoxicity of Manuka honey towards breast cancer MCF-7 cells increases with rising UMF5+ to UMF15+ rating. Further research is in progress to understand the effect of Manuka honey on MCF-7 breast cancer cells.

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