Evaluation of Manuka Honey Estrogen Activity Using the MCF-7 Cell Proliferation Assay

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Authors’ contributions

This work was carried out in collaboration between all authors. Authors ROA and PSN designed the study and wrote the protocol. Authors KH and TA performed the experimental work, managed literature searches, performed data and statistical analyses. Author KH wrote the first draft of the manuscript. All authors read and approved the final manuscript.

Article Information

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ABSTRACT

Aims: To assess the estrogenic activity of Manuka honey using the MCF7 cell proliferation assay.

Study Design: In vitro cell based E-screen.

Place and Duration of Study: Ulster University, Coleraine, UK, September 2015 to September 2016.

Methodology: Manuka honey (UMF15+) was characterized for total phenolic content (TPC) using the Folin-Ciocalteu assay and antioxidant power, using the 2, 2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) assay. Estrogenic activity was assessed using MCF-7 cells cultured in DMEM-F2 phenol red-free media supplemented with 10% charcoal stripped FBS and evaluated using Sulforhodamine B (SRB) colorimetric assay. All experiments were conducted in triplicate (n=12-48) and genistein was the positive control. The effect of Manuka honey (UMF15+) treatment on intracellular reactive oxygen species (ROS) was measured using the 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) assay.

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1. INTRODUCTION

Breast cancer is the major cause of death globally amongst women accounting for 25% of all cancer and 10% cancer deaths in women [1]. Diet is estimated to contribute to nearly 35% of all newly diagnosed breast cancers [2]. Alcohol consumption and obesity have been correlated to breast cancer risk, but attention is also focusing on the possible role of environmental estrogens [1]. There is increasing interest in the detection of plant derived substances which may possess estrogenic characteristics [3]. Circulating levels of estrogens and dysregulated estrogen signaling pathways are implicated in the development and progression of some breast cancers and for these, treatment is often aimed at the estrogen receptor signaling pathway. Tamoxifen, one of the most common chemotherapeutic drugs in the treatment of estrogen receptor positive breast cancer since the 1980s, blocks the estrogenic effects on breast cancer cells [4]. Some breast cancer patients may show resistance to tamoxifen treatment however, increased dosage can lead to increasing risks of side-effects on normal tissues [5]. A diminished effect of chemotherapeutic agents has invited research into the role of alternative treatments and adjuvants in breast cancer treatment.

Honey has medicinal uses due to its antibacterial properties, but is now being considered for its antioxidant and anticancer properties. Honeys from different floral sources are composed of a complex mixture of sugars, protein, minerals, vitamins, flavonoids, organic acids, phenolic compounds and enzymes; the phytochemicals from honey are considered as active bio-compounds [6-8]. Phenolic compounds are amongst the main constituents contributing to antioxidant, anticancer, anti-inflammatory and other beneficial properties of honey [6-8]. Manuka honey has been shown to be high in phenolic content [9] with anti-carcinogenic effects [10]. We demonstrated recently, that Manuka honey inhibition of breast cancer cells was related to antioxidant capacity [11].

Phytoestrogens are plant-derived compounds that mimic the female sex hormone 17β-estradiol; flavonoids were demonstrated to possess estrogenic activity due to the phenolic (ring) structure which is similar to the structure of endogenous hormones [12-17]. Other phytoestrogens, notably genistein in soy, exhibited stimulatory activity towards hormone sensitive breast cancer cell line MCF-7 [14,18-21]. There are currently several In-vitro assays for environmental estrogens, but the E-SCREEN assay which uses MCF-7 (or the MCF-7 Bus) cell strains is considered one of the most reliable, easy, and rapid to perform [22,23]. The MCF-7 proliferation assay requires a medium free of phenol red as this functions as weak estrogen [24].

Greek thyme honey [25] and Tualang honey [26] were reported to show estrogenic activity. With the exception of these two publications honey estrogenic activity towards MCF-7 cells have not been extensively studied. To our knowledge little or no research has appeared on the Manuka honey estrogenic activity. The overall aims of this study are, to address the perceived gap in research regarding estrogenic activity in Manuka honey. The specific aims of this research were, to determine the total phenol content and antioxidant effects of medicinal grade Manuka honey, to evaluate the estrogenic and cancer inhibitory characteristics of Manuka honey (UMF15+) using MCF-7 breast cancer cells, and to examine the effect of honey treatment on
MCF-7 intracellular oxidative stress. The hypothesis tested was that Manuka honey possesses estrogenic activity which can promote MCF-7 cell growth under some circumstances.

2. MATERIALS AND METHODS

2.1 Materials

Manuka honey (UMF15+) was purchased from Comvita Ltd (UK). MCF-7 cells were from American Type Culture Collection (ATCC). Cells were grown in DMEM supplemented with 10% FBS, 1% Penicillin, streptomycin mixture and 1% non-essential amino acids. For estrogenic assays, cells were washed with PBS and transferred to DMEM-F2 media lacking phenol red (Invitrogen Ltd UK) and supplemented with charcoal stripped FBS (Sigma Aldrich). 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 Antioxidant Assays

2.2.1 Instrumentation

Colorimetric measurements were recorded using a UV/Visible spectrophotometer (Ultrospec 2000, Pharmacia Biotech, Uppsala Sweden) in conjunction with 1-cm polystyrene cuvettes (Sarsted Ltd., Leicester, UK). All microplate assays involved a 96-microplate reader (VERS Amax; Molecular devices, Sunnydale, California, USA) with flat-bottomed 96-well microplates (NUNC, Sigma Aldrich, UK). Florence measurements for ROS assay were recorded using a fluorimeter (FLUOstar Omega, BMG Labtech, Germany).

2.2.2 Sample extractions and reference antioxidant preparation

Manuka honey (UMF15+) (1g) was diluted in 9 ml of distilled water and the mixture was analyzed for total antioxidant capacity (TAC) and total phenolic content (TPC) as described below.

2.2.3 The 2, 2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid(ABTS) radical cation de- colorization assay

The ABTS assay was modified from [27]. Briefly, 27.4 mg of ABTS and 20 mg of sodium persulfate were dissolved with 90 ml and 10 ml of phosphate-buffer saline (PBS), respectively. The ABTS working solution was prepared in 100 ml volumetric flask by mixing ABTS and sodium persulfate stock solutions and stored in the dark overnight at room temperature. Prior to use, ABTS working solution was diluted with PBS until an initial absorbance value of 0.85 using a 1cm spectrophotometer at 734 nm was obtained. Manuka honey test compound was prepared at 10% or reference compounds (gallic acid, trolox, vitamin C) were serially diluted and 20 μl were added to separate micro tubes and 1.48 ml of ABTS solution was added. The mixtures were incubated for 30 min at 37°C. Thereafter 200 μl of solutions were distributed to a 96-well plate and absorbance measurements were recorded at 734 nm using a microplate reader.

2.2.4 Total phenolic content- folin assay

2.2.4.1 Analysis of honey total phenols

Total phenolic contents (TPC) were determined using the Folin-Ciocalteu method modified from [28]. Gallic acid (GA) standard samples (0-1000 μl) were added to microcentrifuge tubes in volumes 1000 μl, 500 μl, 250 μl, 125 μl, 62.5 μl and 0.0 μl and topped up to a final volume (1000 μl) with water. For test analysis of honey, 1g honey was diluted with 9 ml distilled water. Thereafter, 50 μl of honey test sample was then transferred to 6 new microcentrifuge tubes followed by 100 μl of Folin-Denis regent and 850 μl sodium carbonate. The samples were then vortexed briefly, incubated at 37°C for 60 min. and centrifuged at 11,000 rpm for 5 min. The clear supernatant (200 μl) was transferred to a 96-microplate and absorbance measured at 760 nm using a microplate reader.

2.3 Cytotoxicity Assay

2.3.1 Cell culture

MCF-7 cells (American Type Cell Culture) were grown in DMEM supplemented with 10% FBS, 1% penicillin, streptomycin mixture and 1% non-essential amino acids. Culture flasks and 96-microwell plates were incubated in a humidified incubator at 37°C in O2 95% 5% CO2. (LEEC research incubator, LEEC, UK). Cells were trypsinized, counted using a NucleoCounter (NC-3000, Chemo Metec, Denmark) and seeded (10,000 cells/well) in 96-microwell plates with 50 μl of phenyl red free culture medium overnight to allow cell attachment. Cell growth was monitored.
using the Sulforhodamine B assay for cell cytotoxicity (see below).

2.3.2 Tests for estrogenic activity

Manuka honey was prepared as above (section 2.2.2) and filter sterilized with 0.20-μm cellulose acetate filters. The sterilized solution was then diluted with phenyl red-free culture medium 10-fold to create the following final concentrations (5, 0.5, 0.05, 0.005, 0.0005, 0.00005% and 0 (control)). Cells were treated with the different concentrations and incubated at 37°C for 72 hrs (3-days) and 144 hrs (6-days). Genistein trials were conducted as positive control for estrogenic activity with final concentrations (0, 0.005, 0.05, 0.5, 5, and 50 μM) and incubated at 37°C for 3 days. From the preceding data, we estimated the estrogenic potency of honey in terms described by Sato et al [21]: relative proliferative potency (RPP) was (100 *Cv/C0) where Cv and C0 refer to the lowest concentrations of honey and estradiol that produce maximum of cell proliferation. The relative proliferative effect (RPE) of honey was determined from, RPP= 100 * (1-Fv) / (1-FE) where Fv or FE are the observed maximum fold-increase of cell proliferation after treatment with honey or estradiol compared with a non-treatment control [21].

2.3.3 In-vitro cytotoxicity tests

Tests were performed as described in section 2.3.2 with exception that honey concentrations were 0, 0.625, 1.25, 2, 2.5, 3.33, 5, 8.5, and 10% and incubated at 37°C for 24 hrs and 72 hrs.

2.3.4 Sulforhodamine B (SRB) assay for cell numbers

The sulforhodamine B (SRB) assay is a colorimetric assay for the quantification of the total protein of cells [29]. Cells were treated as mentioned above (section 2.3.1). Cells were fixed with 100 μl of cold trichloroacetic acid (TCA 10% v/v), incubated at 5°C for 60 minutes and washed with tap water four times. After drying, 100 μl of SRB dye was added and incubated for 30 minutes at room temperature. The microplate was then washed four times with 100 μl of acetic acid (1% v/v). After drying, 200 μl of Trizma base solution (10 mM) was added to each microplate well to solubilize SRB dye, and the plate was shaken using an Orbital Shaker for 5 min (Speed: 180 revs/min). Absorbance was measured at 564 nm using a microplate reader.

2.3.5 ROS assay for intracellular antioxidant capacity

The ROS assay is a dichlorofluorescein assay for the measuring of reactive oxygen species [30,31]. Cells were treated as mentioned above (section 2.3.1). Cells were washed with 200 μl cold Hanks balanced salt solution (HBSS) and then removed leaving cells suspended in 50 μl HBSS. Cells were then treated with 50 μl well filter-sterilised DCFH-DA working solution (49 μl DCFH-DA stock solution + 20 μl HBSS) and incubated for 45 min. (in 5% CO2, 37°C). Cells were then washed with 200 μl culture media and treated with honey concentrations (10%, 1%, 0.1%, 0.01%, 0.001% and 0 (control, media only). The plate was then incubated at 37°C for 60 min. and read on a fluorimeter at fluorescence excitation λ 485 nm and emission λ 520 nm.

2.4 Statistical Analysis

All experiments were conducted in three trials with 12-48 replicates per drug concentration. Routine data analysis was conducted using Microsoft Excel. Mean values and standard error of mean (S.E.M.) are used in figures. Group means were analyzed for statistically significant differences using one-way ANOVA followed by Tukey's or Dunnett's T3 multiple comparisons post-hoc test to locate statistically significant differences between pairs of means. Where variables had unequal variances the Dunnett's T3 post-hoc test was used for the separation of means replacing Tukey's test for non-homogenous variances. Statistical significance was noted with P-values less than 0.05. All analyses were performed using IBM SPSS Statistics v.22 for Windows, Chicago, IL, USA.

3. RESULTS

3.1 Total Phenols and Antioxidant Power of Honey

Fig. 1 shows three ABTS calibrations for antioxidant standard references (gallic acid, trolox, and vitamin C) used in this study. All assays had linear responses with coefficients of regression (R2) > 0.98. The data were fitting a straight-line equation (Y = x. GRAD) where, Y= absorbance and x = concentration of antioxidant, and GRAD = slope of the line.
3.2 Genistein Estrogenic Activity

Fig. 3 shows genistein positive control for estrogen testing using MCF-7 cells for 3-days (72 hrs). The concentrations of genistein in the cell culture media were 0-50 \times 10^{-6} M. The tests showed that MCF-7 cell proliferation increased by a maximum of 238% at concentrations between 1 \times 10^{-8} M, and 5 \times 10^{-7} M compared with cells treated with media only. Therefore, values for RPP and RPE for genistein were estimated as 0.1% and 90% in good agreement with values reported previously [21].

3.3 Genistein Estrogenic Activity

Fig. 2 shows the results for Manuka honey (UMF15+) estrogenic activity testing, monitored from changes in MCF-7 cell proliferation at 3-days (Fig. 2a) or 6-days (Fig. 2b). The concentrations of honey in the cell culture media were 0-0.5% w/v Fig. 2A (x-axis). MCF-7 cell viability significantly increased at a honey concentration 0.000005%. Fig. 2B shows changes in MCF-7 cell viability following 6-day treatment increased significantly at honey concentrations of 0.000005, 0.00005, 0.0005 and 0.005% (corresponding to a TPC of 2.75 \times 10^{-10} M, 2.75 \times 10^{-9} M, 2.75 \times 10^{-8} M, 2.75 \times 10^{-7} M GAE, respectively. Taking the minimum dose of honey that produces maximum cell stimulation as 0.0005% w/v (a conservative estimate) the total phenols content was 2.75 \times 10^{-8} M (see above), and RPP was calculated as ~18%, compared to a minimal dose 5\times 10^{-10} M for estradiol (E2) [21]. The maximum fold-increase in MCF-7 cells numbers was 1.45-1.55 after treatment with honey (Fig. 2) compared to non-treated controls and consequently, RPE was 22.5-27.5% for honey, using a literature maximum-fold-increase of 3.0 for beta-estradiol [22,23].
Table 1. Total phenols content and antioxidant power for Manuka honey (UMF +15)

<table>
<thead>
<tr>
<th>Assay/ reference</th>
<th>Gallic acid</th>
<th>Trolox</th>
<th>Vitamin C</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPC (mg/100 g)</td>
<td>96.7 ± 3.2</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>TPC (mol/L)*</td>
<td>7.95 x 10^{-3}</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>AOP (mg/100 g)</td>
<td>2045.54</td>
<td>12118.59</td>
<td>10121.78</td>
</tr>
<tr>
<td>AOP (mol/L)*</td>
<td>168 x 10^{-3}</td>
<td>679 x 10^{-3}</td>
<td>804.0 x 10^{-3}</td>
</tr>
</tbody>
</table>

*Total Phenols Content (TPC) by Folin-Ciocalteu assay; Antioxidant Power (AOP) by ABTS assay. (mol/L). ND= value not determined. * =values as equivalent concentrations (mol/l of honey) calculated with FW = 170.2 g/ mol (gallic acid), 250.16 g/mol (trolox) or 176.12 g/ mol (vitamin C), e.g. TPC (mol/l) = TP (mg/100g honey) x 10 x density of honey (1.4 kg/L)/ FW

Fig. 3. Genistein estrogenic activity testing using MCF-7 cell viability as index
Values are means and their standard errors (n=12). Mean values with unlike letters were significantly different (p<0.05)

3.4 Cell Cytotoxicity Changes Due to Honey

Cytotoxicity tests for Manuka honey (UMF15+) were performed using treatment durations of 1-day (Fig. 4, right panel) or 3-days (Fig. 4 right panel) to match conditions used previously in our laboratory [11]. The concentrations of honey in the cell culture media were 0-10% w/v or 0-8% honey (Fig. 4; x- axis). With 24 hr treatment (Fig. 4 left panel) MCF-7 cell viability significantly declined at honey concentration 3.33% (184 x 10^{-6} M GAE). Fig. 4 (Right panel) shows changes in MCF-7 cell viability following 3-day treatment with Manuka honey (UMF15+). MCF-7 cell viability significantly declined at honey concentration > 2.5% (~140 x10^{-6} M GAE).

Fig. 4. Effect Manuka honey (UMF15+) on MCF-7 cell viability
24 hrs (Left panel) and 72hrs (Right panel) exposure by sulforhodamine B (SRB) assay.
Values are means and standard errors (n=18). Mean values with unlike letters were significantly different (P < 0.05)
3.5 Intracellular Reactive Oxygen Species Assay

The intracellular ROS for cells treated with different honey concentrations were measured using the cell-permeable and ROS sensitive dye, 2’,7’-dichlorofluorescin diacetate (DCFH-DA). DCFH-DA, originally non-fluorescent compound, is de-acetylated by intracellular esterase, and then oxidized to highly fluorescent 2’,7’-dichlorofluorescein (DCF) by intracellular ROS [30,31]. There was a significant increase in fluorescence intensity at all honey concentrations compared to non-treated cells control (Fig. 5).

4. DISCUSSION

4.1 Total Phenols Content and Antioxidant Power

Several assays have been proposed for the measurement of the antioxidant capacity of honey however the general recommendation is to use more than one, therefore ABTS (gallic acid, trolox and vitamin C) and Folin-Ciocalteu were chosen for this study [32,33,34]. Prior et al [35] noted variations in antioxidant assay methods and suggested a requirement for more than one method for analysis.

The total phenolic content (TPC) is considered an indicator of antioxidant capacity [32,33]. In this study, Manuka honey (UMF15+) exhibited a TPC of 96.7±3.2 mgGAE/100 g which is comparable to values reported from other Manuka honeys (see Table 2). Portokalakis et al. found the same 15+ Manuka honey TPC to be 204.2 mg GAE/100 g [11]. The lower TPC value for honey from this study was suggested to be due to the older age of the product. A high correlation between TPC and antioxidant capacity has been reported in studies of other honeys [32] and also confirmed by Portokalakis et al. [11]. However, it was found that the antioxidant capacity UMF 15+ and 18+ Manuka honey were not significantly different, though the TPC was significantly greater in UMF18+ Manuka honey [11]. From such observations, it was inferred that polyphenols are not the only bioactive compounds influencing antioxidant capacity in Manuka honey as measured by chemical methods. Components including, glucose oxidase, catalase, organic acids, and amino acids may contribute to antioxidant power [7,33].

4.2 Estrogenic Characteristics of Manuka Honey Compared to Genistein

Estrogenic effects of have been noted for a diverse range environmental and dietary compounds [22,23]. Their widely different chemical structures make it difficult to measure estrogenic compounds using simple chemical...
analysis. Animal testing is also undesirable and expensive. The E-SCREEN assay used in this study measures MCF-7 cell growth and is recognized as valid for large-scale screening, reliable, easy, and quick to perform [22,23,37].

The identity of estrogenic compounds in Manuka honey was not investigated in this initial study. However, candidate estrogenic components from honey are likely to be flavonoids. Past research showed that flavonoids mediate estrogenic activity [12-17]. Manuka honey was reported to have a total flavonoid content of 27.7-33.4 mg/Kg consisting mainly of quercetin (13%), isorhamnetin (12.9%), chrysin (12.6%) and luteolin (12.6%); see [38]. The total flavonoid content for UMF5+ Manuka honey was 34.5 mg catechin equivalents/ kg corresponding to ~1.6 x 10⁻⁴M [39]. After 10⁻⁴-fold dilutions employed in this study, then flavonoid concentrations would reach approximately 1x 10⁻⁶M which agrees with the concentrations of flavonoids shown to produce estrogenic responses [12-17].

Genistein utilized as positive control in the current study is well documented for its estrogenic activity [14,18-21,40]. Lippman et al. [41] were first to observe a biphasic effect of estrogen on MCF-7 growth with stimulation maximum at 10⁻⁸M concentration and growth inhibition at 10⁻⁶ M or higher concentrations. Quercetin and genistein also displayed a biphasic effect on MCF-7 cells as potent estrogen agonists at low concentrations (1x10⁻⁸ M) and with growth inhibition at high (2x10⁻⁶ M) concentrations [13,16,42]. The genistein results from this study show this biphasic effect with MCF-7 cell stimulation at concentrations 5x10⁻⁸M, 5x10⁻⁶ M, 5x10⁻⁵ M and cytotoxicity at higher concentrations 5-50x10⁻⁶ M. The stimulation and inhibitory effects seen with MCF7 cells may involve different pathways [14,18-21,40].

A comparison of the estrogenic parameters for honey (RPP= 18% and RPE= 22.5-27.5%) and genistein (RPP= 0.1%, RPE= 90%) is informative. First, Manuka honey produced MCF-7 growth stimulation at a relative dose of 0.18 compared to 0.01 for genistein (on a scale with estradiol= 1.0). The estimates for genistein agree with previous reports which indicate that this agent binds to the estrogen receptor with an affinity 20-100x lower than 17β-estradiol [14,18-21,40]. The values for RPE suggest that honey produced a maximum cell stimulation amounting to 22.5-27.5% of the value observed with estradiol compared to genistein RPE of 70-90% as compared estradiol. The differences in RPP relates to dose/ affinity of different agents for estrogen receptors. However, values of RPE relate MCF-7 cell maximal growth stimulation (regardless of the maximum dose applied) and is likely affected by the proportion of receptor sites occupied when cells are exposed to excess agent [22,42]. The reason for the comparatively low RPE value for honey is not certain at present.

4.3 Cell Viability

Cancer mortality rates remain high despite modern scientific breakthroughs and discoveries. Interest in alternative treatments for breast cancer have increased recently, owing undesirable side effects from chemotherapy, radiotherapy and surgery, as well as decreased sensitivity to commonly used chemotherapeutic drugs such as tamoxifen [10]. The cytotoxic effects observed for Manuka honey, are in good agreement with reports by Fernandez-Cabezudo et al. and Portokalakis et al. showed that treating MCF-7 cells with Manuka honey (UMF5+ - UMF18+) produced dose-dependent declines in cell viability [10,11].

4.4 ROS Assay

Reactive oxygen species (ROS) can disrupt the balance of the intra and extracellular environment of a cell, resulting in carcinogenic effects that can lead to cancer. The free radical scavenging activity of honey has been linked the phenols in Manuka honey to antioxidant activity expressed by scavenging ROS and superoxide anions [32]. In this study, we found that treatment of MCF-7 cells with honey produced significant increases in ROS. Thus, it was hypothesized, that total intracellular ROS rise for MCF-7 cells is related to TPC (Fig. 5).

5. CONCLUSION

The TPC of honey (Folin assay results) was related to antioxidant capacity, determined by the ABTS method, which is in agreement with other studies. Manuka honey showed estrogenic activity monitored using MCF-7 breast cancer cell growth promotion assay. The bi-phasic MCF-7 cell response was seen, and high concentrations of Manuka honey inhibited cell growth, consistent with previous research of estrogens and phytoestrogens [13,16,41,43]. Changes in intracellular ROS increased in a concentration-dependent manner, suggesting ROS formation is related to TPC for honeys. This is surprising in view of past studies linking honeys phenols with ROS scavenging [44].
The main limitations of this study is due to its In vitro design which means the results should not be extrapolated to human or animal models. However, the value of MCF-7 In vitro screening for dietary and environmental estrogens is well recognized. Interestingly, the concentrations of honey (and associated nominal TPC or flavonoid) concentration demonstrated to stimulate MCF-7 growth are within achievable physiological concentrations observed in humans [45]. By contrast, the concentrations required to exhibit cytotoxicity towards MCF-7 cells have yet to be achieved in human models. Further research is needed to understand better the effect of Manuka honey on breast cancer cells.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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