

Manuka Honey-Induced Cytotoxicity Against MCF7 Breast Cancer Cells Is Correlated To Total Phenol Content And Antioxidant Power

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 charcoal 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 UMF 5+ honey to 2358 ±79 mg GAE/ kg for UMF 18+ honey. The antioxidant power for Manuka honey ranged from 170±22 mg GAE/kg for UMF 5+ honey rising to 266±21 mg GAE/kg for UMF 18+ honey. Manuka honey showed dose-dependent cytotoxicity towards MCF-7 cells after 24 hrs treatment. The concentration of honey which produces 50% inhibitory activity (IC₅₀) ranged from 4.7% (w/v) for UMF 5+ honey to 2.2% (w/v) for UMF 18+ honey. The cytotoxicity of Manuka honey was highly correlated with, values for the total phenols content (R²=0.99) and antioxidant power (R²=0.95) of Manuka

Conclusion: The total phenols content and antioxidant power of manuka honey was related to the 24hr cytotoxicity towards MCF-7 breast cancer cells.

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 cyclooxygenase-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 G₀/G₁ 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 pinobanksin 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-MB-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

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 ($\geq 99.0\%$) (TPTZ), gallic acid (97.5-102.5%), sodium carbonate ($\geq 99.5\%$ 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 ~~Fer~~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 (50 μL) were added to Eppendorf tubes, with 100 μL Folin-Denis reagent and 850 μL of sodium carbonate (3.5% w/v) solution. The samples were vortexed briefly and incubated for 20min at 37-40°C. The reacted samples (800 μL) were transferred to cuvettes and absorbance was read at 760 nm using a Shimadzu UV-2700 UV-VIS spectrophotometer. Base-line measurements were carried out using deionized water and blank values were deducted from all measurements. Calibrations were produced using gallic acid 3 mM (0-1000 μM). 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, 75 μL of test sample were added to Eppendorf tubes followed by 1425 μL of FRAP solution. The mixture was vortexed briefly and incubated in 37°C water bath for 30 min. Samples (200 μL) were transferred to 96-wells microplate and absorbance was read at 593 nm 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 (0-500 μM) 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.

2.5. Cytotoxicity and MTT Assay

MCF-7 cells that were cultured in sterile T-75 flasks at 37°C and 3.5% CO₂ atmosphere until 70% confluence, trypsinized and counted using a Neubauer chamber. Sterile 96-well micro-plates were loaded with 10⁴/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 (0.2 μ M) and applied to the plated cells. After 24h honey and media were removed from microplates, cells were washed 2-times with cold PBS and 20 μ L of MTT solution/well was added. Three hours after MTT application DMSO 100 μ L was added to each well to dissolve the blue formazan crystals and optical density (OD) was measured at 570 nm two hours later using a Synergy HT, Bio-TEK microplate reader. Optical density (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

3.1. Total phenols-Phenols and antioxidant-Antioxidant power-Power of Honey Samples

According to data 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 UMF 5+ had almost double the total phenols content than thyme honey, and UMF 18+ had approximately 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 (GAEC 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 \pm 65	58.8 \pm 8
UMF 5+	1367 \pm 152	170 \pm 22
UMF 10+	1747 \pm 52	206 \pm 25
UMF 15+	2042 \pm 49	248 \pm 8
UMF 18+	2358 \pm 79	266 \pm 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 UMF 5+ 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 were performed using treatment durations of 24 h and 48 h. One-way ANOVA for 24 hr data showed there were statistically significant differences between the honey treatments and media-only cell culture control ($F(4,35) = 32.809$, $P = .000$, $\eta^2 = 0.789$) 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 48 h treatment, one-way ANOVA found mean values of Manuka honeys and control groups differed significantly ($F(4,35) = 228.831$, $P = .000$, $\eta^2 = 0.963$). *Post-hoc* analysis revealed that all Manuka sample produced a statistically significant 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 (UMF 5+ vs. UMF 10+ (P -value = .012), UMF 10+ vs. UMF 18+ (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

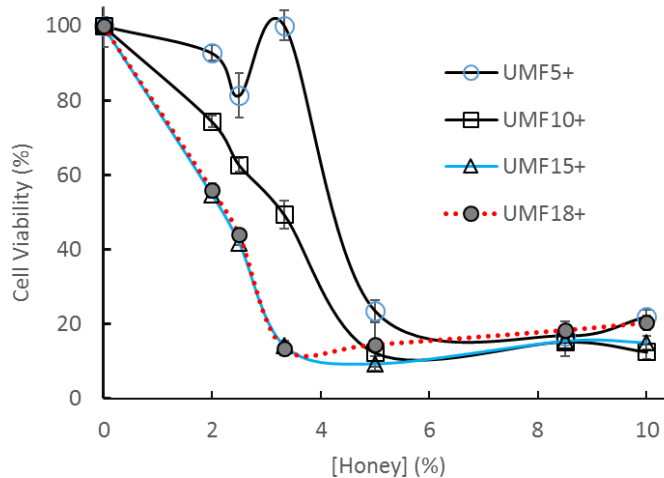


Fig. 1A. Effect of Manuka honey treatment on breast cancer MCF-7 cell viability

Cell were culture with RPMI 1640 with 10% Charcoal stripped FBS, 1% penstrep and assay using the MTT assay. Results are presented as mean values of eight samples of two different days/datasets.

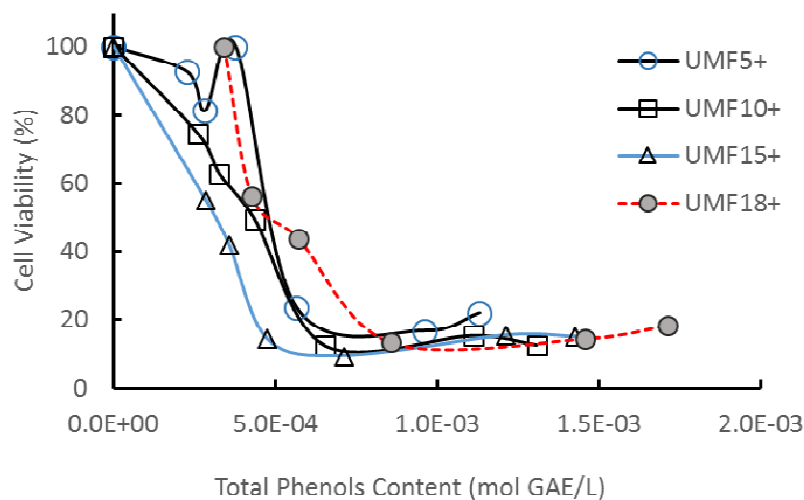


Fig. 1B. Effect of Manuka honey treatment on breast cancer MCF-7 cell viability

Cell were culture with RPMI 1640 with 10% Charcoal stripped FBS, 1% penstrep and assay using the MTT assay. X-axis uses the total phenols content to measure of "active components" for each honey treatment. Results are presented as mean values of eight samples of two different days/datasets.

Figure 1A shows changes of MCF-7 cell viability following 24h treatment with UMF 5+, UMF 10+, UMF 15+ and UMF 18+ Manuka honey. The concentrations of honey in cell culture media was 2-10% w/v as shown in Fig 1 (x-axis). However, each honey has a different total phenols content (Table 1). Fig 1B shows the concentration of "active component" in each treatment, presented as total phenols content. Generally, MCF-7 viability declined at honey concentration of 2-10%. The half-maximal inhibitory concentrations (IC₅₀), determined using log linear 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% w/w honey). The preceding IC₅₀ values were also expressed in terms of the equivalent total phenols content, from which it is evident that UMF 15+ is probably the most potent honey.

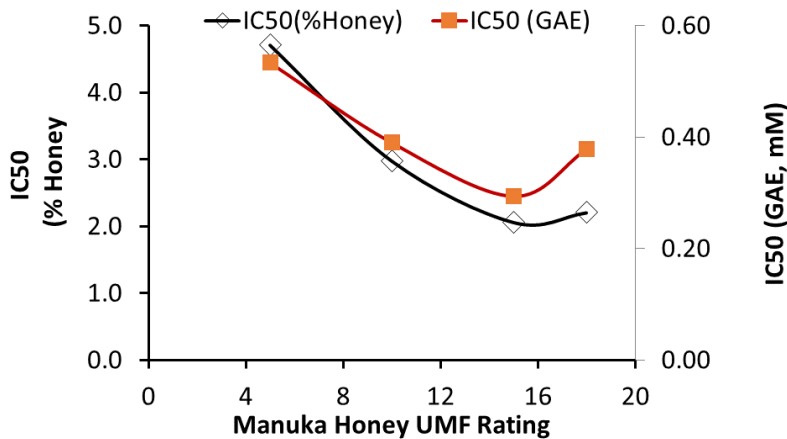


Fig. 2. Effect of Manuka honey UMF rating on the inhibitory concentration (IC₅₀) for breast cancer MCF-7 cells

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 UMF 5+ at 3.33%. The range of honey concentrations were 2-10%w/v. For UMF 5+ to UMF 15+ (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).

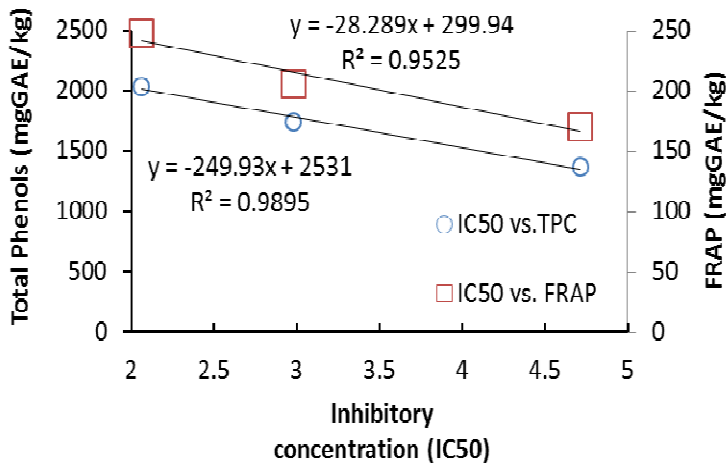


Fig. 3. Relating MCF-7 cell inhibitory concentration (IC₅₀) and honey characteristics. Total phenols content (TPC) and antioxidant power (FRAP) for Manuka honey samples, UMF 5+, UMF 10+ and UMF 15+

4. DISCUSSION

4.1. Total Phenols Content

The total phenolic content of Manuka honey is an indicator of its antioxidant power [17]. Variations in total phenols content for Manuka honey reflect a variety of agronomic and processing factors [21]. In this investigation there was an increase in the total phenols content for the Manuka honey series UMF 5+ <-UMF 10+<-UMF 15 <-UMF 18+ (Table 1). The total phenols values reported in this article (Table 1) are similar to reports for Manuka honey originating from the Northland region (903-2706 mg/kg) of New Zealand [22]. The total phenols content for UMF 5+ 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 500mg GAE/kg or lower [22-38].

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]
Portugal (v)	600-1398	[38]

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

A few honeys contain nearly 1000mg GAE/kg including some from Argentina, Brazil, Italy, Burkina Faso and Portugal (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 mg GAE/kg) compared to, values (510.4-589.2 mg GAE/kg) for Gelam, Borneo, Tualang and pineapple honey produced by *Apis* [23]. Compared to current results some thyme honey samples from Portugal and Morocco had 800-924 mg GAE/kg [33, 38] whilst heather honey had 1150-1398 mg GAE/kg [38]. Overall, it seems that Manuka honey belongs to a rare grouping of “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).

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, protocatechuic, syringic, and vanillic acids) or hydroxycinnamic acids (p-coumaric, ferulic, sinapic and caffeic acids). The flavonoids are also

represented, notably flavan-3-ols (catechins, gallic acid, epicatechin) and flavanols (kaempferol, quercetin, myricetin) [36]. Specific polyphenols identified from Manuka honey are predominantly phenyllactic acid, gallic acid, methyl-syringate or leptosperin [1,3,14,22]. 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, UMF 5+ < UMF 10+ < UMF 15+ < UMF 18+ (Table 1). The differences in antioxidant power were statistically significant and approximately 4-times higher than values reported when the same samples were analysed earlier [17]. Interestingly, there were no differences in the antioxidant power for UMF 15+ and UMF 18+ 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 Activity of Manuka honey Honey with increasing Increasing UMF rating 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 UMF 10+ Manuka honey produced a dose dependent decline in cell viability [11] with the IC50 of >5% w/v and 4% w/v for 24 hrs or 72 hrs exposure, respectively. By comparison, the IC50 for Manuka honey UMF 10+ was 3% w/v after 24 hrs in the present study. We found also that IC50 decreased with increasing UMF rating from UMF 5+, UMF 10+, to UMF 15+ (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. In the previous study [11], Manuka honey 10+ was demonstrated to produce a dose-dependent apoptosis in MCF-7 cells.

5. CONCLUSION

Manuka honeys rated UMF 5+, 10+, 15+, and 18+ exhibit higher apparent total phenols content than most other honey cited in the literature. There is a strong correlation between the total phenols content, antioxidant power, and UMF rating for Manuka honey rated UMF 5+ to UMF 15+. The current study demonstrated for the first time that Manuka honey cytotoxicity towards breast cancer MCF-7 cells increased with rising UMF 5+ to UMF 15+ rating. As a future recommendation further research is needed understand better the effect of Manuka honey on breast cancer cells.

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