In Vitro Analysis for the use of Honey Bee Products In the Treatment of Metastatic Prostate Cancer.

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To Mum, I would never have achieved any of this without your nurturing enthusiasm towards my fascination of science from a young age.

Abstract

The prognosis for men diagnosed with advanced metastatic prostate cancer is poor. A low five-year survival rate of 28% highlights the ongoing need for improved treatment options, with attention to a reduction in prostate cancer metastasis.

Honey bee (*Apis mellifera*) products have been recognised for their medicinal properties for thousands of years, with both honey and bee venom having been used to treat wounds and rheumatoid arthritis. However, only within the last 30 years have honey bee products been investigated for the treatment of cancer. Of the over 200 constituents in honey, phenolic compounds, which include flavonoids and phenolic acids, have been suggested to be responsible for most of the beneficial properties. In addition, bee venom and melittin have been shown to possess anti-cancer effects towards many cell types including prostate. The cytotoxicity of both honey and bee venom have been reported previously in many cancer cell lines, including prostate cancer; however, their anti-metastatic effects are not well documented.

The present study was carried out to examine the *in vitro* anti-metastatic activity of three New Zealand honeys, honey-derived phenolic compounds, and bee venom against the PC3 prostate cancer cell line. Cytotoxicity was assessed using both colourimetric and dye exclusion assays to determine maximal non-lethal concentrations. Quantification of phenolic compounds in the three New Zealand honeys was made using HPLC. Finally, anti-metastatic effects were assessed in PC3 cells using multiple techniques including adhesion assays, scratch wound-healing assays, and Boyden chamber migration and invasion assays.

Honey, quercetin, and the bee venom extract melittin, caused a reduction in cell adhesion, while other phenolic compounds, over a wide range of concentrations, had no effect. Thyme and honeydew honey inhibited migration over 48 h, although manuka did not. However, all honeys inhibited invasion between 50 - 75% over 72 h. Of the five honey-derived phenolic compounds selected, only caffeic acid did not affect cell migration, and only kaempferol did not inhibit invasion. Finally, melittin inhibited both migration and invasion by 60% and 50%, respectively.

These results showed that honey could reduce the migration and invasion of prostate cancer cells and that the anti-metastatic properties of honey may be attributed to the presence of phenolic compounds. Further, the bee venom extract melittin, could also reduce the migration and invasion of prostate cancer cells. Honey and melittin may be beneficial for the prevention

of prostate cancer metastasis, and therefore co-administration with chemotherapeutic agents should be investigated. However, due to the variability of phenolic compounds in honeys of different floral origins, standardisation or supplementation of honey may be recommended.

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List of Abbreviations

ANOVA	Analysis of Variance
BSA	Bovine Serum Albumin
CAF	Cancer-associated Fibroblast
CAPE	Caffeic Acid Phenethyl Ester
CE	(+) Catechin Equivalent (Total Flavonoids)
Col I	Collagen Type I
DAPI	4',6-diamidino-2-phenylindole
DMSO	Dimethyl Sulfoxide
DN	Goethe Diastase Number
DU145	Androgen Insensitive, Moderate Tumourigenic Prostate Cancer Cell Line
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
Em.	Emission Wavelength
EMT	Epithelial-mesenchymal Transition
Ex.	Excitation Wavelength
F-actin	Filamentous Actin
FBS	Fetal Bovine Serum
Fn	Fibronectin
GAE	Gallic Acid Equivalent (Total Phenolics)
GLUT2	Glucose Transporter 2
GTP	Guanosine Triphosphate
h	Hour
HCl	Hydrochloric Acid
HMF	Hydroxymethylfurfural
HO	Hoechst 33342
HUVEC	Human Umbilical Vein Endothelial Cells
LDH	Lactate Dehydrogenase
LLC	Lewis Lung Carcinoma
LNCaP	Androgen Sensitive, Very Low Tumourigenic Prostate Cancer Cell Line
MCa	Mammary Carcinoma Cells
MGO	Methyglyoxal
Milli-Q	Millipore Water - Ultrapure Type 1
min	Minute

MMP	Matrix Metalloproteinase
MRP	Multidrug Resistance-associated Proteins
ms	Millisecond
MSC	Mesenchymal Stem Cell
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NO	Nitric Oxide
NPA	Non-peroxide Activity.
PC3	Androgen Insensitive, High Tumourigenic Prostate Cancer Cell Line
PI	Propidium Iodide
ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute Medium
SD	Standard Deviation
SEM	Standard Error of the Mean
SGLT1	Sodium-glucose Transport Protein 1
SMC	Smooth Muscle Cells
SRB	Sulforhodamine B
TAA	Total Antibiotic Activity
TB	Trypan Blue
TXRed	Texas Red® Sulfonyl Chloride
UMF®	Unique Manuka Factor®
uPA	Urokinase Plasminogen Activator
v/v	Volume per Volume
VCAM-1	Vascular Cell Adhesion Protein 1
w/v	Weight per Volume
λ	Wavelength (nm)

Chapter 1: Introduction

1.1 Prostate Cancer

1.1.1 Epidemiology

According to GLOBOCAN, worldwide there were an estimated 14.1 million new cancer cases and 8.2 million deaths in 2012 (Ferlay *et al.*, 2013). Population growth and ageing have been reported to be the primary cause for the increased incidence of cancers throughout the world (Ferlay *et al.*, 2010). Age standardization revealed that the incidence of cancer was 25% greater in males than females. Within the world, Australia and New Zealand possessed the highest cancer rates per 100,000 people, predominantly attributed to high prostate cancer rates (Ferlay *et al.*, 2013). At the time of prostate cancer diagnosis, primary site or regional lymph node tumour location can confer a five-year survival rate as high as 98.9% (SEER, 2014). However at the time of detection, if metastasis to distant sites has occurred including to the lungs and bone, this survival rate may be as low as 28%. In 2012, a Belgian study examined the survival rates of men who had advanced metastatic prostate cancer (Tombal *et al.*, 2012). They found that the mean five-year survival rate for prostate cancer with bone metastasis was 3%, compared to 56% without bone metastasis. The difference in survival rates highlighted the severity of prostate cancer metastasis, and therefore, the ongoing need for improved treatment options both to prevent the progression and treat the metastasis of prostate cancer.

1.1.2 Cancer Metastasis

The most deadly cancer cell type is one that has acquired an invasive or metastatic phenotype (Nabi *et al.*, 1989; Sethi *et al.*, 2011). Due to the difficulty of treating multiple tumour sites *in vivo*, metastasis is regarded as the most difficult cancer process to treat, and accounts for the greatest proportion of cancer-related deaths (Nabi *et al.*, 1989; Weiss, 1990; Sethi *et al.*, 2011).

Metastasis is defined as the spread of cancer cells from the primary tumour site towards distant sites, resulting in the development of secondary tumours (Weng *et al.*, 2012). Liotta *et al.* (1991) described the processes involved, in which cells must first leave the primary tumour site by invading through the local tissue and enter the circulation, accumulate at a secondary site,

infiltrate the secondary site through the surrounding interstitial fluid and parenchyma, and finally establish tumour growth.

Traditionally, metastasis was thought of as a late-stage cancer process, however, more recently it has been identified to be initiated early on. Malignancy and invasiveness have been linked to an altered protein expression profile that may include the up-regulation of proteases (Wilkins-Port *et al.*, 2012), allowing tumour cells to invade into the surrounding stroma through the extracellular matrix (ECM) (Liotta *et al.*, 1991; Abdel Aziz *et al.*, 2009). Following this, invading primary tumour cells can migrate using integrins that bind to ECM proteins including collagen I, fibronectin, laminin and vimentin (Liotta *et al.*, 1991).

Cell motility is initiated by the formation of pseudopodia; protrusions at the leading edge of a cell that can attach to the ECM (Shankar *et al.*, 2010; Petrie *et al.*, 2012). Initial cell attachment to the ECM is mediated via protein-protein interactions, most often involving integrin receptors such as $\alpha 2\beta 1$ and $\alpha 11\beta 1$ (collagen I), or $\alpha 4\text{-}9\beta 1$ and $\alpha 5\beta 1\text{-}8$ (fibronectin) (Hynes, 2002). Adhesion may also be mediated through the expression of proteins other than integrins. Vascular cell adhesion molecule 1 (VCAM-1) is expressed on tumour cells, and facilitates cellular adhesion through interactions with integrins (Foster, 1996). The expression of VCAM-1 has been linked to an increase in bone metastasis in prostate and breast cancer, therefore may provide a target for anti-metastatic therapy (Chen *et al.*, 2012; Rahim *et al.*, 2014).

Processes throughout the invasion and migration of cells are mediated through the expression of proteases released by both the tumour and surrounding stromal cells, where proteolysis of the ECM is essential for tumour angiogenesis and metastasis (Abdel Aziz *et al.*, 2009) (Sevenich *et al.*, 2014). These proteases can increase cell adhesion to host cells and the surrounding ECM, degrade physical barriers such as the basement membrane, increase cell motility, as well as further facilitate the aggregation of tumour cells to distant tissues (Liotta *et al.*, 1991). Proteases including matrix metalloproteinases (MMPs), urokinase-type plasminogen activator (uPA) and cathepsins have been implicated in cancer metastasis and ECM degradation, while their overexpression has been linked to a worsened prognosis in prostate cancer (Andreasen *et al.*, 1997; Morgia *et al.*, 2005; Woodward *et al.*, 2007; Nalla *et al.*, 2010). As an example of this, MMPs with gelatinase activity (-2 and -9), are used as prognostic markers for metastatic prostate cancer, which have been reported to be upregulated in invading cells and stromal cells (Morgia *et al.*, 2005).

During the progression of prostate cancer towards an aggressive phenotype, alterations in cellular responses to metabolites and androgens often result in a worsened prognosis (Feldman *et al.*, 2001; Sreekumar *et al.*, 2009). Androgens are important in normal prostate tissue homeostasis, where cellular growth and maintenance are regulated through the interaction of androgen ligands with intracellular androgen receptors (Jenster, 1999). As prostate cancer cells are dependent on androgens to mediate growth, anti-androgen therapy is highly effective during the early stages of cancer (Scher *et al.*, 2005). However, as prostate cancer progresses, cancerous cells become less sensitive to androgens, and therefore anti-androgen therapy may no longer be effective (Jenster, 1999; Chlenski *et al.*, 2001). Studies have shown that molecular events and proteins are altered during prostate cancer progression, and therefore, therapy must also be altered to include alternative targets (Huss *et al.*, 2001).

Due to the multifaceted nature of cancer metastasis which involves multiple signalling pathways, inhibition may require the simultaneous targeting of several mechanisms. As opposed to investigating cancer cell metastasis as a whole, it may be beneficial to investigate individual mechanisms that are involved at several points along the way. In the present study, the mechanisms of metastasis investigated were cell adhesion, migration and invasion.

1.1.3 *In Vitro* Cancer Model - PC3 and DU145 Cell Lines

A model for studying cancer metastasis might include the use of an *in vivo* model to assess both the progression of the primary tumour, stromal invasion, and secondary tumour formation in lymph nodes and distant sites. Nevertheless, the use of *in vitro* metastatic models is common when investigating the anti-cancer properties of compounds before they progress to *in vivo* models.

PC3 and DU145 are androgen-insensitive prostate cancer cell lines that demonstrate aggressive behaviour *in vitro*, both of which are analogous to late-stage aggressive clinical prostate cancer cases (Chlenski *et al.*, 2001; Tai *et al.*, 2011). LNCaP is a less metastatic prostate cancer cell line that is androgen sensitive, and more characteristic of early- to mid-stage clinical prostate cancer (Horoszewicz *et al.*, 1980). The present study aimed to assess the effect of compounds for metastatic prostate cancer, therefore, the cell lines PC3 and DU145 were selected for investigation.

The initiation of metastasis is characterised by altered expressions in protein and intracellular messengers. In prostate cancer, it has been found that oxidative stress is required for an aggressive phenotype, with PC3 cells generating higher levels of reactive oxygen species (ROS) compared to DU145 and LNCaP, which may partially account for its metastatic activity (Kumar *et al.*, 2008). In addition, the altered expression of many proteins may confer a more aggressive cell phenotype. Focal adhesion kinase (FAK) is required for the migration of many aggressive cancer cell types, with an increased expression is often correlating to an increased metastatic phenotype (Slack *et al.*, 2001). An increased FAK expression in PC3 and DU145 cells, compared to LNCaP cells, has been attributed to their enhanced metastatic potential both *in vivo* and *in vitro* (Slack *et al.*, 2001).

It is clear from these differences that examination of multiple cell lines *in vitro* is important to assess the effect of drugs and compounds against some of the different clinical presentations of cancer.

1.2 Honey

The majority of honey is produced solely by the honey bee (*Apis mellifera*) through the regurgitation of saccharides derived from the nectar of flowers (Crane, 1975). Alternately, honeydew honey is produced by the honey bees' consumption of honeydew drops: a sugary excretion from insects (such as aphids (*Ultracoelostoma assimile*)) that feed on plant sap (Kirkwood *et al.*, 1960). The enzymes invertase, glucose oxidase and diastase, within the honey stomach of bees, convert plant derived polysaccharides into monosaccharides (Winston, 1991). In the case of honeydew honey, the duel digestion from aphids and bees may result in a darker honey with an increased mineral and enzyme content (White *et al.*, 1980; Astwood *et al.*, 1998). Honey contains compounds other than sugars including: proteins, vitamins, minerals, phytochemicals and enzymes (White, 1978). Of the phytochemicals, phenolic compounds consisting of phenolic acids and flavonoids are thought to be the most beneficial in terms of human health (Del Rio *et al.*, 2013).

Honey was first recognised for its medicinal benefits in Egypt around 3100 BC, however, it has been consumed by humans as far back as 7000 BC in Spain (Crane, 1999). The anti-inflammatory and anti-bacterial properties of honey are still exploited for the treatment of wounds, burns and ulcers (Amaya 2015; Majtanova *et al.*, 2015). Even today, honey alone or in combination with other phenol and anti-oxidant rich products such as coffee has been

demonstrated to be superior to some topical or systemic first line steroids, for the treatment of oral inflammation caused by chemotherapy or infectious coughs (Raeessi *et al.*, 2013; Raeessi *et al.*, 2014). A shift from a curative to a more prophylactic use for honey has occurred only recently, since honey consumption has been found to be beneficial in the prevention of cardiovascular diseases, infections, and even cancer (Namias, 2003; Al-Waili, 2004; Yaghoobi *et al.*, 2008; Alvarez-Suarez *et al.*, 2013).

Honey is a complex matrix composed of 180 - 200 compounds including sugars (75%), water (20%) vitamins (<0.006%), minerals (<0.2%), proteins (0.4%) and phenolic compounds (White *et al.*, 1980; Gheldof *et al.*, 2002; Alvarez-Suarez *et al.*, 2013).

1.2.1.1 Phenolic Acids and Flavonoids

Phenolic containing compounds, or 'phenolics', are found all throughout nature, where their involvement in cellular processes is vital to most plants and organisms (Shahidi *et al.*, 1992). They are generally thought to contribute to the major part of the activity in honey, largely due to their anti-oxidant activity. Because of their widespread expression in most plants, they are ingested at between 25 mg to 1 g per day in most humans; predominantly through the consumption of coffee (Clifford, 1999). Phenolic compounds have been shown to have many therapeutic benefits either alone or in combination, such as cardio- and neuro-protection, as well as reduced inflammation in chronic diseases (Del Rio *et al.*, 2013). Compared to compounds alone, the administration of phenolic-rich dietary vegetables may have added benefit, due to the combination of multiple phenolics (Del Rio *et al.*, 2013). For this reason, the benefits of honey may be comparable or greater than those of vegetables, coffee and tea, due to the large variety and quantity of phenolic compounds present.

Phenolic compounds are divided into two main categories based on the number of phenol groups they contain: phenolic acids (1 group) and polyphenols (2 or more groups) (Bravo, 1998). Polyphenols are divided further based on the number of phenolic rings they contain: flavonoids (2) and tannins (3 or more). With respect to therapeutic phenolics consumed as part of a normal diet, phenolic acids and flavonoids have been identified as the most important (Stalikas, 2007; Alvarez-Suarez *et al.*, 2013). Interestingly, in honey, the total phenolic acid content is greater than the flavonoid content (Yao *et al.*, 2003; Kassim *et al.*, 2010). For this reason, the investigation of phenolic acids with high activity may be beneficial.

A wide variety and concentration of phenolic acids and flavonoids are present in different honey types, and so, the most commonly found phenolic compounds are presented in **Table 1**.

Table 1. Common Phenolic Compounds in Honey.

Phenolic Acid	Flavonoid
Vanillic acid	Tricetin
p-coumaric acid	Isohamnetin
Gallic acid	Quercetin
Ellagic acid	Kaempferol
Syringic acid	Luteolin
Chlorogenic acid	Pinocembrin
Ferulic acid	Chrysin
Caffeic acid	Galangin
	Pinobanksin
	Myricetin
	Apigen
	Genistein

Compounds were selected from a range of studies (Yao *et al.*, 2003; Jaganathan *et al.*, 2009b; Kassim *et al.*, 2010; Khalil *et al.*, 2011; Alvarez-Suarez *et al.*, 2013)

Studies investigating honey phenolic content in New Zealand manuka honey previously reported that quercetin, kaempferol and chrysin represented 13.8%, 5.0% and 12.6% of the total flavonoids, respectively (Yao *et al.*, 2003). Gallic acid and caffeic acid have been reported to represent a large proportion of the total phenolic content of Malaysian honey (Kassim *et al.*, 2010). Another study showed the total phenolic acid and flavonoid content detected in manuka honey to be comparatively higher than 6 Malaysian honeys, where catechin, caffeic acid and *p*-coumaric acid were found at high concentrations (Khalil *et al.*, 2011). A review by Jaganathan *et al*. (2009b) identified some of the pharmacologically promising polyphenols in honey to include: caffeic acid, chrysin, galangin, quercetin, kaempferol, apigen and pinobanksin.

Quercetin, found in fruits, vegetables and honey, is the most consumed flavonoid in the Western world, and therefore, has been the most studied (Hertog *et al.*, 1994). Quercetin alone or in combination with other polyphenols has been shown to have anti-proliferative activity in many cancer cell lines (Gulati *et al.*, 2006; Jaganathan *et al.*, 2009b; Jaramillo-Carmona *et al.*, 2014).

Due to their ubiquitous presence at high concentrations in honey, the following phenolic compounds were selected for further investigation throughout the present study: quercetin, gallic acid, kaempferol, chrysin and caffeic acid (**Appendix 4**). The dietary consumption of the five honey-derived phenolic compounds used in the present study have been comprehensively investigated, with regard to their bioavailability and possible benefits in human diseases including cardiovascular, infection, diabetes and cancer (Alvarez-Suarez *et al.*, 2013; Del Rio *et al.*, 2013; Lall *et al.*, 2015).

1.2.1.2 Carbohydrates

Sugars account for over 99% of the total dry weight of honey (White *et al.*, 1980). Of these sugars, fructose (40.5%), glucose (33.5%), maltose (7.5%) and sucrose (1.5%) are the most common (Cooper *et al.*, 2002; Henriques *et al.*, 2006). The high sugar content in honey, resulting in a high osmolarity, may be partially responsible for the benefit of honey in wound healing (Molan, 1999). Nevertheless, the sugars found in honey may work independently of their effect on cellular osmolarity. Carbohydrates can be used as substrates for the Maillard reaction; the formation of a Maillard product through the interaction between reducing sugars and amino acids, that as an example, produce flavour and browning often seen in cooked meat (Maillard, 1912). Both carbohydrates alone, and Maillard products formed from Maillard reactions, can have *in vitro* anti-oxidant activity (Gheldof *et al.*, 2002). Compared to honey, the oral administration of corn syrup in humans did not increase total plasma phenolic levels, however, did increase the plasma anti-oxidant capacity (Schramm *et al.*, 2003). This further supported the idea that carbohydrates can have anti-oxidant effects through the formation of Maillard products. These findings suggest that honey may exhibit greater benefit compared to other natural phenolic sources, due to the presence of a highly concentrated sugar matrix.

1.2.2 Classification of Honey

As with most honey related studies, including the present study, the use of monofloral honeys is most common. Due to optimal foraging by bees, or human processing, multifloral honey contains many honey types. The classification of monofloral is however not perfect, and relies on indicators such as colour, sugar spectrum, conductivity and pollen content (Alimentarius, 2001). As it is not possible to force bees to forage on one flower type, the variation between honey samples of the same monofloral type is large. For example, to define New Zealand, manuka honey as monofloral, a minimum of 70% of the total pollen must originate from

Leptospermum scoparium (Stephens, 2006). Percentage pollen composition may differ between year, geographical origin and manufacturer, and therefore may confound literature comparisons.

Manuka, thought to be the most clinically beneficial honey, is often used as a positive control or comparison in honey-related research (Yao *et al.*, 2003; Tan *et al.*, 2009). The antibacterial health benefits of manuka honey have been attributed to the presence of methylglyoxal (MGO) in high concentrations; an organic compound which displays both *in vitro* and *in vivo* anti-bacterial and anti-cancer properties (Mavric *et al.*, 2008; Talukdar *et al.*, 2008). Labels including unique manuka factor (UMF®), total antibiotic activity (TAA) and non-peroxide activity (NPA) are used to categorise manuka honey, which give an indication of the phenolic content and potential therapeutic benefit of each manuka sample. Samples above 10 UMF®/TAA are considered to be effective in wound healing, and therefore, manuka with a UMF®/TAA of 10+ was used in the present study (Molan, 1999).

1.2.3 Differences in Honey Depending on Floral Origin

The anti-oxidant and phenolic content of honeys is extremely variable, due to differences in the location of bees and the flower source (florals) (Frankel *et al.*, 1998; Gheldof *et al.*, 2002). This suggests that the therapeutic benefits of honey administration may be unique to certain types of honey. It has been reported that darker honeys possess a stronger anti-oxidant activity and higher phenolic content (Moniruzzaman *et al.*, 2014). Moniruzzaman *et al*. showed a strong positive linear correlation between the colour of honey versus the total phenolic acid content ($r = 0.943$), flavonoid content ($r = 0.926$), and anti-oxidant activity ($r =$ between 0.838 and 0.894). Darker honeys may therefore have a higher phenolic content than lighter honeys, resulting in an increased therapeutic potential (Estevinho *et al.*, 2008; Moniruzzaman *et al.*, 2014; Pontis *et al.*, 2014). Evidence of this increased potential has been shown, where honeys with a higher phenolic content have demonstrated a greater anti-cancer effect *in vitro* (Jaganathan *et al.*, 2009a; Jaganathan *et al.*, 2010b). In contrast, honeys containing low levels of phenolic compounds may increase cancer cell proliferation (Erejuwa *et al.*, 2014).

Reactive oxygen species (ROS) are produced during mitochondrial metabolism to maintain cellular homeostasis, where low concentrations may support proliferation, and high concentrations may cause cell death (Cairns *et al.*, 2011; Ray *et al.*, 2012). ROS may be produced by glucose oxidase (GO), an enzyme found at high concentration in many honeys. In

some honeys, low concentrations of GO, as a result of inadequate storage or floral differences, may produce low concentrations of ROS, which have been shown to increase cancer cell proliferation (Pelicano *et al.*, 2004; Erejuwa *et al.*, 2014). Erejuwa *et al*. showed that the anti-oxidant activity of honey containing high levels of phenolic compounds may prevent the cellular proliferation induced by low levels of ROS. This again suggests that honeys containing high levels of phenolic compounds, which increase their anti-oxidant activity, may provide an increased anti-cancer potential compared to honeys with a reduced phenolic content.

In conjunction with the evidence provided, consultation with local apiarist Mr. Steven Wootton (Alpine Honey, Hāwea, New Zealand) regarding darker honey revealed that, due to their greater anti-oxidant and anti-inflammatory properties, New Zealand thyme, manuka and beechforest honeydew (honeydew) honey types might possess a greater anti-cancer activity compared to lighter honeys such as white clover (*Trifolium repens)* or viper's bugloss (*Echium vulgare)* (**Table 2**).

Table 2. Name and Origin of Selected New Zealand Honeys. See Appendix 1 for map.

Local and Scientific Name	Location	Season Collected
Thyme *(Thymus vulgaris)*	Central Otago	Nov-2013
Manuka *(Leptoseprmum scoparium)*	Central Otago	Jan-2014
Black/Red Beech *(Nothofagus solandri, N. fusca)*	Canterbury	Jan-2014

Vanhanen (2011) showed that both New Zealand honeydew and manuka honey possess a high mineral content, with honeydew containing more than twice that of manuka. Further, Vanhanen (2011) showed that thyme honey had a lower mineral content compared to most other honeys, despite these three honeys being the darkest. Therefore, Vanhanen concluded that colour intensity had a stronger correlation to phenolic content than mineral content, and that thyme honey may still possess increased activity compared to lighter honeys of the same mineral content.

1.2.3 Oral Bioavailability of Honey

Phenolic compounds within the carbohydrate matrix of honey may exist in two forms: either bound to sugars (as glycosides), or unbound (as aglycones). Both the absorption and metabolism of these compounds in either state varies greatly, and may affect the overall plasma

concentration of each compound. Further, the presence of sugars in honey may affect the bioavailability of phenolic compounds, and so these variables were investigated.

Phenolic compounds may be highly reactive and damaging to cells, and therefore, in nature they are often found within vacuoles bound to glucose, cellulose or other polyphenols (Hartley *et al.*, 1976; Zoecklein *et al.*, 2000). The glycosylation of phenolic compounds to sugars may be important in reducing the compound activity, in order to protect the cell from cytoplasmic damage (Cuyckens *et al.*, 2004). Phenolic compounds, including flavonoids, found within the gut of a honey bee are often glycosylated, however, during the production of honey, they may be converted to flavonoid aglycones by glycosidase enzymes from honey bee saliva (Sabatier *et al.*, 1992; Truchado *et al.*, 2009). Further hydrolysis of glycoside-linked flavonoids occurs in the small intestine of humans after oral administration, by both bacteria and β-endoglucosidase enzymes in the intestinal epithelium (Day *et al.*, 1998; Spencer *et al.*, 1999).

1.2.3.1 Absorption of Honey-derived Phenolic Compounds

Unlike most phenolic-containing natural foods, flavonoids in honey exist predominantly as aglycones due to their extensive hydrolysis (Truchado *et al.*, 2009). Interestingly, Truchado *et al.* (2011) reported that honey from the stingless bee (*Apidae: meliponinae)* may contain a higher flavonoid glycoside content compared to their respective aglycones; however, they noted this was not common for most honey types. Due to the presence of flavonoids in both glycosidic and aglycone forms in honey, comparative absorptions of both forms is important. Aglycones have been suggested to have an increased intestinal absorption profile compared to their respective glycosides (Izumi *et al.*, 2000; Scalbert *et al.*, 2000). In contrast, studies have shown that glycosides may have an increased absorption profile compared to their aglycones (Morand *et al.*, 2000; Gee *et al.*, 2001; Setchell *et al.*, 2001). In further contrast, other groups have showed there to be no difference in total absorption, and have suggested that glycoside absorption may only lag due to the need to be hydrolysed by gut bacteria (Richelle *et al.*, 2002; Zubik *et al.*, 2003). Due to the contradictory evidence regarding phenolic absorption, the bioavailability of honey phenolic compounds in either aglycone or glycoside forms needs to be further investigated in order to determine the true benefit of orally absorbed honey. The absorption of phenolic acids may be different to flavonoids, with respect to their glycosylated or aglycone forms. It has been suggested that phenolic-glycosides cannot be absorbed from the small intestine until their glycoside is removed (Perez-Jimenez *et al.*, 2009).

Glycosylated flavonoids may be transported through the intestinal epithelium and into the portal vein either as glycosides or aglycones via three main processes: uptake via the sodium-dependent glucose transporter (SGLT1) and hydrolysis via cytosolic β–glucosidase (aglycone), uptake via the SGLT1 and interaction with the glucose transporter 2 (GLUT2) (glycoside), or luminal hydrolysis via lactase phloridzin hydrolase into an aglycone that can directly diffuse across membranes (Vanacker *et al.*, 1995; Spencer *et al.*, 1999; Gee *et al.*, 2000; Del Rio *et al.*, 2013).

Membrane bound multidrug-resistance-associated proteins (MRPs) may play an important role in the excretion of intestinally absorbed flavonoids (Alvarez-Suarez *et al.*, 2013). MRP-2 is located on the apical membrane of enterocytes, and has been shown to excrete cytosolic flavonoids back into the gut (Akao *et al.*, 2007; Jiang *et al.*, 2012). In contrast, both MRP-1 and MRP-3 may increase the excretion of cytosolic flavonoids from enterocytes into the blood, due to their basolateral expression (Borst *et al.*, 1999; König *et al.*, 1999). Aside from enterocytes, many other cell types express MRPs in order to control the efflux of compounds. Prostate cancer cells express low levels of MRPs compared to many other cancer types, which suggests the efflux of phenolic compounds may be minimal, and therefore may possess greater activity (Nooter *et al.*, 1995). However, as a result of chemotherapy, MRP expression may be increased in prostate cancer cells, which may result in a decreased activity of phenolics through site-specific efflux (Van Brussel *et al.*, 2001). Together, these findings may suggest a benefit for phenolic containing compounds in early stage prostate cancer, however they may be less effective later on as a result of chemotherapeutic treatment.

1.2.3.2 Metabolism of Honey-derived Phenolic Compounds

The bioavailability of single-administered honey-derived phenolic compounds has been reported to be very poor. As an example, the extensive metabolism of phenolic compounds including chrysin by sulphation and glucuronidation may result in a bioavailability of less than 1% (Galijatovic *et al.*, 1999; Saarinen *et al.*, 2001; Walle *et al.*, 2001). The extensive first pass metabolism of kaempferol by phase I oxidation and phase II glucuronidation in the intestine and liver results in a bioavailability of less than 2% (Barve *et al.*, 2009). Like the other flavonoids, the bioavailability of quercetin as an aglycone is also very poor, with no free quercetin detectable in human plasma after oral administration of 4 g quercetin (Gugler *et al.*, 1975). Further, it was shown that over 50% of the orally administered dose was faecally excreted unchanged. Interestingly, gallic acid still had the same relative bioavailability when

orally ingested in a carbohydrate and protein matrix, as shown by comparing black tea to acidum gallicum (gallic acid) tablets (Shahrzad *et al.*, 2001).

Before flavonoids enter the bloodstream, they undergo extensive phase II metabolism within the intestinal epithelium, through glucoronate, methyl and sulfate group conjugation (Manach *et al.*, 2004a). The metabolism of flavonoids may further promote intestinal absorption (Wen *et al.*, 2006). Due to the extensive metabolism and conjugation of polyphenols during absorption, only a very small proportion of the original compound exists in the plasma, and therefore, even less may exist at the desired site of action (Del Rio *et al.*, 2013). Stalmach *et al.*, (2009) demonstrated the complex metabolism of the polyphenol chlorogenic acid to caffeic acid in the gut by esterase enzymes. Multiple conjugations to caffeic acid by catechol-O-methyl transferase, sulfotransferase, esterases, reductase, Co-enzyme A and glycuronosyltransferase were reported (Stalmach *et al.*, 2009). This suggested that the extensive metabolism of polyphenols might result in very low concentrations of the circulating un-metabolised compound. This might suggest that *in vitro* analysis of polyphenols for the treatment of cancer may have little clinical significance, if the circulating compound is so extensively metabolised *in vivo*.

1.2.3.3 Advantages of Honey as a Vehicle for Phenolic Compounds

Despite the poor bioavailability of orally administered phenolics, honey may function as a beneficial vehicle for the administration of phenolic compounds, where the presence of sugars may facilitate intestinal absorption (Alvarez-Suarez *et al.*, 2013). Fructose, the predominant saccharide in honey, may aid in the absorption of phenolic compounds both directly and indirectly. Fructose has been shown to reduce hypoglycaemia in diabetic subjects due to its effects on prolonging gastric emptying and slowing digestion (Vaisman *et al.*, 2006; Kellett *et al.*, 2008). Erejuwa *et al*. (2012) suggested that a prolonged digestion may increase the absorption of other nutrients such as phenolic compounds. Further, fructose and glucose have been shown to increase the expression and localisation of GLUT2 in intestinal epithelial cells (Kellett *et al.*, 2000; Jones *et al.*, 2011). Due to the involvement of GLUT2 in the efflux of flavonoids into the blood, the presence of high levels of fructose may further support the use of honey as a vehicle for phenolic compounds (Manzano *et al.*, 2010).

Taken together, the administration of honey may provide greater benefit compared to other natural products due to the high levels of phenolic acids, flavonoids and sugars.

1.2.4 Possible Anti-cancer Effects of Honey

Multiple proteins are involved in the adhesion of cancer cells to the ECM, and their over-expression has been linked to the initiation and progression of cancer metastasis. As an example of this, the over-expression of vascular cell adhesion molecule-1 (VCAM-1) in prostate cancer has been linked to an enhancement of metastatic potential (Tai *et al.,* 2014). It has previously been reported that apigen and quercetin, both honey-derived flavonoids, could inhibit melanoma lung metastasis through the inhibition of VCAM-1 expression (Piantelli *et al.,* 2006). In addition, the anti-angiogenic activity of flavonoids has been demonstrated through the inhibition of cell adhesion (Gerritsen *et al.,* 1995; Panés *et al.*, 1996; Kobuchi *et al.,* 1999). These findings suggest that honey may possess anti-metastatic activity in prostate cancer, due to a reduction in VCAM-1 expression by honey-derived flavonoids.

As cancers with an aggressive phenotype have been shown to generate more ROS, the anti-oxidant activity of honey may have greater potential in highly metastatic cell lines (Kumar *et al.*, 2008). Honey may therefore be more beneficial in the aggressive PC3 and DU145 cell lines that have a greater dependence on NO and ROS production, compared to the less aggressive LNCaP cell line (Kumar *et al.,* 2008). Hassan *et al*. (2012) reported that NO production and decreased anti-oxidant status caused increased hepatocellular carcinoma cell proliferation, and that honey exhibited cytotoxicity through a reduction in NO and restoration of anti-oxidant status. Further, the authors demonstrated that a reduction in ROS activity could inhibit MMP-9 activity, and therefore might provide a mechanism of action for the inhibition of metastasis. Abdel Aziz *et al*. (2009) demonstrated that honey had anti-metastatic effects in HepG2 hepatocellular carcinoma cells, due to the inhibition of MMP proteolytic and gelatinolytic activity. In addition, the honey-derived flavonoid quercetin has been shown to decrease MMP-2 and -9 expression in PC3 prostate cancer cells (Vijayababu *et al.,* 2006). Together, these findings suggest an increased benefit of honey for the treatment of advanced or highly metastatic cancer types over early stage tumours, however, extensive clinical research must be conducted. Further, due to the involvement of gelatinase MMPs in prostate cancer metastasis, along with the potential inhibition of MMPs by honey, honey may possess anti-metastatic activity in prostate cancer, *in vivo*.

Unfortunately, clinical data available in the use of honey for the direct treatment of cancer is non-existent. A search using the World Health Organization's clinical trial database retrieved a total of 138 clinical trials under "honey" in humans; however, most involved its use as a topical

agent for wound healing, or its protective properties in cardiovascular/obesity related diseases (WHO, 2015). Of those few targeted towards cancer, a large proportion involved its use for the treatment of cancer-related pathologies surrounding the decrease of inflammation (Zidan *et al.*, 2006; Molan, 2012).

Honey may possess anti-cancer effects via multiple mechanisms including through the modulation of inflammation. The involvement of inflammation in the initiation and progression of cancer has been well documented, since inflammation can promote both the tumourigenesis and carcinogenesis of a tumour (Grivennikov *et al.*, 2010). Prednisolone, an orally administered corticosteroid, is often given alongside conventional chemotherapeutics during prostate cancer treatment due to its immunosuppressant effects. Its proven anti-inflammatory effects have been shown to lower white blood cell migration and accumulation around the tumour site (Baay *et al.*, 2011). Honey has been shown to exhibit anti-inflammatory activity through the suppression of leukocyte infiltration (Leong *et al.*, 2012). Interestingly, honey was shown to be as effective as prednisolone treatment in an inflammatory model of colitis (Bilsel *et al.*, 2002). Honey has been shown to inhibit cyclooxygenase-1 and -2, prostaglandins and tumour necrosis factor-α (Kassim *et al.*, 2010; Vallianou *et al.*, 2014). The inhibition of these inflammatory mediators may be due to the inhibition of NF-κB (Hussein *et al.*, 2013). For these reasons, co-administration of honey with chemotherapeutics may also be beneficial, due to the anti-inflammatory of effects of honey (Molan, 2012).

Currently, there exist no human clinical trials for the use of honey for the primary treatment of cancer. Similar to the lack of clinical evidence for honey in cancer, the amount of *in vivo* animal evidence is also limited. Despite this, both the oral and intravenous administrations of honey has been used for the prophylactic and cytotoxic treatment of a range of cancer types (Oršolić *et al.*, 2005; Attia *et al.*, 2008; Alvarez-Suarez *et al.*, 2013; Erejuwa *et al.*, 2014). The anti-metastatic effect of honey was examined in mice subcutaneously inoculated with either mammary carcinoma or methylcholanthrene-induced fibrosarcoma. Honey (2 g/kg) was orally administered for either 10 days prior to (preventative) or 2 days after (curative) cancer cell inoculation (Oršolić *et al.*, 2005). Authors reported that only the preventative administration of honey caused a reduction in lung metastasis for both cancer cell types after 21 days ($p>0.01$). In contrast, the curative administration of honey increased lung metastasis. They explained the preventive treatment decrease by the ability of polyphenols present in honey to stimulate the host immune defence against the tumour. Further, they speculated that the pro-metastatic effect of curative honey was due to the increase in vitamins and minerals available for tumour growth,

as well as the osmolarity of honey increasing lymph flow (Oršolić *et al.*, 2005). Bioavailability was not discussed by the authors, since the difference in metastasis between the preventative and curative dosing regimens could have been due to differences in plasma phenolic concentrations at the time of cell implantation. Attia *et al.* (2008) investigated the effect of honey oral pre-treatment every second day for 4 weeks in mice, at concentrations between 10 to 1000 mg honey per 100 g body weight. After treatment, mice were inoculated with Ehrlich ascites tumours. The authors reported that the anti-cancer effect of honey in orally pre-treated mice was due to increases in bone marrow cells, phagocytic macrophages, T- and B-cells at the site of inoculation (Attia *et al.*, 2008). Together, these results suggest that honey may be beneficial for the treatment of cancer *in vivo*.

There have also been no human clinical trials for the primary treatment of cancer through the combination of honey with conventional anti-cancer treatments such as chemotherapy or radiation therapy. Further, few animal model experiments have been conducted which investigated this. *In vitro*, manuka honey displayed a greater cytotoxicity than paclitaxel, where concentrations as low as 0.6% (w/v) induced apoptosis through the activation of the caspase-9 intrinsic pathway (Fernandez-Cabezudo *et al.*, 2013). Manuka honey was given alone or in combination with paclitaxel for the treatment of melanoma in mice, where honey at 50% (w/v) was intravenously infused twice a week for 3 weeks. Alone, intravenous manuka honey reduced tumour growth by 33%, and paclitaxel alone reduced tumour growth by 61%. The combination with paclitaxel also reduced tumour growth by 61%, and improved mouse survival and organ protection (Fernandez-Cabezudo *et al.*, 2013). Similarly, Gribel and Pashinskiĭ (1990) reported that honey alone or in combination with either cyclophosphamide or 5-fluorouracil could demonstrate *in vivo* anti-metastatic and anti-tumoural effects in both rat and murine tumours. These results suggested that honey may be beneficial as an adjuvant to chemotherapy side effects, however, may not affect overall tumour size during chemotherapy. Again, further clinical research must be conducted to demonstrate the benefits of honey compared to current cancer therapies.

1.3 Bee Venom

Aside from honey, other honey bee products have been recognised for their anti-cancer effects. Bee venom has been recognised for its therapeutic properties for thousands of years by the Chinese, Egyptians, Ancient Mexicans and Greeks (de Conconi *et al.*, 1988; Ali, 2012; Kim, 2013). The therapeutic use of bee venom or bee pollen is called apitherapy. Apitherapy has

most commonly been used for the treatment of rheumatoid arthritis (Kwon *et al.*, 2001). Only within the last 30 years have bee venom, and its most abundant constituent melittin, been demonstrated to be effective for the treatment of cancer models both *in vitro* and *in vivo* (Hait *et al.*, 1985; Son *et al.*, 2007; Park *et al.*, 2011; Oršolić, 2012).

Bee venom contains as many as 18 peptides including melittin (40 - 50%), apamin (2 - 3%), mast-cell-degranulating peptide (MCD) (2 - 3%), protease inhibitors (<0.8%), enzymes such as phospholipase A2 (PLA_2) (10 - 12%), amines such as histamine, dopamine and noradrenaline (0.1 - 1%) and carbohydrates (1.5%) (Son *et al.*, 2007; Oršolić, 2012). Melittin, the 26-amino acid peptide in bee venom, has been suggested to be responsible for the anti-tumour activity of bee venom, and therefore, has been most widely investigated (Son *et al.*, 2007; Jeong *et al.*, 2014).

Research on compounds contained in bee venom has mainly focused on melittin or soluble PLA_2. Melittin interacts with cytosolic PLA_2 produced by cells, and therefore the combination of both compounds may be important. PLA_2 is an enzyme family that facilitates the hydrolysis of fatty acids from membrane-bound phospholipids, resulting in the release of lipid mediators (Burke *et al.*, 2009). Released lipids may act as substrates in pathways involved in the regulation of cell survival, proliferation and motility, all of which are involved in the progression of cancer (Scott *et al.*, 2010). PLA_2 is over expressed in the PC3 and DU145 prostate cancer cells, and may be partially responsible for the metastatic potential of both cell lines. Further, the inhibition of PLA_2 has been shown to decrease the metastatic activity of both cell lines (Lansky *et al.*, 2005b). The role of PLA_2 in cancer development is complex, since either activation or inactivation may increase tumourigenesis, suggestive of a tissue-specific bimodal regulation (Scott *et al.*, 2010). The release of fatty acids as pro-tumour mediators by PLA_2 is evident in most cancer cell lines, however, increased PLA_2 activation can cause membrane disruption and cytotoxicity (Lad *et al.*, 1979). As melittin may decrease MMP-2, -3 and -9 expression in some cell lines as well as PLA_2, it may possess anti-metastatic activity in prostate cancer cells that are known to overexpress these (Nah *et al.*, 2007; Park *et al.*, 2010).

Other studies examining bee venom and melittin have reported the anti-proliferative activity of bee venom (1 - 10 µg/mL) and melittin (0.5 - 2.5 µg/mL) against prostate cancer cells through the activation of caspases by NF-κB inactivation (Park *et al.*, 2011). Further, Park *et al.* demonstrated that the *in vivo* administration of intravenous bee venom (3 - 6 mg/kg) in nude mice inoculated with PC3 cells resulted in a decrease in tumour growth, related to caspase

activation and NF-κB inactivation. NF-κB can regulate the expression of the androgen receptor (AR) in prostate cancer growth, with inhibition reducing cell growth and demonstrating therapeutic potential (Zhang *et al.*, 2009). Bee venom and its constituents have been shown to cause tumour cell cytotoxicity either directly or indirectly through immune system modulation, both of which have been attributed to reductions in NF-κB and calcium-calmodulin complex activity (Oršolić, 2012). NF-κB and the calcium-calmodulin complex may be implicated in the metastasis of cancer cells, therefore, bee venom and melittin may increase apoptosis, while inhibiting proliferation as well as angiogenesis, invasion and metastasis in prostate cancer (Tsai *et al.*; Huber *et al.*, 2004; Liu *et al.*, 2014).

1.4 Hypothesis

Hypothesis I. It was hypothesised that administration of honey would lead to a reduction in the *in vitro* metastatic activity and viability of prostate cancer cells. Further, it was predicted that phenolic compounds would be responsible. To address this, three dark New Zealand honeys (thyme, manuka and beech forest honeydew) were selected for their high anti-oxidant and phenolic content. Due to their high activity and ubiquitous expression in honey, the following phenolic compounds were selected for separate investigation: quercetin (3,5,7,3',4'-pentahydroxyflavone), gallic acid (3,4,5-trihydroxybenzoic acid), kaempferol (3,4',5,7-tetrahydroxyflavone), chrysin (5,7-dihydroxyflavone) and caffeic acid (3,4-dihydroxycinnamic acid) (**Appendix 4**).

Hypothesis II. It was hypothesised that bee venom would lead to a reduction in the *in vitro* metastatic activity and viability of prostate cancer cells. To address this, both bee venom and an extract, melittin, were used.

1.5 Aims

In order to explore the hypotheses, the study was divided into three specific aims:

Aim I. ***To assess the cytotoxic potential of honey, bee venom, and honey-derived phenolic compounds and determine a maximal non-lethal concentration.***

PC3 and DU145 prostate cancer cells were exposed to compounds or honey, and multiple proliferation and cytotoxicity assays were used to characterise cytotoxicity in order to establish a maximal non-lethal concentration. Assays included the colourimetric MTT and SRB assays, the TB and HO/PI dye exclusion assays, as well as the LDH assay.

Aim II. ***To identify and quantify important phenolic acids and flavonoids in honey.***

Chemical extraction of phenolic compounds from the three honeys was carried out, with HPLC being used to quantify the five phenolic compounds selected (quercetin, gallic acid, kaempferol, chrysin and caffeic acid).

Aim III. ***To assess the anti-metastatic potential of honey, bee venom, and honey-derived phenolic compounds through changes in the adhesion, migration and invasion of prostate cancer cells.***

Using concentrations established from Aim I, prostate cancer cells were exposed to non-lethal amounts of compounds. Adhesion to collagen I and fibronectin were quantified, and Boyden chambers were used to evaluate changes in migration and invasion of cells.

Chapter 2: Materials and Methods

2.1 Materials

Dimethyl sulphoxide (DMSO) was purchased from Scharlau (Barcelona, Spain). Trypan Blue (0.4%), RPMI-1640 Medium, Fetal Bovine Serum (FBS), Penicillin Streptomycin (PS), PBS (8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4; pH 8.0) and Trypsin (2.69 mM EDTA, 1 g/L trypsin, 0.14 M NaCl, 76.78 mM Tris HCl; pH 8.0) were purchased from Gibco (Carlsbad, CA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Tris-base, Quercetin (95% HPLC), Caffeic acid (98% HPLC), Sucrose, Maltose, fructose, D-(+)-glucose Trichloroacetic acid (TCA), Sulphorhodamine B (SRB), Acetic acid (CH_3COOH), Formic Acid (HPLC grade), Methanol (HPLC grade), Sodium bisulfite, and Ethyl acetate were purchased from Sigma-Aldrich (St. Louis, MO). Thyme, Manuka and Honeydew Honey were gifted by New Zealand Honey Specialties Limited (Mosgiel, New Zealand). NADH (95% HPLC), Kaempferol (98% HPLC), Chrysin (99.8% HPLC) and Melittin (85% HPLC) were purchased from Sapphire Biosciences (NSW, Australia). Gallic acid (98% HPLC) was purchased from Abcam (Cambridge, England). Bee venom was purchased from Shaanxi Pioneer Biotech (Xi'an, China). Pyruvate, Propidium Iodide (Excitation/Emission 536 nm/617 nm) and Hoechst 33342 (Excitation/Emission 352 nm/461 nm) were purchased from Life Technologies (California, USA). Fibronectin, Collagen I and Matrigel® were purchased from Corning (New York, USA)

2.2 Treatment Compounds

2.2.1 Honey

Thyme and manuka (10+ Total Antibiotic Activity) honey was collected from the Central Otago/Wanaka region of New Zealand, produced from the thyme bush (*Thymus vulgaris)* and manuka tree *(Leptospermum scoparium).* Honeydew honey was collected in the Canterbury region of New Zealand, from the beech forest tree *(Nothofagus fusca)* (**Appendix 1**). All honeys were produced by the European honey bee (*Apis mellifera).* (Cooper *et al.*, 2002; Henriques *et al.*, 2006). Raw honeys were stored at room temperature, with minimal light exposure. For cell treatments, honeys were dissolved in 37°C serum-free RPMI-1640 medium to a final stock

concentration of 55% (w/v) and sterilized using a syringe filter unit (0.22 µm). Prior to all experiments, fresh preparations of honey stock solutions were made.

2.2.1.1 The Construction of an Artificial Honey Control

It was important that a sugar control (artificial honey) was employed, so that the effects observed by honey could be attributed to constituents other than sugars. Heat treatment may have been a possible method to produce an artificial honey, as it has been used to inactivate proteins and enzymes, and destroy active compounds in many natural extracts (Czipa, 2010; Ropa, 2010; Siripongvutikorn *et al.*, 2012). In contrast to both studies, other reports found that due to glycoside release and the Maillard reaction between sugars and proteins, heat treatment may lead to an increase in the anti-oxidant capacity of phenolic-containing natural products such as honey, citrus peels and cabbage (Jeong *et al.*, 2004; Turkmen *et al.*, 2006; Kusznierewicz *et al.*, 2008). Maillard reaction products known as high molecular mass melanoidins were identified to be primarily responsible for the radical scavenging capacity of honey exposed to heat (Brudzynski *et al.*, 2011a). In further contrast to the observed increases and decreases in honey activity and content, studies have shown that heat treatment may have no effect on the total phenolic content nor anti-oxidant capacity (Wang *et al.*, 2004; Šarić *et al.*, 2013). Due to the inconclusive evidence surrounding heat treatment of honey to remove phenolic and protein content, it was decided that the artificial honey control should be synthetically made using sugars in their respective concentrations, rather than using heat-inactivated honey. An artificial honey control in the present study was made by combining 1.5 g sucrose, 7.5 g maltose, 33.5 g glucose and 40.5 g fructose in 17 mL of deionized water (Cooper *et al.*, 2002; Henriques *et al.*, 2006).

2.2.2 Honey-derived Phenols

Gallic acid, quercetin and caffeic acid were stored at room temperature. Kaempferol and chrysin were stored at -20°C. Prior to each experiment, all compounds were dissolved in 100% DMSO and syringe filtered (0.22 µm).

2.2.3 Bee Venom and Melittin

Lipolyzed bee venom was stored at -20°C. Bee venom was dissolved in Millipore Water Milli-Q (55 µg/mL) and stored as aliquots at -80°C. Melittin was dissolved in Milli-Q (5 mg/mL) and

stored as aliquots at -80°C. Prior to each experiment, stock aliquots were dissolved in warmed RPMI-1640 medium and syringe filtered (0.22 µm).

2.3 Cell Maintenance

Human prostate cancer cell lines, PC3 and DU145, were gifted by Prof. Rosengren (University of Otago, New Zealand). Cells were maintained in RPMI-1640 medium (pH 7.4), supplemented with 5% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, and 2 g/L sodium bicarbonate (growth medium). Cells were incubated in 5% CO_2/95% O_2 humidified air at 37°C. At 90% confluency, cells were passaged by the addition of 5 mL trypsin solution (2.69 mM EDTA, 1 g/L trypsin, 0.14 M NaCl, 76.78 mM Tris HCl; pH 8.0) followed by a 2 min incubation at 37°C, to allow cells to detach from the bottom of the flask. Cells were rescued using fresh supplemented growth medium (10 mL). Cell-trypsin-medium solution was centrifuged at 1800 rpm for 4 min at 4°C. Supernatant was removed, and cells were resuspended in supplemented RPMI-1640 medium (20 mL).

2.4 Honey pH

To determine the effect of pH on cell viability, only thyme honey was investigated. Solutions of thyme honey (0 - 100%, w/v) were dissolved in 5% FBS supplemented RPMI-1640 medium and incubated for either 0 or 24 h in 5% CO_2/95% O_2 humidified air at 37°C. pH was measured using a Mettler Toledo SevenEasy S20 pH Meter.

2.5 Cytotoxicity

Prior to the assessment of cytotoxicity, growth curves were constructed to determine optimal seeding density. In 96-well plates for a maximum seeding time of 72 h, cells were seeded in growth medium at $5x10^3$ cells/well (DU145) and $3x10^3$ cells/well (PC3) **(Appendix 2)**. Prior to each cytotoxicity experiment, cells were allowed to adhere for 24 h.

2.5.1 Assessment using the MTT Assay

The reduction of the tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), to a blue formazan crystal is facilitated by mitochondrial dehydrogenase and

NADPH-dependent cellular oxidoreductase enzymes, and is often used to quantify cell viability (Mosmann, 1983; Berridge *et al.*, 1993). Cells were treated as shown in **Table 3**. Following treatment for 0, 24, 48 or 72 h, MTT (0.5 mg/mL) was added to each well, and incubated for 3 h at 37°C. MTT was then removed, and formazan crystals were dissolved in 100 µL DMSO. Absorbances were measured at 560 nm using a BioRad benchmarkplus microplate spectrophotometer. Cell viability was calculated as percentage of vehicle control using GraphPad Prism v.5.0.

Table 3. Compound Concentrations for Cytotoxicity Assay

Compound	Final Concentrations
Honey (incl. Artificial)	0.5, 1, 2, 3, 4, 5 % (w/v)
Bee Venom and Melittin	1, 2, 3, 4, 5 µg/mL
Quercetin	5, 10, 25, 30, 50 µM
Gallic Acid	5, 10, 25, 50, 75, 100 µM
Kaempferol	10, 25, 30, 50, 75, 100, 150 µM
Chrysin and Caffeic Acid	5, 10, 25, 50, 100, 150 µM

In order to assess the intrinsic MTT reductive capacity of honey, bee venom, melittin and honey compounds, control experiments were repeated with the honeys and compounds as above, however in the absence of cells. Assessment of honey MTT interaction was repeated 5 months later to determine whether honey age effected the reduction of MTT. To further examine whether honey could cause a shift in the MTT absorbance spectrum, PC3 cells were exposed to either thyme or artificial honey (5% w/v). Spectra analysis was performed using a spectrophotometer at wavelengths between 200 and 900 nm (Jenway, Staffordshire, UK). Data were plotted using Origin Pro v.8.0 software (Northampton, USA). Honey compound photographs were captured using a mobile phone camera (LG G2, 13 Megapixels, 4160x3120; 4:3).

2.5.2 Assessment using the Sulforhodamine B (SRB) Assay

The sulforhodamine B (SRB) assay is a colourimetric assay that is used to quantify cell cytotoxicity through the binding of SRB to cell proteins (Vichai *et al.*, 2006). Cells were treated as shown in **Table 3**. Following either a 24, 48 or 72 h treatment incubation, medium was aspirated and the cells were fixed with trichloroacetic acid (TCA) 10% and kept at 4°C for 1 h. Plates were washed gently with ddH_2O and dried. Cells were stained for 10 min by adding SRB

(0.4% SRB in 1.0% CH_3COOH) at room temperature. Unbound SRB stain was removed by washing three times in CH_3COOH (1.0%) until clear. After drying, SRB dye was solubilised by the addition of Tris base (100 µL, 10 mM, pH 10.5). Absorbances were measured at 510 nm with a 630 nm background, using a spectrophotometer. Cell viability was calculated as percentage of vehicle using GraphPad Prism v.5.0.

To further examine whether compounds had the intrinsic ability to cause a shift in the SRB absorbance spectrum, spectra analysis was performed using a spectrophotometer. Data were plotted using Origin Pro v.8.0 software.

2.5.3 Assessment using the Lactate Dehydrogenase (LDH) Assay

LDH is a cytoplasmic enzyme that is responsible for the catalytic conversion of pyruvate to lactate which converts NADH to NAD^+ (Decker *et al.*, 1988). When a cell dies, the membrane becomes permeable and intracellular LDH is released into the surrounding environment which can then be assessed as a marker of cytotoxicity. LDH release, and therefore compound cytotoxicity, can be calculated from a decrease in the reduced form of the coenzyme nicotinamide adenine dinucleotide (NADH) (Decker *et al.*, 1988). PC3 and DU145 cells were seeded in 24-well plates at a density of $3x10^4$ cells/well and allowed to adhere overnight. Either thyme or artificial honey solutions were added to each well so that final concentrations were 0, 1, 2, 3, 4 and 5% (w/v). After a 24 h treatment incubation, cell medium was removed and centrifuged at 1800 rpm for 4 min, then the supernatant was transferred and held on ice. PBS (650 µL) and medium sample (350 µL) with pyruvate (10 µL, 100 mM in PBS, determined using the Beer-Lambert law) were combined in a cuvette to establish a baseline using a spectrophotometer at 340 nm (Swinehart, 1962). NADH (20 µL, 10 mM in PBS) was then added to the cuvette, which was inverted and read for 300 s to obtain a kinetic profile. Data were analysed using Origin Pro v.8.0 software in order to obtain an equation for each profile. R^2 criteria for sample equation selection were set at no less than 0.9. Linear gradients for all samples were plotted using GraphPad Prism v.5.0.

To examine whether honey had intrinsic LDH activity, the above experiment was repeated with thyme honey (0 - 55% w/v) in the absence of cells. Data were plotted using Origin Pro v.8.0 software.

2.5.4 Assessment using the Trypan Blue (TB) Exclusion Assay

The loss of membrane integrity of non-viable cells allows for the permeation of dyes such as trypan blue (TB) into the cell. Based on this principle, it is possible to determine cell viability by the ability of a viable cell to exclude TB, and a dead cell to incorporate blue (Strober, 2001). PC3 and DU145 cells were seeded in 24-well plates at a density of $3x10^4$ cells/well. Either thyme or artificial honey solutions were added to each well so that final concentrations were 0, 1, 2.5 and 5% (w/v). Following treatment and incubation for either 24 or 48 h, medium containing non-adherent and dead cells was removed and set aside. Adhered cells were trypsinised and added to the medium/cell suspension. Samples were centrifuged at 1800 rpm for 4 min, at 21°C. Pellets were resuspended in growth medium (200 µL). TB (5 µL, 0.4%) was added to cell suspension (20 µL), resulting in a final concentration of 0.08%, as adapted from Strober (2001). Cells were counted in 8 quadrants of a haemocytometer with a light microscope, with cells stained blue counted as dead, and results were expressed as a percentage of total cells using GraphPad Prism v.5.0.

2.5.5 Assessment using Hoechst 33342 and Propidium Iodide

Propidium iodide (PI) exclusion works via the same principle as TB, since only viable cells are able to exclude the dye due to the preserved membrane integrity. Hoechst 33342 (HO) stains all cell nuclei, and therefore acts to determine a total cell number from which a percentage of PI stained cells can be calculated (Ciancio *et al.*, 1988). PC3 and DU145 cells in growth medium were seeded in 24-well plates at a density of $6x10^4$ cells/well. Thyme honey solutions were added to each well so that final concentrations were 0, 1, 2, 3, 4 and 5% (w/v). After 24 h, both HO (2 mg/mL, DMSO) and PI (5 mg/mL, PBS) were added to each well to give a final concentration of 2 µg/mL and 50 µg/mL, respectively. After 10 min, wells were gently aspirated to remove excess fluorescent agents. Ca^{2+}/Mg^{2+} supplemented-PBS was added immediately and cells were visualised (200 x magnification) using a Nikon Eclipse TI Inverted Microscope with a digital DS-U3 camera and a Nikon intensilight C-HGFI mercury lamp (Olympus, Japan). PI was observed using a TXRed filter (540 - 580 nm Ex., 600 - 666 nm Em.) for 500 ms. HO was observed using a DAPI filter (340 - 380 nm Ex., 435 - 485 nm Em.). Images were processed using NIS elements viewer software v.4.0. Following this, ImageJ v.1.48 (National Institute of Health: Bethesda, Maryland, USA) was used to quantify PI stained cells by the use of image thresholds. Data were expressed as mean percentage PI stained cells using GraphPad Prism v.5.0.

Hoechst nuclear staining was also used to assess cell death morphology. Cell nuclei were categorised as either normal, apoptotic (condensed, shrunken, fragmented or pyknotic) or late stage apoptotic/necrotic (swollen).

2.6 Assessment of Cell Adherence via an Attachment Assay

In order to assess the ability of honey to affect PC3 prostate cancer cell adherence *in vitro*, an attachment assay was used (Jin *et al.*, 2000). 96-well plates were coated with either collagen I (20 µL, 5 g/mL, 0.01M HCl in PBS) or fibronectin (20 µL, 5 µg/mL in PBS) and dried for 1 h. Plates were washed twice in PBS to remove excess protein. Bovine serum albumin (1%, PBS) (BSA) was added to each well for 1 h to block non-specific binding. Wells were again washed twice with PBS and left to dry for a further 2 h. $1x10^4$ PC3 or DU145 cells suspended in growth medium were added to each well. Cells were treated as shown in **Table 4** and were left to incubate for 30 and 60 min (PC3) or 30, 60 and 90 min (DU145). Wells were aspirated and washed twice in PBS. Growth medium with MTT (5 mg/mL) was added to each well and incubated for 3 h. Formazan crystals were solubilised in DMSO (100 µL) and absorbances were measured at 560 nm using a spectrophotometer. Data were expressed as a percentage of vehicle control adhered cells using GraphPad Prism v.5.0.

Table 4. Compound Concentrations for Adherence Assay.

Compound	Final Concentrations
Honey (incl. Artificial)	0.5, 1, 2, 3, 4, 5 % (w/v)
Bee Venom and Melittin	1, 2, 3, 4, 5 µg/µL
Quercetin	5, 10, 25, 30, 50 µM
Gallic Acid	5, 10, 25, 50, 75, 100 µM
Kaempferol	10, 25, 30, 50, 75, 100, 150 µM
Chrysin and Caffeic Acid	5, 10, 25, 50, 100, 150 µM

2.7 Scratch Wound Healing Assay

The following assay was adapted from Liang *et al.* (2007), and was used to demonstrate the *in vitro* migratory behaviour of PC3 prostate cancer cells in the presence of thyme honey. $3x10^5$ PC3 or DU145 cells were seeded in 6-well plates and left to adhere for ~30 h to establish a confluent monolayer. Following this, a scratch was made in the centre of the well using a 10 µL pipette tip. Plates were washed twice in growth medium to remove scratched cells, then medium was replaced. Scratches were identified using a Nikon Eclipse Ti inverted microscope

(Olympus) and captured using a Digital DS-U3 camera. Thyme honey was added to each well so that final concentrations were 0, 1, 2, 3, 4 or 5% (w/v). Plates were then incubated for 20 h. Following treatment incubation, scratches were identified, and photographs were captured (40 x magnification) in the same location as pre-treatment. Colour corrections were made using Microsoft Publisher 2013 (60% brightness, 60% contrast).

2.8 Boyden Chamber Migration Assay

PC3 cells are a more aggressive and invasive prostate cancer cell line compared to DU145, and therefore, PC3 cells were used for the assessment of migration and invasion (Kumar *et al.*, 2008). The Boyden chamber assay was used to determine the *in vitro* migration cells towards a chemoattractant through a layer of collagen I, when exposed to various treatments (Chen, 2005). Migration of cells from the upper compartment towards the lower compartment of the Boyden chambers was measured while the cells were exposed to various compounds at their maximal non-lethal doses (**Table 5**).

The undersides of the polycarbonate membranes (8 μm pore size) were coated with collagen I (15 μL, 150 μg/mL 0.01 M HCl in PBS), left to dry under sterile conditions for 1 h, washed twice with PBS and dried for a further 1 h. $30x10^3$ PC3 cells suspended in serum free growth medium were seeded into the upper chamber. The bottom chamber contained 5% serum-containing growth medium to facilitate migration as a chemoattractant. A pictorial example of the assay setup is shown (**Figure 1**). Both upper and lower chambers were treated for 48 h with compounds at concentrations shown in **Table 5**. Following treatment incubation, MTT solution (5 mg/mL) was added to the upper and lower chambers, and plates were incubated for a further 3 h. Following this, inserts were removed, and a cotton tip was used to remove formazan crystals from the upper chamber. The undersides of the inserts were exposed to DMSO (150 μL) to dissolve formazan crystals from migrated cells. Absorbances were measured at 560 nm using a spectrophotometer. Data were expressed as a percentage of migrated cells compared to a vehicle control, and were presented using GraphPad Prism v.5.0.

Table 5. Compound Concentrations for PC3 Migration Assay.

Compound	Concentration
Honey (incl. Artificial)	1 % (w/v)
Bee Venom and Melittin	5 µg/µL
Quercetin	25 µM
Gallic Acid	10 µM
Kaempferol	150 µM
Chrysin	100 µM
Caffeic Acid	50 µM

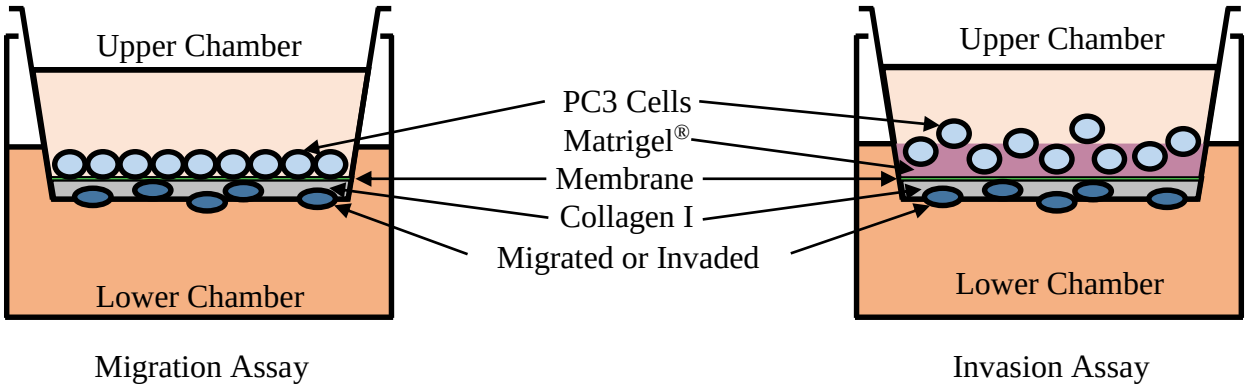


Figure 1. Boyden Chamber Setup for Migration and Invasion Assays.

2.9 Boyden Chamber Matrigel® Invasion Assay

This assay was used to determine the *in vitro* invasive ability of PC3 prostate cancer cells through Matrigel®, a solution containing extracellular matrix and basement membrane proteins resembling the structural parts of the tumour-surrounding stromal environment (Hughes *et al.*, 2010). Boyden chambers coated with Matrigel® were used to measure the invasion of cells from the inside of the insert towards the underside while exposed to various compounds at their maximal non-lethal doses (**Table 6**). The undersides of the polycarbonate membranes were coated with collagen I as per the methods stated in **2.8**. Upon coating, Matrigel® was kept on ice, and pipettes and pipette tips were cooled. Matrigel® (30 µL) was added to the upper chamber of each insert, and after 1 h, was washed with serum free growth medium. All further methods including cell seeding, treatment steps and the MTT assay were as described in **2.8**, with the exception that treatment duration was 72 h. A pictorial example of the assay setup is shown (**Figure 1**). Data were expressed as a percentage of migrated cells compared to a vehicle control, and were presented using GraphPad Prism v.5.0.

Table 6. Compound Concentrations for PC3 Invasion Assay.

Compound	Concentration
Honey (incl. Artificial)	0.5% (w/v)
Bee Venom and Melittin	5 µg/µL
Quercetin and Gallic Acid	10 µM
Kaempferol	150 µM
Chrysin	100 µM
Caffeic Acid	50 µM

2.10 High Performance Liquid Chromatography (HPLC)

2.10.1 Absorbance Spectrum of Standards

Stock solutions of standards were dissolved in DMSO (1 - 2 mg/mL). Both high (100:1, v/v) and low (5:950, v/v) concentrations of each solution were read using a spectrophotometer to produce a spectral profile. High concentrations were used to identify peaks in the upper visible and infrared (IF) spectrum (500 - 900 nm), whereas low concentrations were used to identify peaks in the lower visible and ultraviolet (UV) spectrum (190 - 500 nm), where most phenolic absorbance peaks are found (Holser, 2012). Maximal peaks for each standard were used later to identify compounds in each honey sample using HPLC.

Calibration curves for all compounds (1 - 100 µg/mL, 100% methanol (HPLC grade)) were made by injecting different concentrations of each compound standard. Peak areas were calculated versus standard concentrations to give a final concentration for individual compounds from each of the honey samples.

2.10.2 Sample Preparation for Chromatography.

2.10.2.1 Ethyl Acetate Extraction of Phenolic Compounds from Honey

With help from Dr. Allan Gamble and Mr. Sumit Dadhwal (Department of Pharmacy, University of Otago, New Zealand), the following method was used (adapted from Wahdan, 1998, which previously described the separation of caffeic acid and ferulic acid from honey). Thyme, manuka and honeydew honeys were dissolved in warmed MilliQ water to give a final

concentration of 20% (w/v) (10 mL or 15.48 g honey in 40 mL MilliQ). After extraction, dried samples were redissolved in methanol (600 µL).

2.10.2.1.1 Free Phenol Extraction

Each honey solution (50 mL) was adjusted to pH 3.5 using concentrated HCl. Sodium bisulfite (1 g) and ethyl acetate (50 mL) were mixed with the honey solution in a separating funnel. The mixture was shaken for 1 min so that phenolic compounds moved into the organic phase. The honey solution was poured off, and the ethyl acetate layer was transferred into a separate beaker for further extraction. A further 50 mL ethyl acetate was added to the honey solution. The above extraction steps were repeated 6 times. The ethyl acetate mixture (300 mL total) was concentrated using a rotary evaporator under vacuum (~240 mbar) at 30°C. Dried compounds were reconstituted (methanol:ethyl acetate, 1:1), and dried under nitrogen to be stored at -15°C.

2.10.2.1.2 Total Phenol Extraction

Extra preparation was required in order to hydrolyse phenolic compounds that were bound to sugars via ester bonds. Each honey solution (25 mL) was combined with NaOH (25 mL, 3N) and allowed to hydrolyse at room temperature for 4 h. Following this, pH was adjusted to 3.5 using concentrated HCl, and extraction was carried out as in **2.10.2.1.1**.

2.10.3 HPLC Analysis

Phenolic separation was carried out using a CBM-20Alite Prominence HPLC (Shimadzu Corporation, Japan) on a reversed-phase Gemini 5 µm C_{18} 110Å, LC column (150 x 4.6 mm) (Phenomenex, USA).

2.10.3.1 Gradient Elution

The following method was adapted from Kassim *et al*. (2010), and consisted of a gradient system used to identify and quantify selected phenols in honey samples, previously extracted as stated in **2.10.2.1**. The mobile phase was a binary solvent solution consisting of A = 0.25% formic acid and 2% methanol in water, and B = methanol. Each sample and standard (20 µL) was injected with a flow rate of 1 mL/min at 30°C. Honey phenolic compounds were detected

using a diode array where the UV absorption spectra were monitored at: 370 nm (kaempferol and quercetin), 325 nm (caffeic acid), and 270 nm (gallic acid and chrysin) as determined in **2.10.1**. The gradient method was as follows:

Table 7. HPLC Gradient System. A = 0.25% formic acid and 2% methanol in water, B = 100% methanol.

Time (min)	Flow Rate (mL/min)	A (%)	B (%)
0	1.00	90	10
15	1.00	90	10
20	1.00	60	40
30	1.00	55	45
50	1.00	40	60
52	1.00	20	80
60	1.00	10	90
62	1.00	90	10
65	1.00	90	10

Calibration curves of standards run in **2.10.1** were used to identify phenolic compounds within the honey samples. Identification was completed by comparing standard retention time and absorbance spectrums against samples. Sample spiking was used to increase the confidence of peak identification. To resolve quantification issues due to split and shouldered peaks, the valley-to-valley integration method was employed. Compound concentration (x) was expressed in µg/100 g of honey:

$$x = 0.6y\left(\frac{100}{w}\right)$$

Where: y = concentration of compound (µg/mL) determined by calibration curve in **Table 11.**

w = weight of honey used in the extraction method (15.48 g in free and 7.74 g in total phenol extracted honey)

2.11 Statistical Analysis

All experiments included 3 pseudo-repeats (n = 1). All results were expressed as mean percentage of control ± S.E.M using GraphPad Prism v.5.0. Data were considered significant at $p<0.05$ (*), where $p<0.05$ was the only significance probed for because the aims were to look for a difference between control and treatment only. The addition of added significance

($p<0.01$) may have led to the false assumption that one group was more significant than another to control; an interaction not independently investigated. For all cytotoxicity experiments, a two-way ANOVA followed by a Bonferroni post-hoc test ($p<0.05$) was used to determine the effect of both concentration and time on cell death. Where normality was assumed, outliers were identified using the Online GraphPad Prism Grubbs' Test, and were removed from data sets according to recommendations based on calculated critical Z values.

For migration and invasion assays, all honeys and compounds were investigated at the same time on the same plate, where all data were expressed and analysed as mean percentage of control ± SEM. However, data were presented on separate graphs in their respective chapters for ease of viewing. As a gatekeeper test, a one-way ANOVA was used to determine whether there was a statistical difference between groups ($p<0.05$). Following the acquisition of significance, individual unadjusted pairwise comparisons were made for each group against vehicle control, where an individual error rate for each comparison of 0.05 (α) was satisfactory. Therefore, the significance of each group to control was determined using a two-tailed Student's t-test ($p<0.05$).

2.11.1 Explanation for the Chosen Statistics

As with most cell culture studies, the present study was comprised of experiments repeated between 2 - 4 times which were recorded as independent experiments (n = 2 - 4). However, all independent experiments also comprised 3 intra-experimental replicates (pseudo-repeats). When presenting data error as SEM rather than standard deviation (SD), a greater sample size (n) will reduce the SEM; where $\mathrm{SEM} = \frac{\mathrm{SD}}{\sqrt{\mathrm{n}}}$. The temptation to falsely increase the sample size is an error that may occur in some cell culture experimental designs, where the inclusion of pseudo-replicates can reduce the SEM and increase statistical significance.

The ANOVA is the most common statistical test used to analyse data sets in pharmacology (Lew, 2007b). The ANOVA has many assumptions, one of which is the assumption of independence (Scariano *et al.*, 1987). Intra-experimental replicates are not independent as they were made from the same cell and drug stock, and therefore are commonly labelled pseudo-replicates or non-independent observations (Cumming *et al.*, 2007). For this reason, all statistical analyses for the determination of cytotoxicity in present study were made using data comprising between 2 to 4 mean values derived from their respective 3 pseudo-replicates.

In this study, the reductions in migration and invasion for each compound compared to untreated control were measured using a two-tailed Student's t-test, rather than the usual ANOVA. All migration and invasion assays were run simultaneously, where treatment with honey, phenolic compounds, bee venom or melittin, occurred on the same plate, however, the results were displayed individually in their respective chapters. It is uncommon for multiple two-tailed Student's t-tests to be used in biological statistics, and due to comparisons not being mathematically independent ('orthogonal'), a one-way ANOVA followed by a Dunnett's post-hoc test is often used. The decision to use two-tailed Student's t-tests for planned comparisons was taken as the Dunnett's post-hoc test may have a reduced power when making pairwise comparisons between multiple groups, therefore, may reduce the ability for a significant result to be found. The hypotheses for the present study were that each compound would decrease the migration and invasion of PC3 cells, independently of the other compounds. Nevertheless, a one-way ANOVA was still used as a gatekeeper test, to ensure that there were significant group differences to control ($p<0.05$). Following a significant result from the gatekeeper one-way ANOVA, an error rate of 0.05 from planned comparisons using individual two-tailed Student's t-tests was deemed satisfactory for each individual group comparison to control. As no hypotheses were made for comparisons between individual groups, two-tailed Student's t-tests were used to compare treated cells to untreated cells.

Chapter 3: Honey Results and Discussion

3.1 Honey pH

Since alterations in medium content or properties during an assay could affect the growth and metastatic properties of the cancer cells, it was important to assess the changes in growth medium over time when incubated with honey (Choi *et al.*, 2014). In order to determine whether thyme honey affected medium acidity, pH was measured over 24 h. Over a range of concentrations from 0 - 55% (w/v), it was evident that incubation of thyme honey with medium did result in a concentration and time-dependent decrease in pH (**Figure 2**). Administration of the highest concentration of 55% (w/v) thyme honey resulted in a pH decrease of 2.92 (from 6.92 to 4.00) over 24 h. However, as 5 % (w/v) was the maximal concentration of honey used in the present study, measuring a pH change over this concentration range was more important.

The pH of growth medium without honey was found to increase by 0.35 (from 7.53 to 7.88) over 24 h. The addition of 5% (w/v) thyme honey resulted in a smaller pH increase of 0.1 (from 7.44 to 7.540) over 24 h. The pH change comparing growth medium only to growth medium with 5% (w/v) thyme honey over 24 h (as used in later experiments) was only 0.44. This demonstrated that the pH of growth medium may be decreased with an increasing concentration of honey, but this change was so small that it was likely to be insignificant.

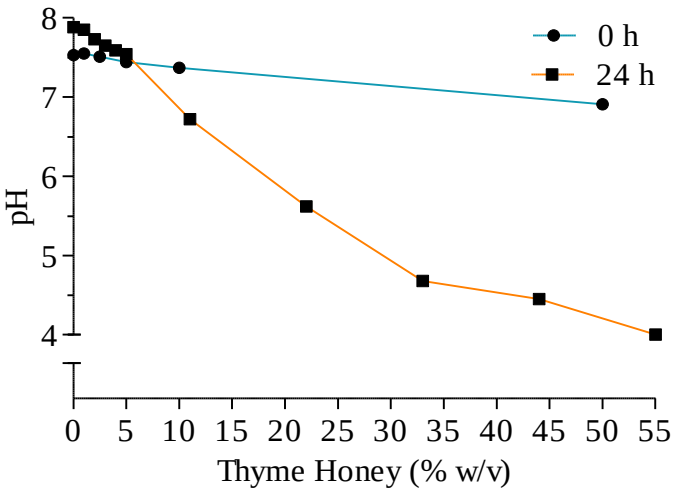


Figure 2. Effect of Incubation Time on Thyme Honey pH in Growth Medium. Thyme honey (0 - 55%, w/v) was dissolved in growth medium and incubated in 5% CO_2/95% O_2 humidified air at 37°C. pH was measured at both 0 and 24 h, (n = 1).

It has been well documented that pure honey has a low pH, and therefore the results from the present study were not novel. Acquarone *et al*. (2007) demonstrated that among 19 floral honeys, the pH varied between 3.25 and 4.00, however, they did not determine the effect of time on pH. A report by the U.S. Department of Agriculture presented data comparing the composition and properties of 490 floral honeys and 14 honeydew honeys (White, 1962). They showed that floral honey pH was 3.91 (3.42 - 6.10) and honeydew honey pH was 4.45 (3.90 - 4.88). The difference in honey pH could be due to compositional variations from floral origin, as well as the addition of enzymes and phenolics from insects (such as aphids) involved in the production of honeydew.

Fernandez-Cabezudo *et al*. (2013) reported that concentrations of manuka honey up to 5% (w/v) did not affect the *in vitro* osmolality or pH of B16.F1 melanoma cells. A concentration of 5% (w/v) honey used in the present study elicited a pH reduction of only 0.44 over 24 h (**Figure 2**), therefore it was assumed that any cellular changes observed by honey treatment would not be attributed to pH changes. In agreement with previous literature, all subsequent experiments were completed using honey at concentrations of 5% (w/v) or less.

3.2 Cytotoxicity of Honey

3.2.1 MTT Assay Optimisation

3.2.1.1 Honey Alone Reduction of MTT

In order to determine the effect of honey on PC3 and DU145 prostate cancer cell viability, the metabolic colourimetric MTT assay was used. MTT is reduced by mitochondrial dehydrogenase enzymes, therefore is was used as a measure of metabolic or mitochondrial activity as a proxy for viability or relative quality of metabolically active cells. It was particularly important to optimise this method, since it was used as part of the migration, invasion and adhesion assays used in later chapters.

Preliminary experiments involved the quantification of cell viability via the treatment of PC3 cells with thyme honey (0 - 7.50% w/v) for 0, 24 and 48 h (**Figure 3**). Administration of thyme honey resulted in a concentration and time-dependent decrease in cell viability over 24 and 48 h (<0.05). Interestingly, thyme honey treatment showed a concentration-dependent increase in MTT reduction, and therefore apparent cell viability, immediately after addition (0 h) with

7.50% (w/v) honey resulting in a 'viability' increase of 58.90% ($p<0.05$). A near 60% increase in cell viability at time 0 h is unrealistic, and suggested that either thyme honey was interfering with the MTT assay by reducing MTT on its own, or that thyme honey rapidly increased the mitochondrial activity of cells. This interference suggested that the MTT assay may underestimate cytotoxicity following honey treatment, and that the use of the MTT assay in this context would require that cell viability be normalised to a 0 h time point for all concentrations.

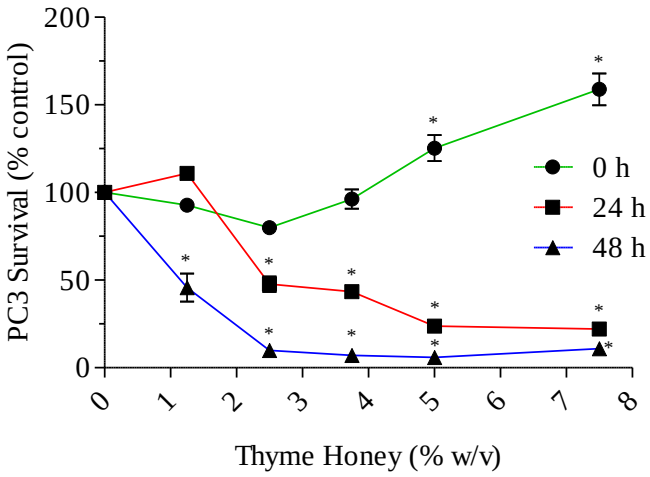


Figure 3. Underestimated Cytotoxicity using the MTT Assay. PC3 cells were seeded at $3x10^3$ cells/well and treated with 0 - 7.5% (w/v) thyme honey for 0, 24 and 48 h. After treatment, viability was established using the MTT assay, where MTT (5 mg/mL) was directly added to each well. Results were completed in triplicate, and values were expressed as mean percentage cell viability ± S.E.M (n = 3). Data were analysed using a one-way ANOVA followed by a Bonferroni post-hoc test, where $p<0.05$ was required for a statistically significant difference. * represents a significant difference between control (0 % w/v) and individual treatment.

It was important to further investigate whether the MTT assay was underestimating cytotoxicity, as maximal non-cytotoxic concentrations were to be used for migration and invasion assays. If cytotoxicity was underestimated, then a non-cytotoxic concentration may actually cause toxicity, and therefore, a reduction in migration or invasion may incorrectly be attributed to anti-metastatic activity.

Many articles have been published with the *in vitro* cytotoxicity of honey measured via the MTT assay. Of these publications, a large proportion involve the addition of MTT directly to honey-containing culture medium (Swellam *et al.*, 2003; Kannan *et al.*, 2009; El-Gendy, 2010; Ghashm *et al.*, 2010; Samarghandian *et al.*, 2011b; Samarghandian *et al.*, 2013). The reduction of MTT by honey as shown here suggested that many reports may have previously underestimated the cytotoxicity of honey. Samarghadian *et al*. (2011b) reported the IC_{50} of honey against the PC3 cell line to be 2.5% and 1.8% (w/v) at 24 and 48 h, respectively. The

method used for the determination of cell viability included the direct addition of MTT to honey-containing wells, and therefore IC_{50} values from the authors were comparable to those seen in **Figure 3** (2.5% at 24 h, 1.3% at 48 h). The 0 h evidence presented here may suggest that the authors underestimated the cytotoxicity of honey in the PC3 cell line. Later, a paper by Samarghadian *et al*. (2013) reported that the IC_{50}s of the honey against the PC3 cells line were 14%, 10% and 4% (w/v) at 24, 48 and 72 h, respectively. Due to the interaction between honey and the MTT, the increase in IC_{50} values presented by the authors over two years may indicate that different honeys may reduce MTT different amounts.

Methods involving the direct addition of MTT to honey-containing wells for cytotoxicity assessment have not been limited to prostate cancer cell studies, but have also be seen in oral squamous cell carcinoma cells and human osteosarcoma cells (Ghashm *et al.*, 2010), human and murine bladder cancer cell lines (Swellam *et al.*, 2003), breast, hepatocellular and colorectal (El-Gendy, 2010), renal cell carcinoma (Samarghandian *et al.*, 2011a) and human osteoblast cells (Kannan *et al.*, 2009).

To further assess the ability of honey to interfere with the accuracy of the MTT assay, characterisation of the reductive capacity of all four honeys in the absence of cells was completed (**Figure 4**). Upon addition of MTT to honey in the absence of cells, it was found that honey caused a concentration-dependent increase in MTT reduction compared to both artificial honey and a medium only control ($p<0.05$). Artificial honey is a mixture of sugar which mimics that found in honey and acts as a control for the effects of the honeys' sugar components alone. Compared to artificial honey, the following percentage increases in absorbance were measured for each honey at 5% (w/v): 421.80 ± 30.73% (thyme), 95.46 ± 4.02% (manuka), and 221.40 ± 5.49% (honeydew) ($p<0.05$). The 5% (w/v) artificial honey caused a 57.02 ± 4.77% increase in MTT absorbance at 560 nm ($p<0.05$). This was the first time that the reduction of MTT by honey in the absence of cells has been reported.

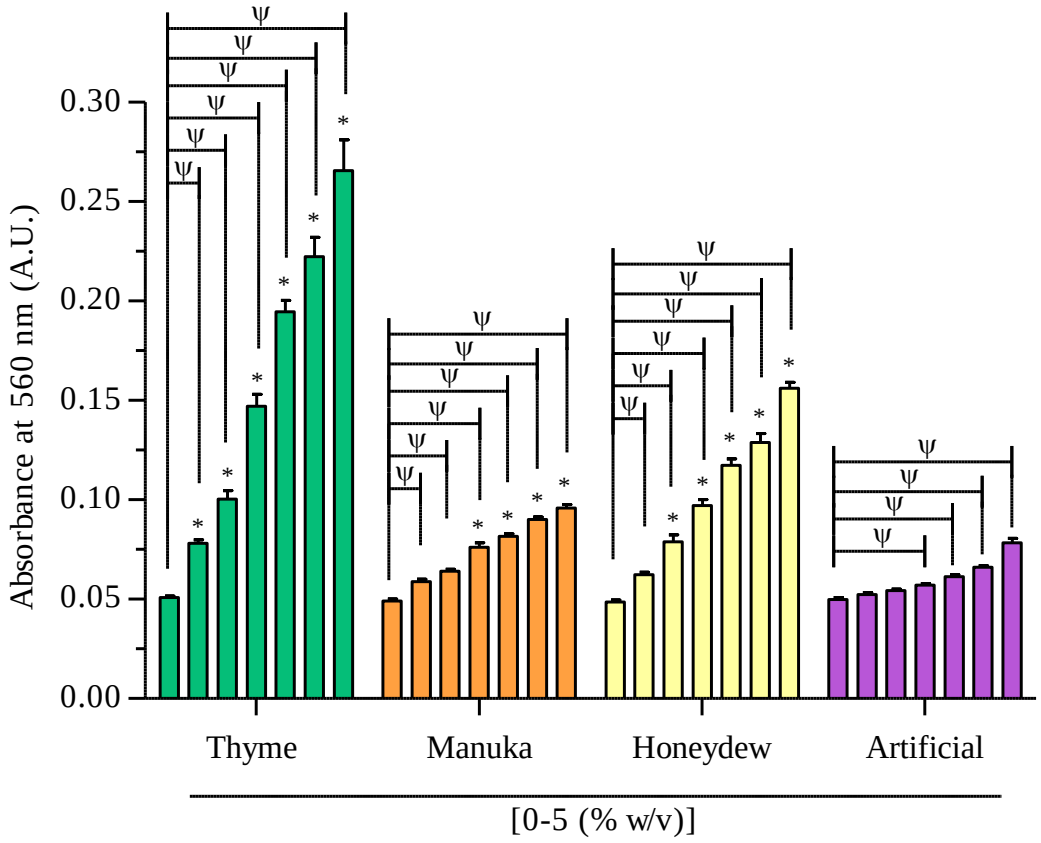


Figure 4. Intrinsic Reduction of MTT by Honey. In the absence of cells, MTT (5 mg/mL) was treated with either thyme, manuka, honeydew or artificial (sugar only) honey (0 - 5% w/v). After 3 h, formazan crystals were solubilised using DMSO. Absorbances were measured at 560 nm. Data were expressed as mean absorbance at 560 nm ± S.E.M (n = 9). Data were analysed using a two-way ANOVA followed by a Bonferroni post-hoc test, where $p<0.05$ was required for a statistically significant difference. * represents a significant difference between an individual treatment vs the corresponding artificial concentration. Individual honey data were further analysed using a one-way ANOVA followed by a Dunnett's post-hoc test, where $p<0.05$ was required for a statistically significant difference. ψ represents a significant difference between individual treatment and 0% (w/v).

One solution to the reduction of MTT by a treatment is the removal of medium before the addition of MTT. Many publications include this method to prevent the interaction between MTT and drugs. In light of this discovery, all future experiments involved the removal of honey before MTT addition.

The use of the artificial honey control demonstrated that sugars were partially responsible for the reduction of MTT. A reducing sugar is a sugar that contains an aldehyde group, or can produce one when in solution. Further, sugars containing ketones, such as fructose, have the ability to isomerize to form aldehydes in solution (Zhu *et al.*, 2010). Glucose, fructose, maltose and sucrose represent the major sugars found in honey (White *et al.*, 1980). All of these sugars, with the exception of sucrose, have the ability to become reducing sugars (Chargaff *et al.*,

1948). The ability for sugars to become reducing agents may explain the reduction of MTT seen for higher artificial honey concentrations (**Figure 4**). As well, it is known that the Maillard reaction can occur when reducing sugars come into contact with amino acids, both of which are found in honey (Maillard, 1912; White *et al.*, 1980). It has been reported that Maillard reaction products in honey are correlated to honey colour, phenolic content and anti-oxidant activity, and therefore may partially account for the total activity of honey both *in vitro* and *in vivo* (Brudzynski *et al.*, 2011a). Therefore, the presence of either reducing sugars or Maillard reaction products may be partially responsible for the reduction of MTT.

As artificial honey only accounted for a small percentage of MTT reduced by honey, honey may contain different compounds which are responsible. Further, the different reductive potentials of each honey suggested that different honeys may possess these compounds at different concentrations.

3.2.1.2 Change in Honey MTT Reductive Capacity Over Time

It has previously been reported that honey storage time can affect the composition of honey, specifically with regard to water, sugars, free phenolic compounds, and total anti-oxidant activity (Šarić *et al.*, 2012). To explore this, the concentration-dependent reduction of MTT by honey was measured before and after a 5 month period from the 17th March, 2014 until the 21st August, 2014 **(Figure 5**). Gradients of MTT absorbance for both fresh and aged honeys were calculated (**Table 8**). MTT reduction by honey was time- and concentration-dependent, with manuka showing the greatest percent increase in MTT reduction when comparing the two time points of 257.68%, followed by honeydew (170.46%) then thyme (139.71%).

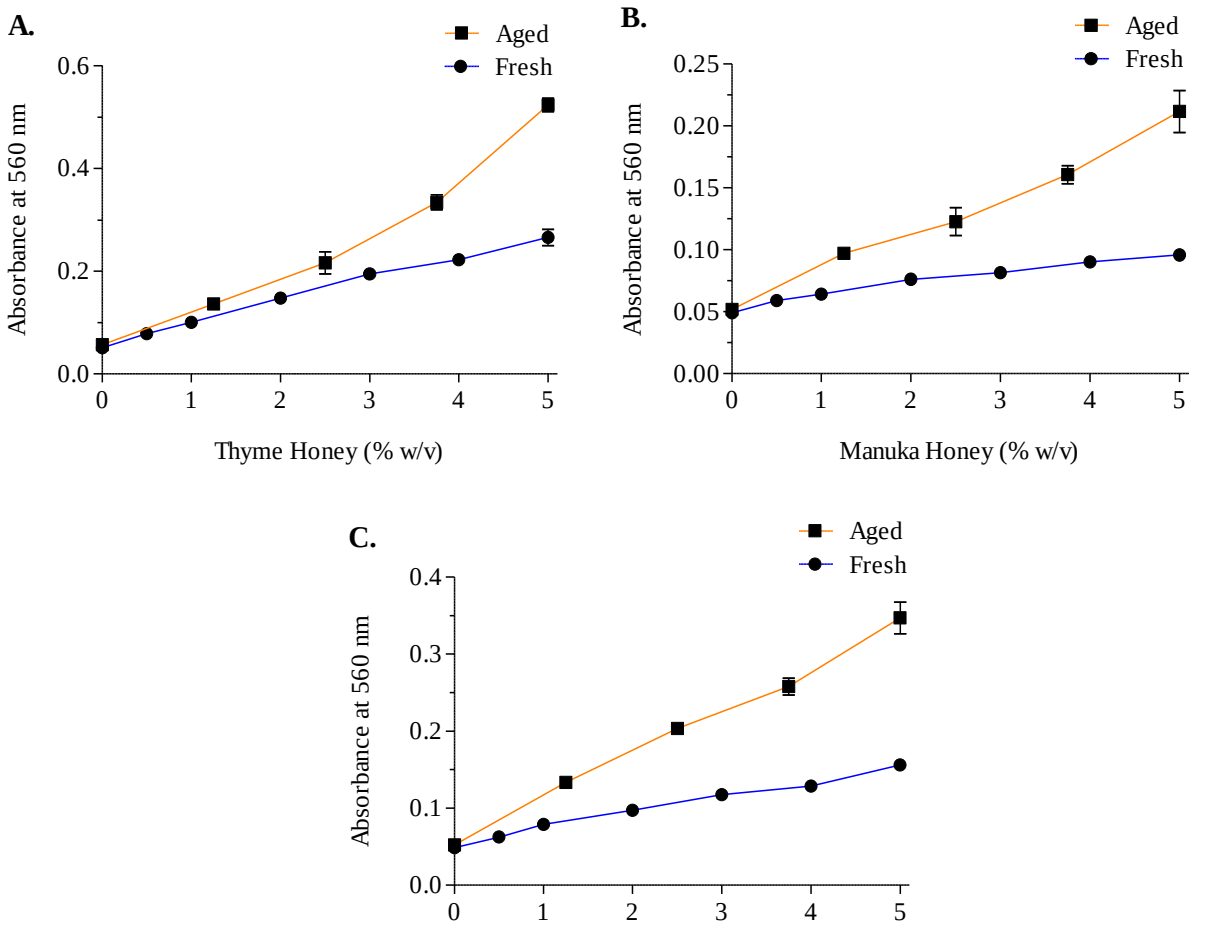


Figure 5. Effect of Honey Age on the Intrinsic Reduction of MTT. In the absence of cells, MTT was treated with either (0 - 5% w/v) (**A**) thyme, (**B**) manuka or (**C**) honeydew honey. Experimental setup was identical to **Figure 4**, however was conducted before and after a 5 month period. 'Fresh' represents honey measured at the beginning of the study (n = 9), 'aged' represents honey measured 5 months into the study (n = 3). Values were expressed as mean absorbance at 560 nm ± S.E.M.

Table 8. Efficacy of MTT reduction by Fresh Honey Compared to Aged. Linear regression gradients for fresh honey compared to aged (5 months) from **Figure 5.** Equations were calculated using GraphPad Prism v.5.0, ($R^2 > 0.9$). Linear regression analysis was carried out to test for a significant difference in gradients over time ($p<0.001$).

Honey	Fresh Gradient	Aged Gradient	Percent Change (%)	Significance
Thyme	0.04251	0.10190	139.71	p<0.001
Manuka	0.00898	0.3212	257.68	p<0.001
Honeydew	0.02038	0.05512	170.46	p<0.001

Contrary to results from Šarić *et al.* (2012) who demonstrated that storage time decreased multifloral honey phenolic content (88.6% reduction) and anti-oxidant content (72.5% reduction), the results in **Table 8** suggest that storage time could increase the reductive capacity

of honey. The changes in reductive capacity and anti-oxidant capacity of honey were assumed to be caused by changes in phenolic compounds, as assessed by Šarić *et al.* One explanation could be the release of phenolic aglycones; compounds that have had the sugar moiety removed. In some honeys, phenolic compounds exist bound to sugars as glycosides, with a lesser amount existing as free aglycones (Campillo *et al.*, 2015). Hui (2006) investigated the effect of temperature and storage time on foods containing high concentrations of phenolic compounds such as isoflavones. It was reported that over 5 months at 30°C, aglycones increased from 1% to 80% of the total isoflavone content. A proposed hypothesis for this may involve the release of phenolic aglycones by hydrogen peroxide in honey. The oxidation of glucose from glucose oxidase in honey produces hydrogen peroxide, which in turn can produce hydroxyl radicals (Brudzynski *et al.*, 2011b). Many radicals including hydroxyl radicals, hydrogen atoms and hydrated electrons may produce phenolic aglycones through the breaking of their glycosidic bonds (Hussein *et al.*, 2011). Further, the enzyme β-glucosidase that is present in honey, can hydrolyse glycosides to aglycones (Low *et al.*, 1986; Sporns *et al.*, 1992; Kaya *et al.*, 2008). If honey-derived phenolics are responsible for the reductive capacity of honey, then the release of phenolic compounds as aglycones from honey sugars over time could explain the increased reduction of MTT seen in **Figure 5**. Further, as sugars may possess reductive capacity, the released sugars may also explain the increased reduction of MTT over time.

3.2.1.3 Absorbance Spectra of MTT in the Presence of Honey

In order to use the MTT assay to quantify the cytotoxicity of cells treated with honey, assessment of the MTT absorbance spectrum was required to ensure the maximal absorbance (λ max) still occurred at 560 nm. Previous reports have indicated that alterations in pH or the incomplete removal of medium, serum or drug can reduce absorbances by causing a shift of the absorption maxima (Plumb *et al.*, 1989; Sieuwerts *et al.*, 1995).

Absorbance profiles were constructed for PC3 cells treated with either 5% (w/v) thyme or artificial honey for 24 h (**Figure 6**). Thyme honey was selected based on the increased MTT interaction it demonstrated compared to other honeys (**Figure 4**). The results suggested that honey did not cause a shift in the absorbance spectrum of solubilised formazan crystals, and therefore, MTT could be used for the determination of cell viability, given that honey was removed prior to MTT addition.

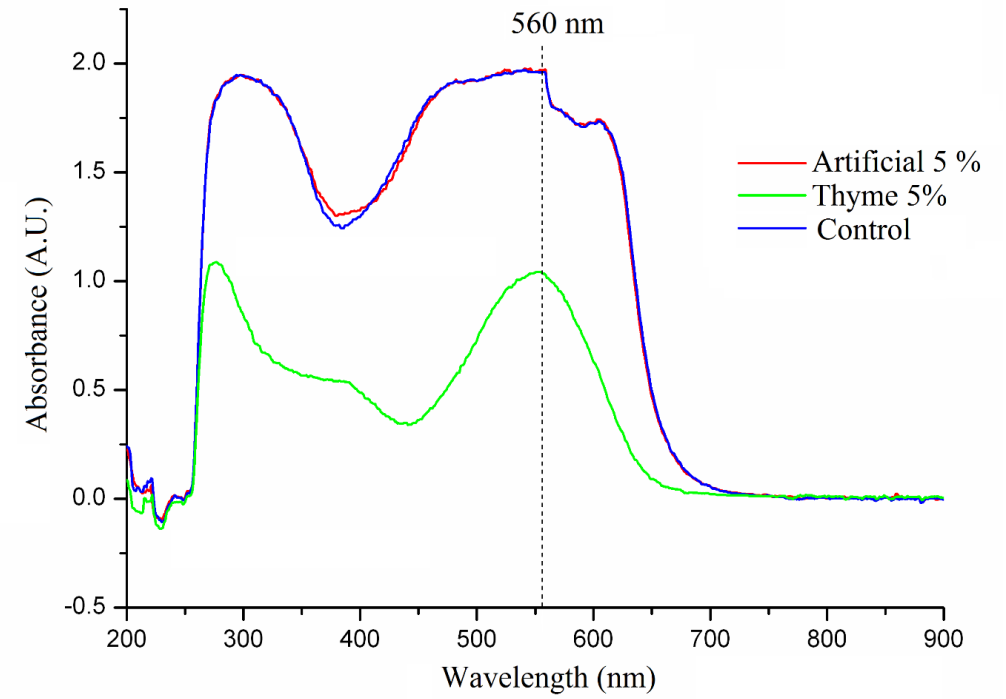


Figure 6. Absorbance Spectra of MTT from Honey Treated PC3 Cells. PC3 cells seeded at a density of $3x10^4$ cells/well (24-well plate) were treated with either 5% thyme honey, artificial honey, or a medium only control for 24 h. After treatment, honey was removed, and MTT (5 mg/mL) was added for 3 h. An absorbance spectral profile was constructed of the DMSO solubilised formazan crystals using a spectrophotometer.

3.2.2 Cytotoxicity of Honey Measured by the MTT Assay

The MTT assay was used to determine the maximal non-cytotoxic concentrations of honey for use in the migration and invasion assays. In PC3 cells, thyme, manuka, honeydew and artificial honey decreased cell viability in a concentration-dependent manner (**Figure 7**) ($p<0.05$). No time-dependent effect on cell viability was detected, using a two-way ANOVA followed by a Bonferroni post-hoc test. At 24 h, administration of 2.00% (w/v) honeydew honey resulted in a mean cell viability of 34.74 ± 6.03%, whereas thyme honey and manuka resulted in 39.90 ± 10.17%, and 61.14 ± 4.00%, respectively ($p<0.05$). To achieve a similar percentage cell viability at 24 h through the administration of artificial honey, a higher concentration of 4.00% (w/v) was required (38.62 ± 6.77%) ($p<0.05$). The maximal non-cytotoxic concentration for thyme, manuka and honeydew honey was 1.00% (w/v) at 24, 48 and 72 h. In contrast, the maximal non-cytotoxic concentration of artificial honey was 2.50% (w/v) at 24 h, and 1.25% (w/v) at 48 and 72 h.

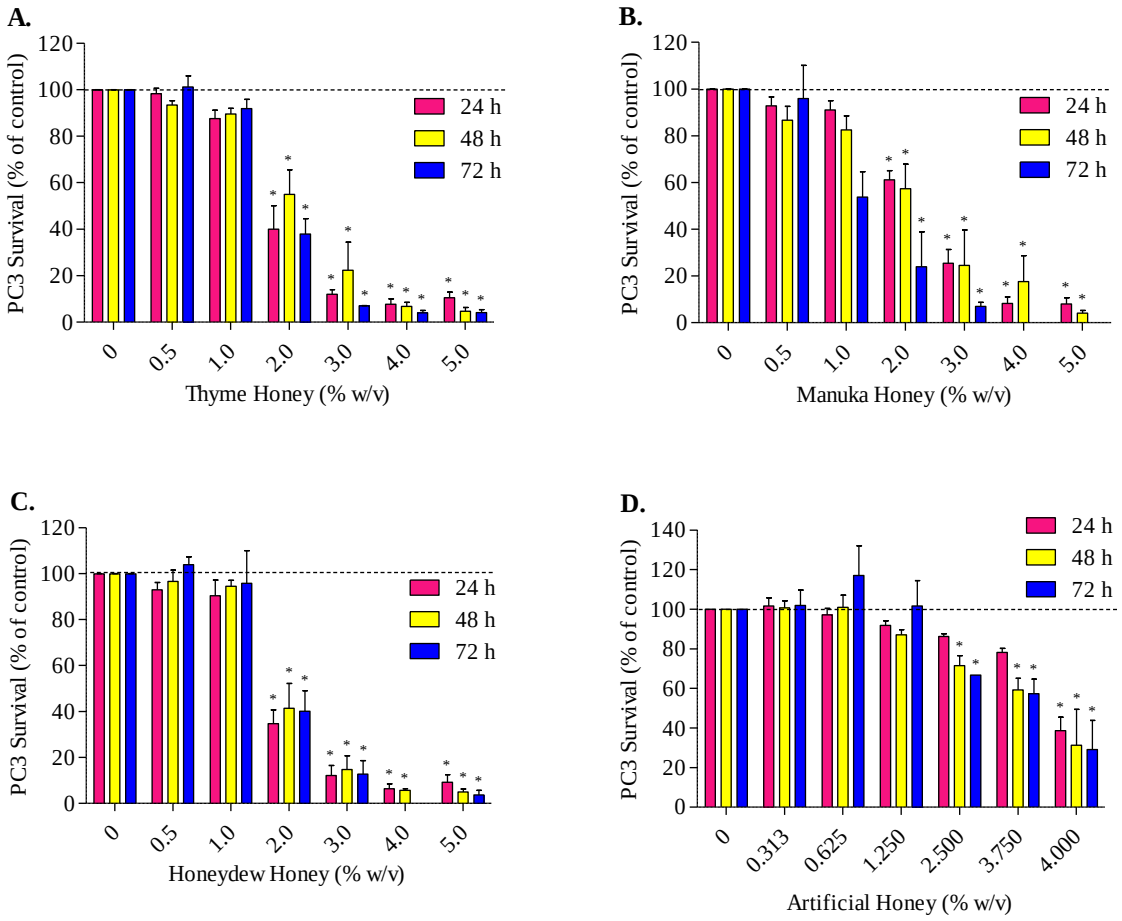


Figure 7. Honey Cytotoxicity Quantified by the MTT Assay in PC3 Cells. PC3 cells were seeded at a density of $3x10^3$ cells/well and treated with 0 - 5% (w/v) (**A**) thyme, (**B**) manuka, (**C**) honeydew or (**D**) artificial honey for 24, 48 and 72 h. After treatment, honey was removed and viability was established via the MTT assay. Experiments were completed in triplicate, and values were expressed as mean percentage cell viability ± S.E.M (n = 3). Data were analysed using a two-way ANOVA followed by a Bonferroni post-hoc test, where $p<0.05$ was required for a statistically significant difference. * represents a significant difference between control and individual treatment.

In DU145 cells, thyme, manuka, honeydew and artificial honey also decreased cell viability in a concentration-dependent manner **(Figure 8**) ($p<0.05$). Again, no time-dependent effect on cell viability was detected, using a two-way ANOVA followed by a Bonferroni post-hoc test. At 24 h, administration of 2.00% (w/v) honeydew honey resulted in a mean cell viability of 58.45 ± 2.73%, whereas thyme honey and manuka resulted in 64.94 ± 5.81%, and 65.69 ± 5.99% cell viability, respectively ($p<0.05$). To achieve a similar percentage cell viability at 24 h through the administration of artificial honey, a higher concentration of 5.00% (w/v) was required (64.70 ± 9.85%) ($p<0.05$). The maximal non-cytotoxic concentration for manuka honey was 0.5% (w/v), whereas thyme and honeydew honey was 1.00% (w/v) at 24, 48 and 72 h. In contrast, the maximal non-cytotoxic concentration of artificial honey was 3.00% (w/v) at

24, 48 and 72 h. Both thyme and honeydew honey were more cytotoxic towards PC3 cells, however, the cytotoxicity of manuka or artificial honey was comparable across both cell lines.

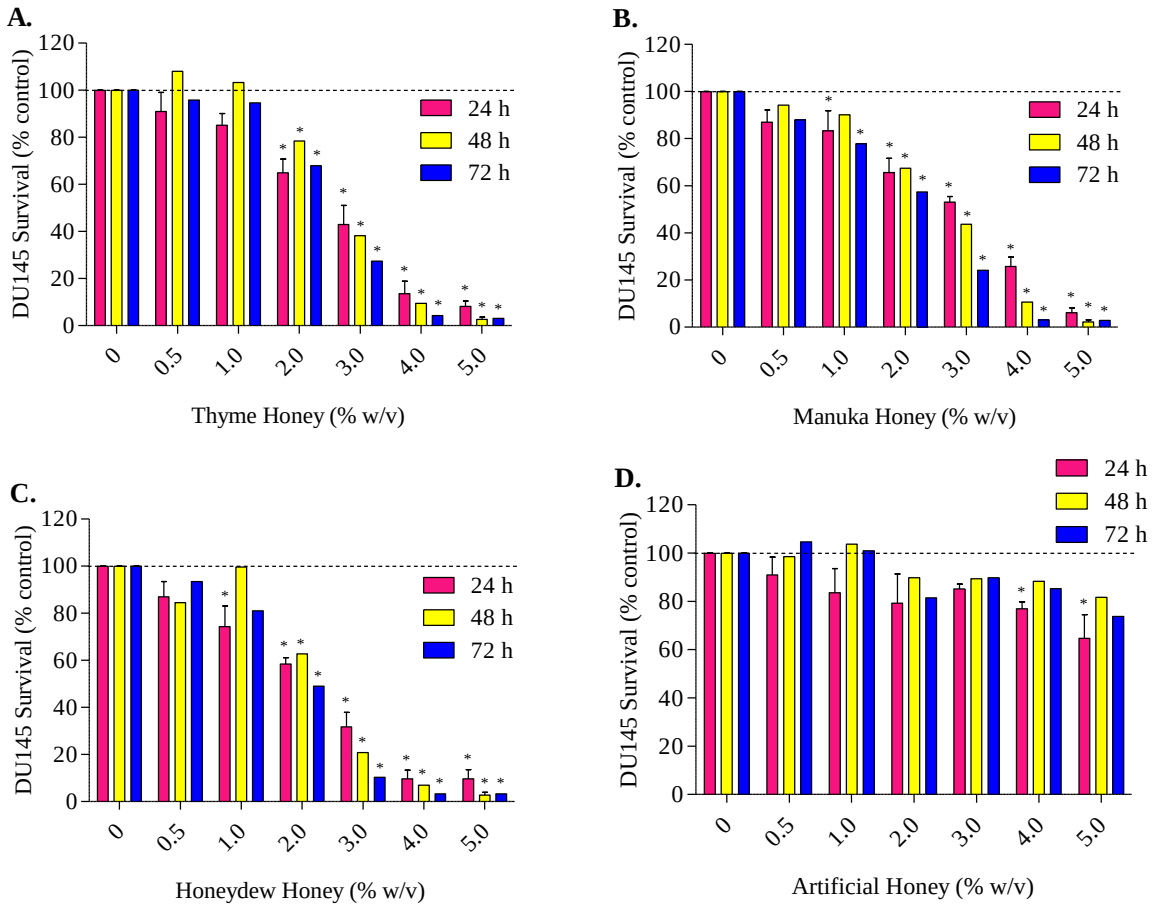


Figure 8. Honey Cytotoxicity Quantified by the MTT Assay in DU145 Cells. DU145 cells were seeded at a density of $5x10^3$ cells/well and treated with 0 - 5% (w/v) (**A**) thyme, (**B**) manuka, (**C**) honeydew or (**D**) artificial honey for 24, 48 and 72 h. After treatment, honey was removed and viability was established via the MTT assay. Experiments were completed in triplicate, and values were expressed as mean percentage cell viability ± S.E.M (24 h, n = 3; 48 and 72 h, n = 1). Data were analysed using a two-way ANOVA followed by a Bonferroni post-hoc test, where $p<0.05$ was required for a statistically significant difference. * represents a significant difference between control and individual treatment.

Few studies have previously been conducted for the assessment of honey cytotoxicity on PC3 or DU145 cells. Samarghadian *et al*. (2011, 2013) have presented data for the cytotoxicity of honey towards PC3 cells, however, due less than optimal use of the MTT assay, as explained above, their data were determined to be inaccurate and therefore were not compared to the findings from the present study. Tsiapara *et al*. (2009) reported the cytotoxicity of Greek thyme, fir and pine honey in PC3 cells. The MTT method they used included the removal of honey-containing medium before the addition of MTT, and therefore in the context of the present

study, their conclusions were assumed to be more accurate. The authors concluded that thyme honey significantly reduced the viability of PC3 cells at concentrations between 0.2 - 125 µg/mL. However, the data presented did not demonstrate an overall decrease in viability, since although 25 µg/mL thyme honey caused a 20% reduction in viability, the viability was restored by the administration of 125 µg/mL honey. This may suggest that although medium containing a high concentration of honey was removed from the wells, remaining honey may still interact with the MTT assay. Alternatively, honey may alter mitochondrial function and cause a biphasic effect on PC3 cell viability as determined using the MTT assay. No other studies using the MTT assay were found for comparison.

3.2.3 Cytotoxicity of Honey Measured by the SRB Assay

In order to confirm the cytotoxicity of honey as determined via the MTT assay, the colourimetric SRB assay was also used. SRB is a protein-determination assay that is used to indirectly determine cell number, and therefore cytotoxicity. No interaction between honey and the SRB assay was investigated, as the assay method involved multiple washing steps before the addition of SRB.

In PC3 cells, thyme, manuka, honeydew and artificial honey decreased cell viability in a concentration- and time-dependent manner (**Figure 9**) ($p<0.05$). At 24 h, administration of 2.00% (w/v) thyme honey resulted in a mean cell viability of 33.92 ± 3.07%, whereas manuka and honeydew honey resulted in 50.33 ± 2.34% and 40.26 ± 1.39%, respectively ($p<0.05$). To achieve a similar percent cell viability at 24 h through the administration of artificial honey, a concentration of 5.00% (w/v) was required (65.47 ± 2.40%) ($p<0.05$). The maximal non-cytotoxic concentration for thyme honey was 0.50% (w/v), whereas manuka and honeydew honey was <0.50% (w/v) at 24, 48 and 72 h. In contrast, the maximal non-cytotoxic concentration of artificial honey was 3.00% (w/v) at 24, and 2.00% (w/v) at 48 and 72 h.

In DU145 cells, thyme, manuka, honeydew and artificial honey also decreased cell viability in a concentration- and time-dependent manner **(Figure 10)** ($p<0.05$). At 24 h, administration of 2.00% (w/v) honeydew honey resulted in a mean cell viability of 76.08 ± 4.46%, whereas thyme and manuka honey resulted in 84.73 ± 1.68% and 83.58 ± 1.33%, respectively ($p<0.05$). To achieve a similar percent cell viability at 24 h through the administration of artificial honey, a concentration of 5.00% (w/v) was required (75.69 ± 14.26%) ($p<0.05$). The maximal non-cytotoxic concentration for thyme, manuka and honeydew honey was 1.00% (w/v) at 24, 48

and 72 h. In contrast, the maximal non-cytotoxic concentration of artificial honey was 4.00% (w/v) at 24 and 48 h, and 3.00% (w/v) at 72 h. Similar to the MTT assay, thyme and honeydew honey were more cytotoxic towards PC3 cells compared to DU145 cells, however, the cytotoxicity of manuka or artificial honey were comparable between both cell lines.

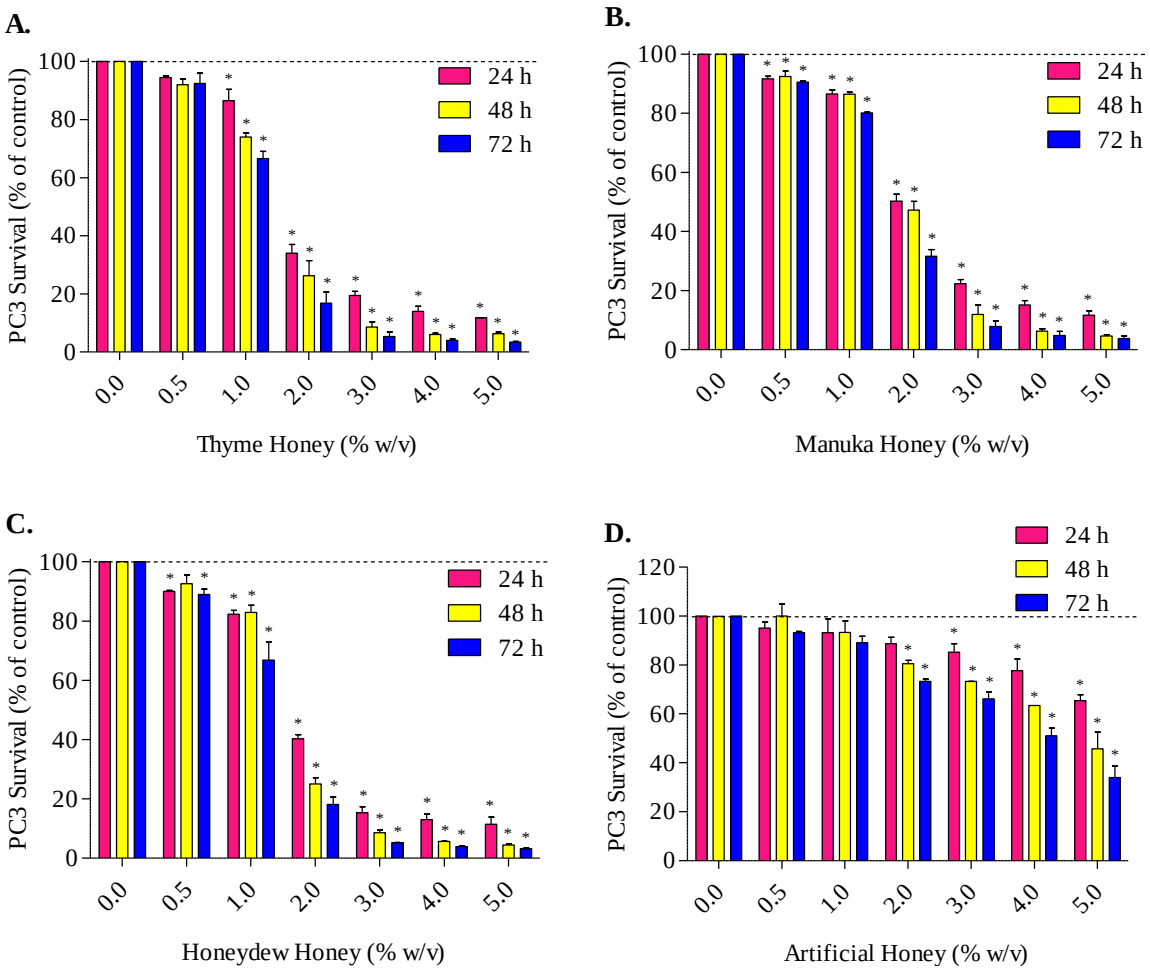


Figure 9. Honey Cytotoxicity Quantified by the SRB Assay in PC3 Cells. PC3 cells were seeded at a density of $3x10^3$ cells/well and treated with 0 - 5% (w/v) (**A**) thyme, (**B**) manuka, (**C**) honeydew or (**D**) artificial honey for 24, 48 and 72 h. After treatment, honey was removed and viability was established via the SRB assay. Experiments were completed in duplicate, and values were expressed as mean percentage cell viability ± S.E.M (n = 2). Data were analysed using a two-way ANOVA followed by a Bonferroni post-hoc test, where $p<0.05$ was required for a statistically significant difference. * represents a significant difference between control and individual treatment.

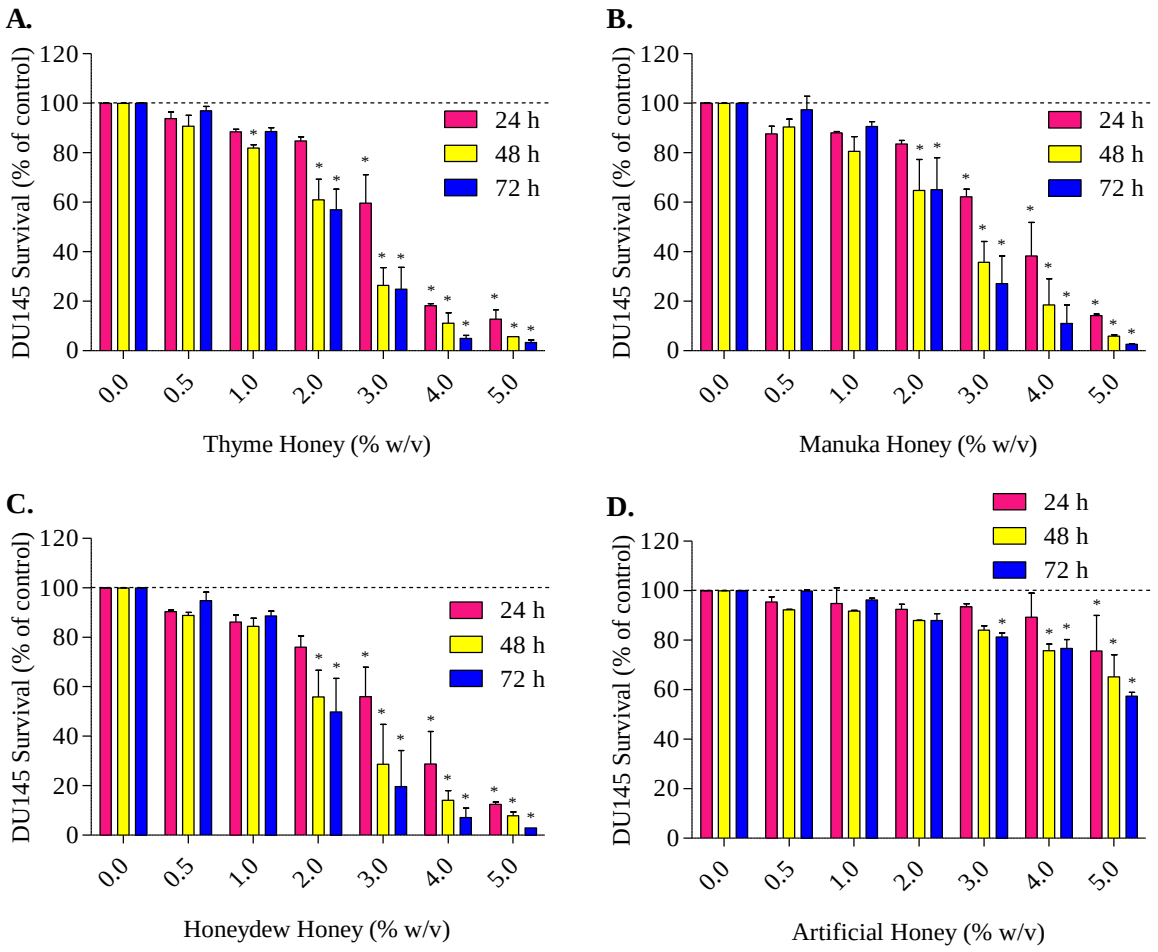


Figure 10. Honey Cytotoxicity Quantified by the SRB Assay in DU145 Cells. DU145 cells were seeded at a density of $5x10^3$ cells/well and treated with 0-5% (w/v) (**A**) thyme, (**B**) manuka, (**C**) honeydew or (**D**) artificial honey for 24, 48 and 72 h. After treatment, drug was removed and viability was established via the SRB assay. Experiments were completed in duplicate, and values were expressed as mean percentage cell viability ± S.E.M (n = 2). Data were analysed using a two-way ANOVA followed by a Bonferroni post-hoc test, where $p<0.05$ was required for a statistically significant difference. * represents a significant difference between control and individual treatment.

3.2.4 Cytotoxicity of Honey Measured by the LDH Assay

To further characterise the cytotoxicity of honey towards prostate cancer cells, the lactate dehydrogenase (LDH) assay was used to detect cells undergoing necrotic or late-stage apoptotic cell death. Throughout the subsequent cytotoxicity discussions, the terms late-stage apoptosis and secondary necrosis have been used interchangeably, and they both represent the same process (Poon *et al.*, 2010). In response to cellular damage, LDH is released into the surrounding environment where it can be detected. Because LDH is highly stable in cell culture medium, its release profile has been used to determine necrotic and late-stage apoptotic cell death (Stoddart, 2011; Kaja *et al.*, 2015). As honey had previously interfered with the MTT

assay, it was important that a potential interaction with the LDH assay was probed for, prior to its use.

Spectrophotometric analysis suggested that thyme honey caused a concentration-dependent decrease in both NADH and NAD^+ in the absence of cells (**Figure 11)**. NADH was treated with 0 - 55% (w/v) thyme honey and pyruvate and it was shown that thyme honey caused a concentration-dependent decrease in NADH (340 nm) and NAD^+ (260 nm). Administration of 11% (w/v) thyme honey resulted in a complete abolishment of NAD^+ from the assay (**Figure 11A**).

In a normal situation, the inversely proportional stoichiometric equilibrium between NADH and NAD^+ means that a decrease in NADH would increase NAD^+ (Farrington *et al.*, 1980). Surprisingly, thyme honey caused a concentration-dependent decrease in both NADH and NAD^+. The balance between NADH and NAD^+ in mitochondrial cell energetics is important for the regulation of cancer progression (Santidrian *et al.*, 2013). Jaganathan and Mandal (2010) reported that the cytotoxic mechanism of honey may involve the dysfunction of the mitochondria, however they demonstrated this was due to the depletion of intracellular non-thiol proteins. A depletion of both NAD species and resultant imbalance of the NADH/NAD^+ redox equilibrium by honey may suggest a mechanism of action for honey involving mitochondrial dysfunction. Mitochondrial alterations by honey may account for the problems with the MTT assay, however, as cellular NAD species and mitochondrial function were not investigated further in the present study, no conclusions about this mechanism could be made.

Despite an absence of literature regarding an interaction of this assay with honey, this was not the first time that an interaction between honey constituents and the LDH assay has been made. Quercetin, a prevalent flavonoid in many honeys, was reported to inhibit LDH activity, with the authors demonstrating that 1.66 µg quercetin resulted in a complete inhibition of LDH and pyruvate kinase, and therefore concluding that the LDH assay was not suitable for cytotoxic assessment of cells treated with quercetin (Grisolia *et al.*, 1975). Redox enzymes, such as oxidases and dehydrogenases, may utilise NADH or NAD^+ as a cofactor, therefore the concentration-dependent depletion of both NADH and NAD^+ by honey might be explained due to the presence of enzymes. In addition, fructose in honey may attenuate the redox reactions of NADH/NAD^+, and therefore may prevent or confound the results (Tygstrup *et al.*, 1965; Onyesom, 2008).

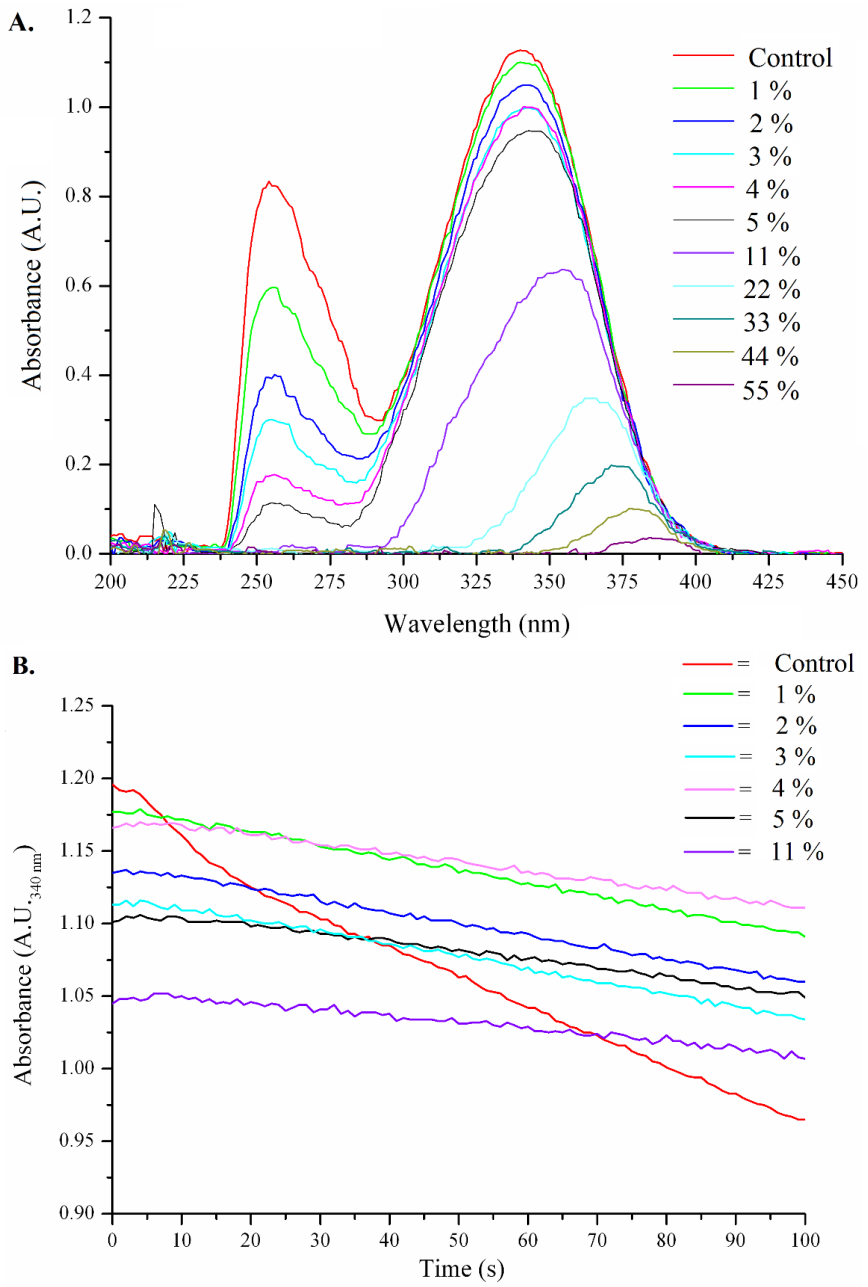


Figure 11. Effect of Thyme Honey on Spectra Profile of NADH and NAD$^+$. Honey was dissolved in PBS and supplemented with pyruvate (1 mM) to mimic LDH conditions. After the addition of NADH (200 µM), samples were read immediately using a spectrophotometer. (**A**) In the absence of cells, spectral analysis was conducted for NADH treated with 0 - 55% (w/v) thyme honey. (**B**) Rate of NADH (340 nm) oxidation treated with 0 - 11% (w/v) thyme honey.

As with all the assays, it was important that controls were performed in order to verify that honey did not interfere with the assay at the concentrations used. The maximal effective concentration of 5% (w/v) honey used throughout the current study resulted in a concentration-dependent increase in the oxidation of NADH in the absence of cells (**Figure 10B**). However, 5% (w/v) honey did not fully abolish either NADH or NAD$^+$, therefore the LDH assay was considered suitable to identify the presence of necrotic or late-apoptotic cell death, although it

was not used to quantify cytotoxicity. It was important to account for the increase in NADH oxidation, as it might have confounded the cytotoxic conclusions of the assay. Therefore, the LDH activity of prostate cancer cells treated with thyme honey was normalised to each corresponding honey concentration in the absence of cells, as shown in **Figure 11**, to account for the oxidation of NADH.

The results reported from the LDH assay were not used to quantify the cytotoxicity of honey in the present study; however they were used to characterise the mechanism of action. Thyme honey caused a concentration-dependent increase in LDH release from both PC3 and DU145 cells, seen as in increase in LDH activity (**Figure 12**) ($p<0.05$) at 24 h. Administration of 5.00% (w/v) thyme honey resulted in a 103.50 ± 2.20% (PC3) and 85.56 ± 6.87% (DU145) increase in LDH activity ($p<0.05$). Administration of 5.00% (w/v) artificial honey had a lesser effect on LDH activity, with a 14.23 ± 1.13% increase in PC3 cells, and a 19.20% reduction in DU145 cells ($p<0.05$). The percent of cell cytotoxicity could not be quantified as the LDH activity of control cell lysate was not measured, however, the increase in LDH activity was suggestive of necrotic or late-stage apoptotic cell death. If 5% (w/v) was assumed to produce a maximal cytotoxicity and LDH release, as seen in the MTT and SRB assay, then the IC_{50} of thyme honey would lie between 2 - 3% (w/v) for both cell lines.

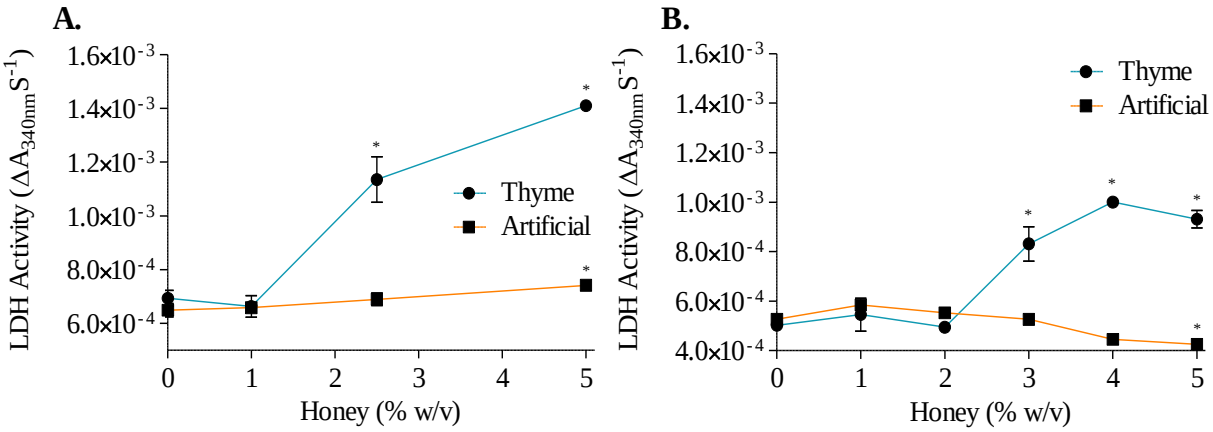


Figure 12. Honey Cytotoxicity Quantified by the LDH Assay. (**A**) PC3 and (**B**) DU145 cells were seeded at a density of $3x10^3$ and $5x10^3$ cells/well, respectively. Cells were treated with either thyme or artificial honey (0-5% w/v) for 24 h. NADH (200 µM) was added to LDH-containing cell medium with pyruvate (1 mM) and read at 340 nm using a spectrophotometer for 300 s. Inclusion criteria for individual kinetic data were *set al* $R^2>0.9$. Experiments were completed in duplicate, and values were expressed as mean percentage cell viability ± S.E.M (n = 2). Data were processed using a two-way ANOVA followed by a Bonferroni post-hoc test, where $p<0.05$ was required for a statistically significant difference. * represents a significant difference between control and individual treatment.

Fauzi *et al*., (2011) showed that tualang honey caused an increase in LDH release in breast (MCF-7 and MDA-MB-231) and cervical (HeLa) cancer cell lines, however, was non-cytotoxic in a normal breast cancer cell line (MCF-10A). At 72 h, the IC_{50} of tualang honey was reported between 2.4 - 2.8 % (w/v); similar to that seen in **Figure 12**. Authors commented that LDH release was due to secondary necrosis, and further supported their claims of apoptotic cell death via flow cytometry and the activation of caspase-3/7 and -9 (Fauzi *et al.*, 2011).

3.2.5 Cytotoxicity of Honey Measured by the Trypan Blue Exclusion Assay

It was observed that both PC3 and DU145 cells became detached after the administration of a wide range of honey concentrations. If honey causes a loss of cell adhesion, then all previous cytotoxicity assays may have overestimated their cytotoxicity, as cell adhesion was required for the detection of cell viability. To investigate this, the TB exclusion assay was used, which involves total cell counting, in which both attached and detached cells are assessed. When a cell dies, the loss of membrane integrity and increased permeability allows for the entry of the TB dye, and blue and uncoloured cells can be counted. The TB assay has been suggested to be more sensitive than indirect colourimetric assays such as the MTT or SRB assay, as it involves the direct counting of cells (Pichichero *et al.*, 2010).

In contrast to all previous cytotoxicity assays, with the exception of the LDH assay, the administration of 1% or less (w/v) thyme honey for 24 or 48 h did not show any cytotoxicity in either PC3 or DU145 cells with the TB assay (**Figure 13**). 5% (w/v) thyme honey resulted in an almost total cell death in PC3 cells at 48 h. Thyme honey was more cytotoxic towards PC3 cells, with the 5% (w/v) thyme honey resulting in a further 48.94 ± 4.73% (24 h) and 34.34 ± 21.88% (48 h) decrease in cell viability compared to DU145 cells ($p<0.05$). Interestingly, thyme honey only caused a time-dependent decrease in cell viability in PC3 cells ($p<0.05$), however, an increase in sample size may have shown one for DU145 cells. In further contrast to the previous cytotoxicity assays used, 5% (w/v) artificial honey caused no significant reduction in cell viability at 24 of 48 h, in either cell line compared to control.

In agreement with the LDH assay results, the TB assay findings suggested a cell death mechanism for honey involving either necrosis or secondary necrosis, although an apoptotic pathway may have preceded this.

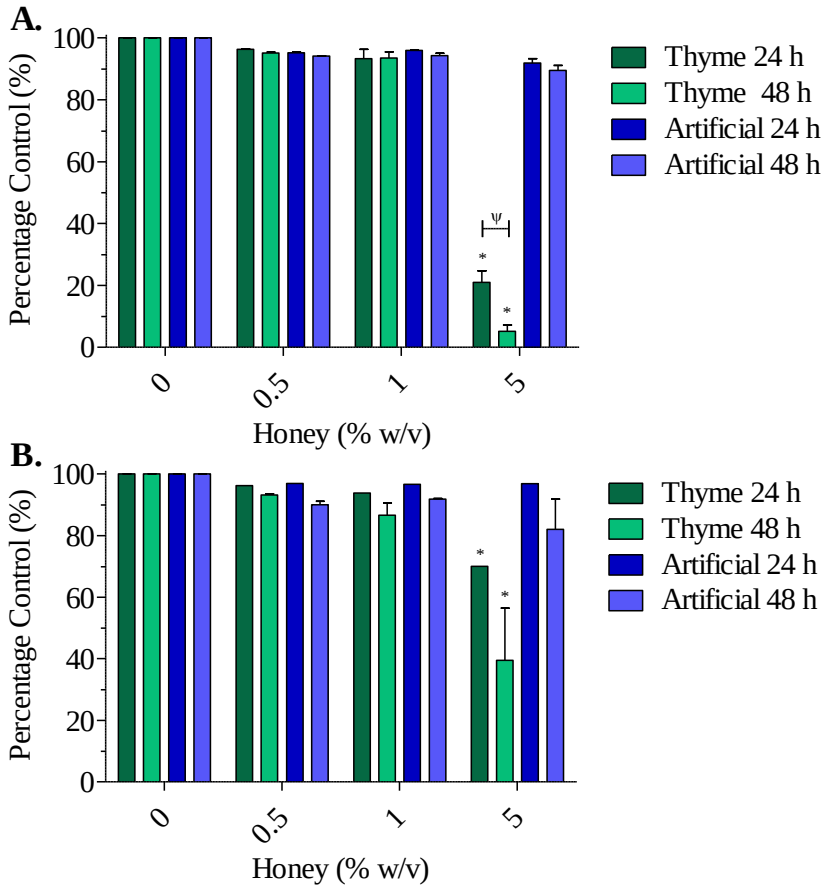


Figure 13. Honey Cytotoxicity Quantified by the TB Exclusion Assay. (A) PC3 and (**B**) DU145 cells were seeded at a density of $3x10^4$ cells/well. Cells were treated with either thyme or artificial honey at 0, 0.5, 1 and 5% (w/v) for 24 and 48 h. Following treatment, adhered cells were trypsinised and were combined with non-adherent cells from the medium. Cells were stained with TB (0.08%) and counted under a light microscope, and where cells with internalised TB dye were described as dead. Experiments were completed in duplicate, and values were expressed as mean cell viability ± S.E.M (n = 2). Data were analysed using a two-way ANOVA followed by a Bonferroni post-hoc test, where $p<0.05$ was required for a statistically significant difference.* represents a significant difference between individual treatment and control. ψ represents a significant difference between different time points at the same concentration.

It was difficult to compare this data with results obtained in the literature, as the TB exclusion assay had not been previously reported for the determination of cell viability in PC3 and DU145 cells treated with honey. Hassan *et al*. (2012) showed that 5% (w/v) Egyptian clover honey loss of HepG2 hepatocellular carcinoma cell viability by 10% (24 h) and 20% (48 h). Similar to the results shown in **Figure 13**, Hassan *et al*. also demonstrated that artificial honey had no effect on the cytotoxicity of HepG2 cells over 72 h, as determined via the TB assay. In melanoma cells, Pichichero *et al*. (2010) used the TB assay to show that at 48 h, 5% (w/v) acacia honey caused an 88% (B16-Fi) and 90% (A375) reduction in cell viability. Inter-cellular and intra-floral differences between studies highlights the difficulty in classifying a cytotoxic concentration for honey.

3.2.6 Cytotoxicity of Honey Measured by the Hoechst/Propidium Iodide Assay

To further support the findings from both the LDH and TB assays which showed that honey may cause a greater proportion of necrosis or secondary necrosis in PC3 cells compared to DU145 cells, the Hoechst/propidium iodide (HO/PI) assay was used. Using the same principle as the TB assay, PI (red) enters the cytoplasm of cells due to the increased membrane permeability that occurs during necrosis and late-stage apoptosis. Unlike PI, HO is a membrane-permeable nuclear stain (blue) for both viable and non-viable cells. Both dyes fluoresce at different wavelengths, which is exploited for their detection via fluorescent microscopy.

The results reported from the HO/PI stain assay were not used to quantify the cytotoxicity of honey in the present study; however they were used to further characterise the mechanism of action. Fluorescent microscopy for both PC3 and DU145 cells indicated that thyme honey caused a concentration-dependent increase in PI stained cells (**Figure 14, 15**). PI stained cells were expressed as a percentage of total (HO) stained cells (**Figure 16**). Analysis of the overlaid images showed that the administration of thyme honey over 24 h caused a greater proportion of PI stained cells PC3 cells compared to DU145 cells at all concentrations, with 5% (w/v) resulting in 32.61 ± 4.52% PI staining in PC3 cells compared to 26.12 ± 8.92% in DU145 cells ($p<0.05$).

It was observed that honey caused a concentration-dependent reduction in the number of HO stained and optically visualised PC3 and DU145 cells (**Figure 14, 15, 17**). This may be explained by the fact that 5% (w/v) honey caused a near 90% loss of cell viability at 24 h seen using the MTT and SRB assays (**Table 9**), therefore, most cells will have undergone primary or secondary necrosis, and been fully lysed. An alternative explanation could be that honey caused the loss of cell adhesion, resulting in fewer cells remaining on the plate.

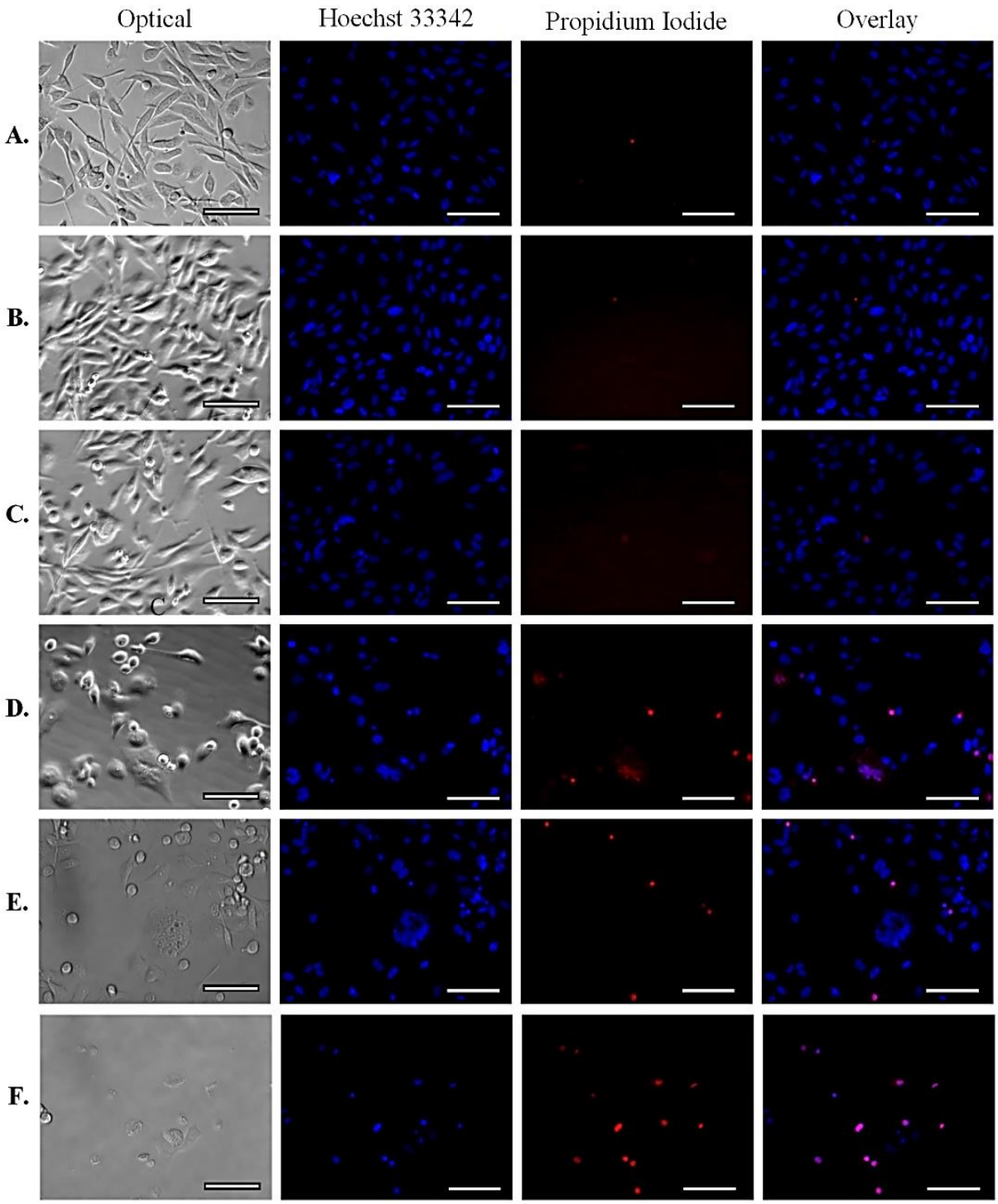


Figure 14. Effect of Thyme Honey on Staining with Propidium Iodide in PC3 Cells. PC3 cells were seeded at a density of $6x10^4$ cells/well. Cells were treated with (**A**) 0, (**B**) 1, (**C**) 2, (**D**) 3, (**E**) 4, or (**F**) 5% (w/v) thyme honey for 24 h. Following treatment, cells were incubated with the fluorescent stains Hoechst 33342 (HO) (2 µg/mL) and propidium iodide (PI) (50 µg/mL). Cells were visualised using an inverted microscope (200x magnification) with fluorescent filters to detect HO (blue) and PI (red). Fluorescent images were overlaid to determine a percentage of PI stained cells compared to control. Experiments were completed in triplicate, and images were selected as representative (n = 3). Scale bars (white) represent 50 µm.

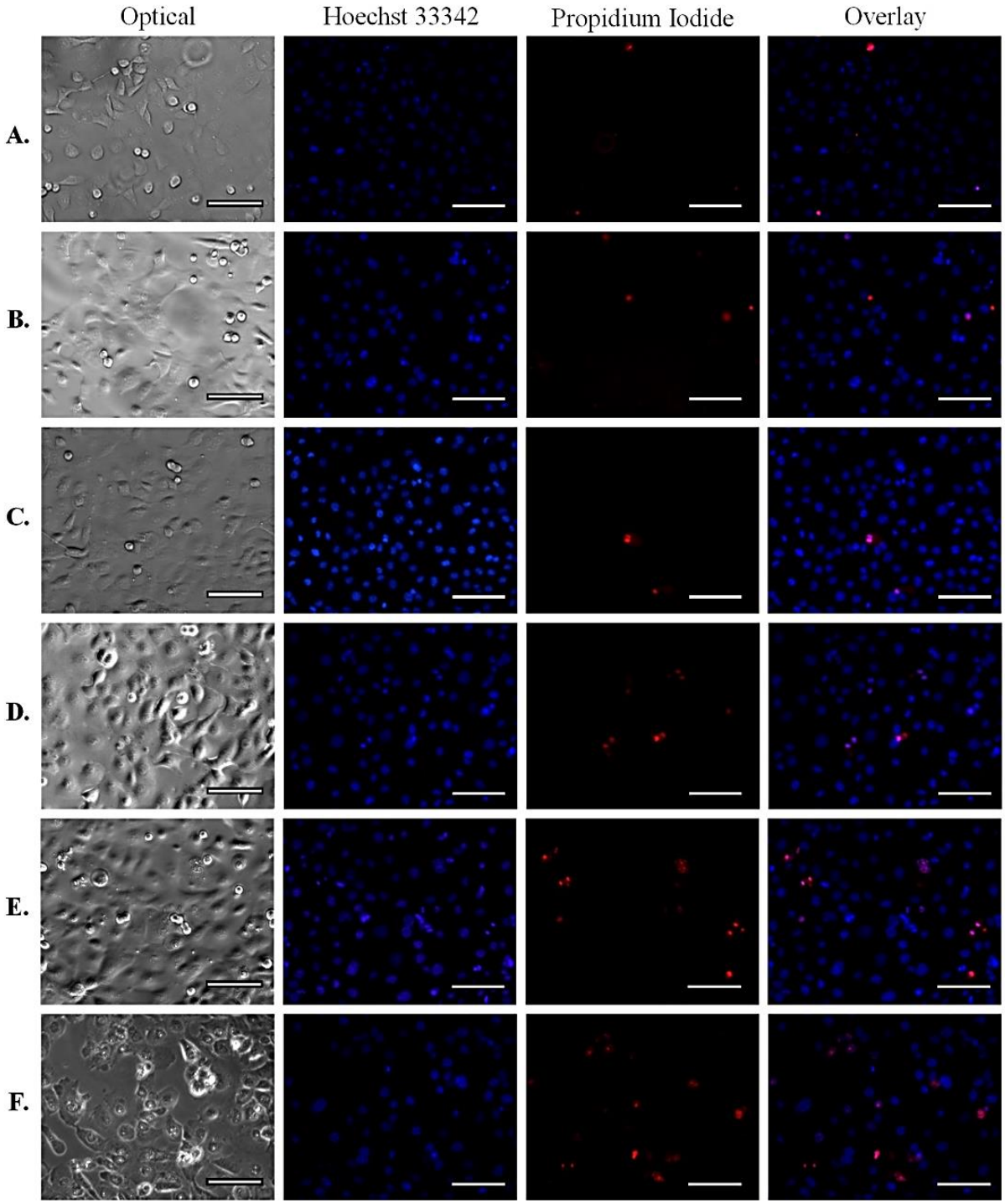


Figure 15. Effect of Thyme Honey on Staining with Propidium Iodide in DU145 Cells. DU145 cells were seeded at a density of $6x10^4$ cells/well. Cells were treated with (**A**) 0, (**B**) 1, (**C**) 2, (**D**) 3, (**E**) 4, or (**F**) 5% (w/v) thyme honey for 24 h. Following treatment, cells were incubated with the fluorescent stains Hoechst 33342 (HO) (2 µg/mL) and propidium iodide (PI) (50 µg/mL). Cells were visualised using an inverted microscope (200x magnification) with fluorescent filters to detect HO (blue) and PI (red). Fluorescent images were overlaid to determine a percentage of PI stained cells compared to control. Experiments were completed in triplicate, and images were selected as representative (n = 3). Scale bars (white) represent 50 µm.

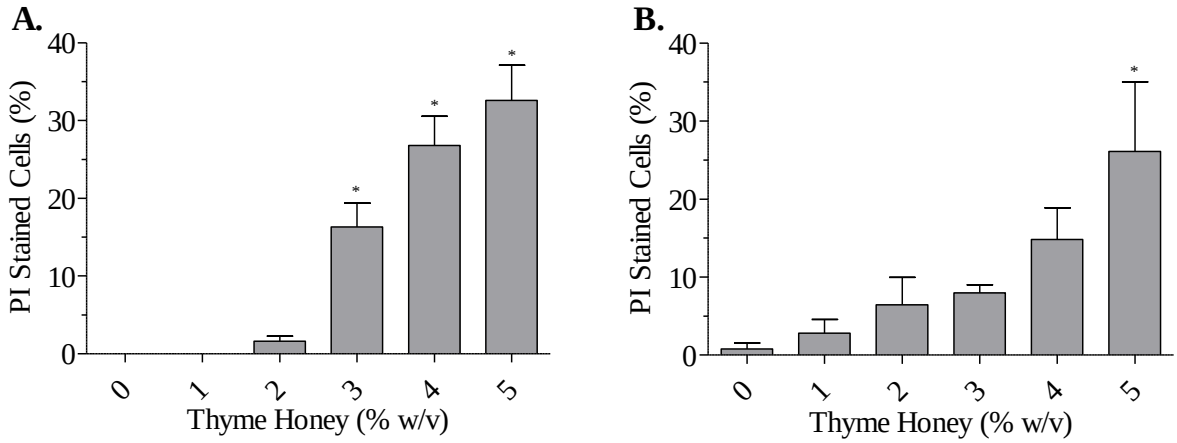


Figure 16. Percentage of PC3 and DU145 Cells Stained with Propidium Iodide. (**A**) PC3 and (**B**) DU145 cells were seeded at a density of $6x10^4$ cells/well, and were treated with 0 - 5% (w/v) thyme honey for 24 h. Cells which stained for either HO or PI were counted by using image thresholds. Graphs represent the percentage of PI stained cells, quantified from all photos taken, examples of which are shown in **Figure 13** and **Figure 14.** Experiments were completed in triplicate, and values were expressed as mean percentage of PI stained cells ± S.E.M (n = 3). Data were analysed using a one-way ANOVA followed by a Dunnett's post-hoc test, where $p<0.05$ was required for a statistically significant difference.* represents a significant difference between individual treatment and control.

The three main cell death pathways are apoptosis, necrosis and autophagy. Classical apoptosis can be simplistically described as a programmed cell death by caspase enzymes which eventuates in the packaging of cellular contents to be phagocytosed by host immune cells such as macrophages (Elmore, 2007). Autophagic cell death is a less common mechanism, and is characterised as self-engulfment in which cellular contents are delivered to the lysosome for degradation (Glick *et al.*, 2010). In contrast, necrotic cell death is often thought of as a spontaneous and unregulated cell death mechanism which involves the swelling and lysing of the cell, releasing cellular content into the extracellular space, although more recently, regulatory signalling pathways have been described (Zong *et al.*, 2006; Linkermann *et al.*, 2013; Berghe *et al.*, 2014). Inflammation as a result of content release by necrotic cell death can often contribute to the pathogenesis of cancer, and therefore apoptosis has been suggested to be a preferred mechanism for chemotherapeutics (Rock *et al.*, 2008).

In cell culture, when no immune cells are present, cells undergoing apoptotic cell death will progress to a necrosis-like swelling followed by the release of cellular contents into the extracellular medium, known as secondary necrosis or late-stage apoptosis (Krysko *et al.*, 2008a). This loss of membrane integrity, which is detectable by dye inclusion assays or other fluorometric assays represents a death endpoint, and does not tell us whether the cells first underwent apoptosis or not. The HO/PI duel-stain assay can easily be used to identify cells

undergoing necrotic or secondary necrotic cell death. However, since the assay also provides a snapshot in time, the Hoechst staining of DNA may be used to observe cells undergoing apoptosis through the presentation of apoptotic markers such as nuclear budding, membrane blebbing, or nuclear condensation (Syed Abdul Rahman *et al.*, 2013). This may explain the lower than expected 30 - 40% of PI stained cells from 5% (w/v) thyme honey seen in **Figure 16,** compared to the >90% cell death seen from the MTT and SRB assays.

Apoptotic-like nuclei were observed, suggesting that honey may exhibit toxicity through multiple cell-death mechanisms (**Figure 17**). Thyme honey resulted in a concentration-dependent increase in the presentation of apoptotic-like nuclei. Nuclear condensation and shrinking were the most common apoptotic characteristics seen; both of which are distinct stages in apoptotic cell death (Johnson *et al.*, 2000; Bortner *et al.*, 2002). Nuclear swelling was also commonly observed, and may be indicative of either primary or secondary necrosis. Further, the presence of nuclear pyknosis and fragmentation were observed, which are also suggestive of an apoptotic cell death mechanism (Dini *et al.*, 1996; Doonan *et al.*, 2008). Despite the presentation of a few morphological and nuclear characteristics of both apoptotic and necrotic cell death, it is important to note that no assumption of primary apoptosis or primary necrosis can be made as no further supporting assays were performed. The appearance of apoptosis may be one reason for the cell death discrepancies between the HO/PI assay and the MTT and SRB assays, however, another reason may be that cells have lysed and fully undergone secondary necrosis.

In support of these conclusions from the Hoechst staining, apoptosis as a cell death mechanism by honey has been widely reported (Jaganathan *et al.*, 2009b; Fauzi *et al.*, 2011; Samarghandian *et al.*, 2011a; Fernandez-Cabezudo *et al.*, 2013; Erejuwa *et al.*, 2014). For example, Jaganathan and Mandal (2009b) reported that Indian honey caused apoptotic cell death in HT-15 and HT-29 colon cancer cells at 24 h through an increased caspase-3 expression, as well as sub G_1 accumulation due to PI staining. The relative lack of cytotoxicity towards many non-cancerous cell types suggests a targeted cancer-specific cell death pathway (Erejuwa *et al.*, 2014).

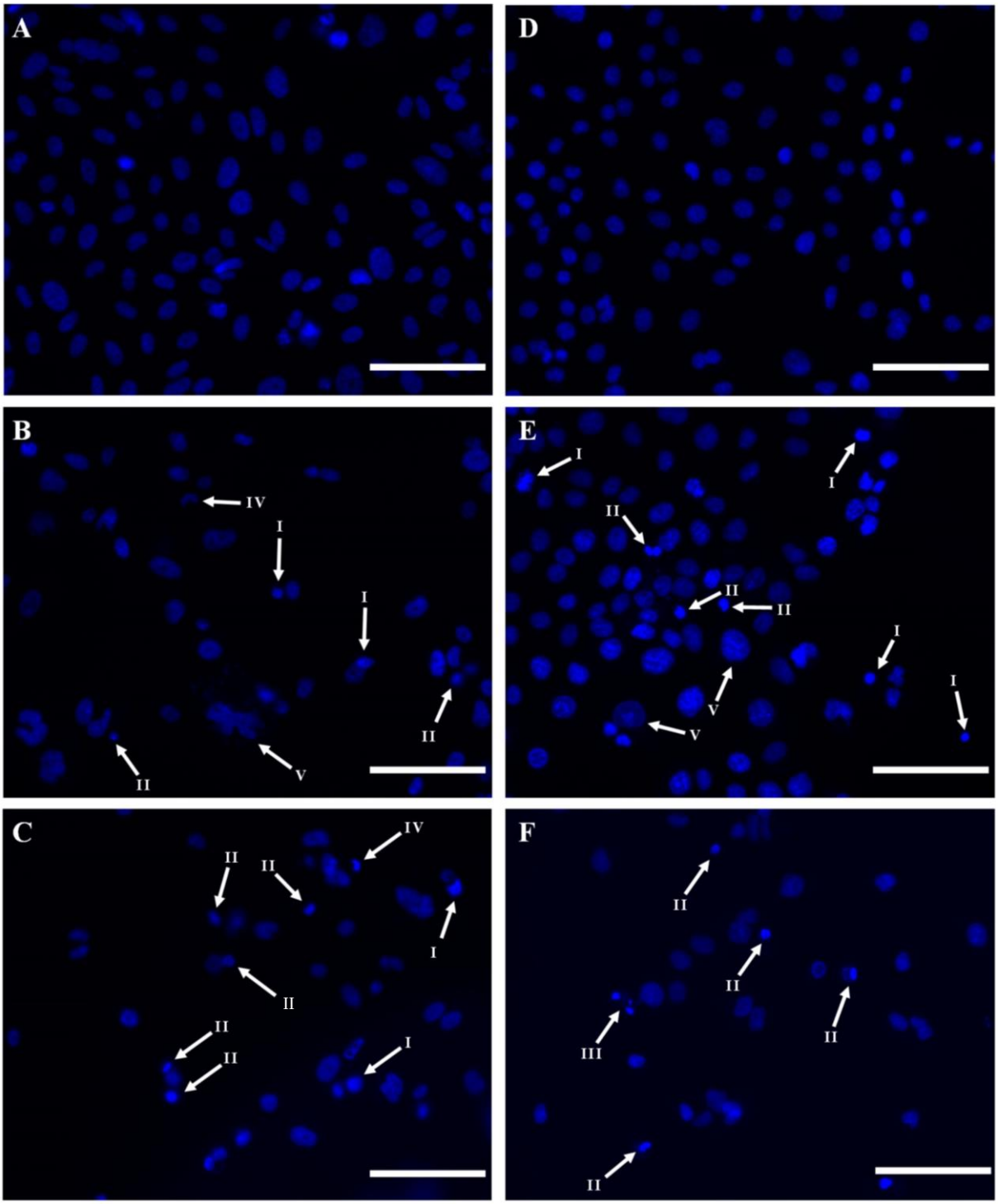


Figure 17. Examples of Apoptotic Nuclei from Thyme Honey. (A, B, C) PC3 or (**D, E, F**) DU145 cells were seeded at a density of $6x10^4$ cells/well. Cells were treated with (**A, D**) 0, (**B, E**) 3, or (**C, F**) 5% (w/v) thyme honey for 24 h. Following treatment, cells were incubated with the fluorescent stains Hoechst 33342 (HO) (2 µg/mL). Cells nuclei were visualised using an inverted microscope (200x magnification) with fluorescent filters to detect HO (blue). Nuclear characteristics of apoptosis were as follows: (**I**) condensation, (**II**) shrinkage, (**III**) fragmentation, (**IV**) pyknosis, and (**V**) swelling. Experiments were completed in triplicate, and images were selected as representative (n = 3). Arrows indicated cells undergoing nuclear apoptosis. Scale bars (white) represent 50 µm.

3.2.7 Cytotoxicity of Honey Summarised

Regardless of which assay was used, the present study provided evidence for both a time- and concentration-dependent cytotoxic effect for honey against both PC3 and DU145 cell lines. The results were in agreement with the proposed hypothesis.

Because honey contains compounds that may interact with the MTT assay, it was important to use multiple assay to characterise its cytotoxicity. The MTT assay was used to determine cell viability via mitochondrial enzyme function, and hence cytotoxicity is recorded by measuring tetrazolium reduction. The SRB assay may therefore report a more accurate representation of cell number, as it measures total protein. Further, the TB assay may be more accurate than both colourimetric assays as it does not require cell adhesion; a requirement that may be altered by honey treatment. In order to summarise the cytotoxicity of honey as reported by different assays, a table was constructed that compared cell viability as determined by the MTT, SRB and TB assay for 2% and 5% (w/v) honey at 24 h (**Table 9**). Compared to the other assays, the HO/PI and LDH assays were only used to characterise a cell death mechanism, and therefore were not summarised in Table 9.

In DU145 cells treated with honey, the MTT assay reported on average, an increased cell death of 20% (2% w/v) and 32% (5% w/v) at 24 h, compared to the SRB assay ($p<0.05$). Further, in PC3 cells treated with 5% (w/v) honey, the MTT assay reported on average, an increased cell death of 25% at 24 h, compared to the SRB assay ($p<0.05$). In contrast, in PC3 cells treated with 2% (w/v) honey, the MTT assay reported on average 5% less cell death at 24 h, compared to the SRB assay. These differences highlighted the need for multiple assays when quantifying cell death by honey.

Compared to the indirect colourimetric MTT and SRB assay, honey treatment showed less cytotoxicity when measured by the direct TB assay (**Table 9**). The discrepancy in test sensitivity could be explained by the differences in measured outcomes, whereby the TB assay is more sensitive to membrane permeability, and hence may underestimate early stage cell death via apoptosis. This discrepancy may also be evident when comparing the MTT and SRB assays to the LDH and HO/PI stain assays, due to what they measure. Therefore, consideration must be made before concluding a maximal non-cytotoxic concentration, and the employment of multiple assays is recommended.

Table 9. Summary of Honey Cytotoxicity at 24 h. All values were expressed as mean percent cell viability ± S.E.M

			Cell Viability at 24 h (%)	
Cell Line	Honey	Assay	2% (w/v)	5% (w/v)
PC3	Thyme	MTT	39.90 ± 10.17	10.49 ± 2.50
		SRB	33.92 ± 3.07	11.71 ± 0.05
		TB	–	21.01 ± 3.66
	Manuka	MTT	61.14 ± 4.03	8.04 ± 2.68
		SRB	50.33 ± 2.34	11.63 ± 1.57
	Honeydew	MTT	34.74 ± 6.03	9.25 ± 3.29
		SRB	40.26 ± 1.39	11.37 ± 2.51
	Artificial	MTT	86.22 ± 2.54	38.62 ± 6.77
		SRB	88.80 ± 2.55	65.47± 2.40
		TB	–	91.83 ± 1.44
DU145	Thyme	MTT	64.95 ± 5.81	8.11 ± 2.32
		SRB	83.58 ± 1.68	12.76 ± 3.83
		TB	–	69.95 ± 0.00
	Manuka	MTT	65.64 ± 5.99	6.17 ± 1.96
		SRB	83.58 ± 1.33	14.14 ± 0.59
	Honeydew	MTT	58.45 ± 2.73	9.72 ± 3.78
		SRB	76.08 ± 2.85	12.48 ± 0.92
	Artificial	MTT	79.28 ± 12.12	64.70 ± 9.85
		SRB	92.45 ± 1.97	75.69 ± 14.26
		TB	–	96.83 ± 0.00

In contrast to these findings, Pichichero *et al*. (2010) showed that the MTT assay reported a higher IC_{50} when compared to the TB assay for melanoma cells treated with acacia honey. They explained that the increased IC_{50} observed via the MTT assay might be due to either alterations in intracellular dehydrogenases, or the direct reduction of MTT by flavonoids (Pichichero *et al.*, 2010). Upon further investigation of their methods, it was found that cytotoxic analysis was carried out through the direct addition of MTT to each well before the honey was removed. Due to the evidence provided in the present study, it could be suggested that Pichichero *et al*. (2010) had underestimated the cytotoxic effects of honey through the reduction of MTT by honey.

Overall, honey was 30% more cytotoxic towards PC3 cells compared to DU145 cells. In PC3 cells, thyme and honeydew honey elicited a 2.2 and 2.4 fold greater cytotoxicity compared to

the artificial honey control, whereas manuka honey only elicited a 1.5 fold greater cytotoxicity. In DU145 cells, honeydew honey elicited a 1.3 fold greater cytotoxicity compared to the artificial honey control, whereas thyme and manuka honey were only 1.2 fold greater. These result suggest that honeydew honey may be more cytotoxic to prostate cancer cells compared to thyme and manuka honeys. These findings were supported by literature showing that honeydew honey may elicit a greater cytotoxicity due to its increased mineral and phenolic content (White *et al.*, 1980; Majtan *et al.*, 2011; Vanhanen *et al.*, 2011). Further, the increased cytotoxicity of honey towards PC3 cells may be due to the different protein expression profiles between the two cell types.

Compared to the SRB assay, the MTT assay showed an overall 20% greater cytotoxicity of honey towards DU145 cells, however, the cytotoxicity towards PC3 cells was comparable. These findings may suggest a greater ability of honey to effect DU145 cell mitochondrial enzyme function. Alternatively, as adhesion is required for both assays, and the SRB measures cell number through the qualification of protein, honey may cause a greater loss of cell adhesion in DU145 cells compared to PC3 cells.

In order to determine the relationship between time and honey cytotoxicity, a compilation was made of all data representing the percentage cell viability (calculated using the MTT assay) of PC3 cells exposed to 5% (w/v) thyme honey, over 72 h (**Figure 18**). Results demonstrated a time-dependent reduction in PC3 cell survival when exposed to 5% (w/v) thyme honey. Dose-response analysis of the normalised and log-transformed data suggested the time at which 50% cell death occurred to be 2.53 ± 1.07 h (R^2 = 0.9555) (**Figure 18**). Interestingly, this time-dependent effect was not found for the MTT assay when only 24, 48 and 72 h data were compared previously in **Figure 7** and **Figure 8**. This may have been due to the time-dependent cytotoxicity of PC3 plateauing at 10 h post treatment (**Figure 18A**).

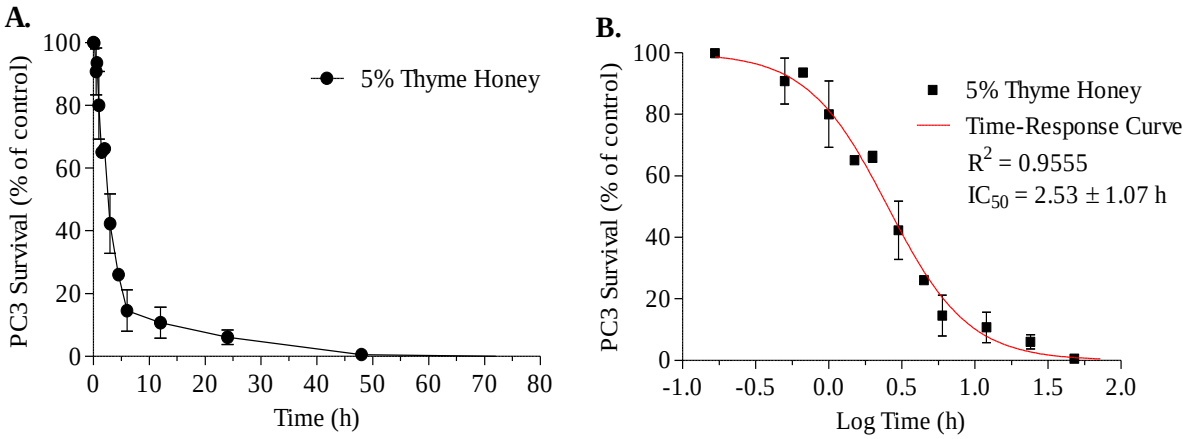


Figure 18. Time-dependent Reduction in PC3 Cell Survival from Thyme Honey. Compilation of all percentage of control cytotoxic values for thyme honey using the MTT assay, between 0 - 72 h. (**A**) Normalised percentage cell survival. (**B**) Normalised and log-transformed percentage cell survival. Experiments were completed in triplicate, and values were expressed as mean percentage cell viability ± S.E.M (n = 2 - 8). Data were analysed and curve fitted using a log (inhibitor) vs. response - variable slope (four parameters) (R^2 = 0.9555).

The early time of 2.53 ± 1.07 h, at which 50% cell death occurred for the administration of 5% (w/v) thyme honey, may be merely an indication of the limitations of the MTT assay to determine cell survival from honey exposure. Thyme honey may have caused a rapid loss of cell adhesion, and therefore the MTT assay may have recorded a false-positive suggesting that non-adherent cells were non-viable. Alternatively, this early 50% cell death time may be suggestive of a very fast cell death onset and may present information about honey-driven cell death mechanisms. Apoptosis is regarded as a regulated cell death that involves an altered protein and enzyme expression within a cell, and therefore may take several hours. The time from apoptosis initiation to completion may require 2 - 3 h, however, the activation of some caspases may take longer (Sundquist *et al.*, 2006; Elmore, 2007). The specific mechanism of apoptotic cell death (intrinsic vs extrinsic) will determine which caspases are activated, and therefore will determine the speed of cell death.

Another explanation is that honey may be cytotoxic via multiple mechanisms involving both necrosis and apoptosis. Necrotic cell death is a differently regulated mechanism, and may occur very quickly as it does not require the activation of caspases, but relies on the loss of membrane integrity. Therefore, the mechanism of cell death by honey may present as rapid necrosis (0 - 3 h), followed by apoptosis (>3 h). It was not possible to determine the cell death mechanism in the present study, as markers for different mechanisms were not investigated. The proposed mechanism of early apoptosis followed by necrosis carried little weight due to the confounding factor that all cells in culture undergo secondary necrosis; an inevitable progression which

shares morphological similarities to primary necrosis. Future investigations of apoptotic markers such as caspase activation, DNA fragmentation, and cytochrome c release may better elucidate the mechanism. Krysko *et al*. (2008b) suggested the employment of flow cytometry or transmission electron microscopy may help to define the mechanism of cell death in cell culture. Further, they suggested that analysis of cytokeratin 18 and caspase release into the supernatant can be used to distinguish between primary and secondary necrotic cells (Krysko *et al.*, 2008b).

Fernadez-Cabezudo *et al*. (2013) demonstrated that the mechanism of cell death from the administration of manuka honey was via the intrinsic apoptotic pathway, where caspase-9, but not caspase-8, was induced. They further supported their findings through the loss of Bcl-2, activation of PARP and DNA fragmentation; all indicative of apoptotic cell death. Finally, they demonstrated that the IC_{50} of manuka honey was 2.5 fold higher in MCF-7 cells with reduced caspase-3 expression. Fernandez-Cabezudo *et al*. did however mention the possibility of a secondary cell death mechanism occurring, as manuka honey was still cytotoxic in a caspase-3 reduced cell line (Fernandez-Cabezudo *et al.*, 2013). Due to other reports of apoptosis not accounting for the total toxicity of honey in renal cell carcinoma cells, non-apoptotic cell death mechanisms could not been eliminated (Samarghandian *et al.*, 2011a). This would suggest that honey causes apoptotic cell death at low concentrations, however, may shift to a more necrotic mechanism at higher concentrations.

If honey caused necrotic death only, then results from PI staining would suggest that 5% (w/v) thyme honey resulted in a 40% loss in cell viability at 24 h. However, both the MTT and SRB assay showed that 5% (w/v) thyme honey resulted in a greater than 90% loss of cell viability at 24 h. This further suggests that honey causes apoptosis, and that the LDH and HO/PI assay are detecting cells undergoing secondary necrosis, and that the other 50% of non-viable cells have not progressed to secondary necrosis yet. However, discrepancies between different cytotoxic mechanism sensitivities from assays in the present study meant that no conclusion could be made on the cell death mechanism of honey in PC3 and DU145 cells. In order to determine a mechanism of cell death, techniques such as flow cytometry should be employed in the future.

A specificity of honey for cancerous cells has previously been reported, where honey has been shown to have little or no effect on the viability of normal cell lines (Du Toit *et al.*, 2009; Al-Refai, 2014; Erejuwa *et al.*, 2014) Other studies have shown that honey had an IC_{50} of 70% (w/v) in normal hepatocytes (WRL-68) but an IC_{50} of 25% (w/v) in hepatocellular cells

(HepG2) (Jubri *et al.*, 2012). Despite the absence of a normal cell line in the present study, honey was shown to be toxic to both PC3 and DU145 prostate cancer cells at low concentrations.

To further characterise the anti-metastatic effects of honey, a maximal non-toxic concentration was established. In order to summarise the maximal non-cytotoxic concentrations of the 3 honeys as reported by different assays, a table (**Table 10**) was constructed to compare cell viability as determined by the MTT, SRB, LDH, TB and HO/PI assay, at 24, 48 and 72 h. Maximal non-cytotoxic concentrations obtained were used for all subsequent experiments to assess the anti-metastatic properties of honey. Concentrations of honey selected were: 1% (w/v) for the 48 h migration assay and 0.5% (w/v) for the 72 h invasion assay. Even though artificial honey had a higher maximal non-cytotoxic concentration (1.25-3% w/v), concentrations of 1% (w/v) for the 48 h migration assay and 0.5% (w/v) for the 72 h invasion assay were used in order to provide a more comparable control.

3.2.7.1 Evaluation of Data Presented as Percentage of Control

The decision to present and analyse data as a percentage of the control throughout the entire study was made to reduce inter-experiment variation. For experiments other than migration and invasion assays, a one-way between-group or two-way between-group repeated-measures ANOVA was used to determine statistical significance. Lew (2007a) reported that when using a one-way ANOVA, analysis of data expressed as percentage of control may have a higher statistical power compared to those expressed as raw values. However, when using a two-way ANOVA, analysis of data expressed as raw values may have a greater power, compared to data expressed as percentage of control (Lew, 2007b). The analysis of data expressed as a percentage of control can also result in the heterogeneity of variance, and therefore may violate the assumptions of the ANOVA (Lew, 2007a). For this reason, data within the present study that required a two-way ANOVA should later be reanalysed with raw values, to increase the statistical power. As the manipulation and re-analysis of these data sets would require such a large amount of time, the current analysis can be listed as a study limitation.

Table 10. Summary of Maximal Non-cytotoxic Concentrations

			Maximal Non-cytotoxic Concentration (% w/v)		
Cell Line	Honey	Assay	24 h	48 h	72 h
PC3	Thyme	MTT	1	1	1
		SRB	0.5	0.5	0.5
		LDH	1	–	–
		TB	1	–	–
		HO/PI	1	–	–
	Manuka	MTT	1	1	0.5
		SRB	<0.5	<0.5	<0.5
	Honeydew	MTT	1	1	1
		SRB	<0.5	<0.5	<0.5
	Artificial	MTT	2.5	1.25	1.25
		SRB	3	2	2
		LDH	4	–	–
		TB	5	–	–
DU145	Thyme	MTT	1	1	1
		SRB	1	1	1
		LDH	2	–	–
		TB	1	–	–
		HO/PI	1	–	–
	Manuka	MTT	0.5	0.5	0.5
		SRB	1	1	1
	Honeydew	MTT	1	1	1
		SRB	1	1	1
	Artificial	MTT	3	3	3
		SRB	4	4	3
		LDH	5	–	–
		TB	5	–	–

3.3 Anti-metastatic Properties of Honey

Cancer cell metastasis is a multi-staged process that involves ECM adhesion, migration and invasion. Phenolic compounds, that can be found in honey, have been reported to possess anti-metastatic activity in a wide range of cancer cell lines, due to the inhibition of adhesion molecules such as VCAM-1 (Piantelli *et al.*, 2006; Jin *et al.*, 2013), as well as the inhibition of migration and invasion through the inhibition of proteases (Vijayababu *et al.*, 2006; Abdel Aziz *et al.*, 2009). Further, the administration of whole honey has been shown to reduce the migration of many cancer cell types (Erejuwa *et al.*, 2014; Moskwa *et al.*, 2014). For these reasons, the *in vitro* anti-metastatic activity of New Zealand honey was assessed. Throughout the present study, the mechanisms of metastasis investigated were divided into cell adhesion, migration and invasion.

3.3.1 Anti-metastatic Activity of Honey Measured by the Adhesion Assay

Observations during the performance of the cytotoxic assays suggested that honey causes a loss of cell attachment, therefore, cell adhesion was assessed. To best replicate the extracellular stromal environment of a cancer, plates were coated with the ECM proteins collagen I or fibronectin prior to cell seeding and treatment. Non-adhered cells were removed after 30 - 120 min, and results were expressed as a percentage of untreated cells.

Thyme, manuka and honeydew honey caused a concentration-dependent decrease in cell adhesion to collagen I and fibronectin in both PC3 and DU145 cells ($p<0.05$) (**Figure 19, 20**). Further, the results showed that high concentrations (4 - 5% w/v) of artificial honey were required to significantly reduced cell adhesion. With respect to collagen I, artificial honey caused a loss of cell adhesion in both cell lines only at 5% (w/v) ($p<0.05$) (**Figure 19**). Interestingly at 90 min, 1 - 4% (w/v) artificial honey caused an increase in fibronectin adhesion ($p<0.05$), however, this returned to control levels by 120 min (**Figure 20**).

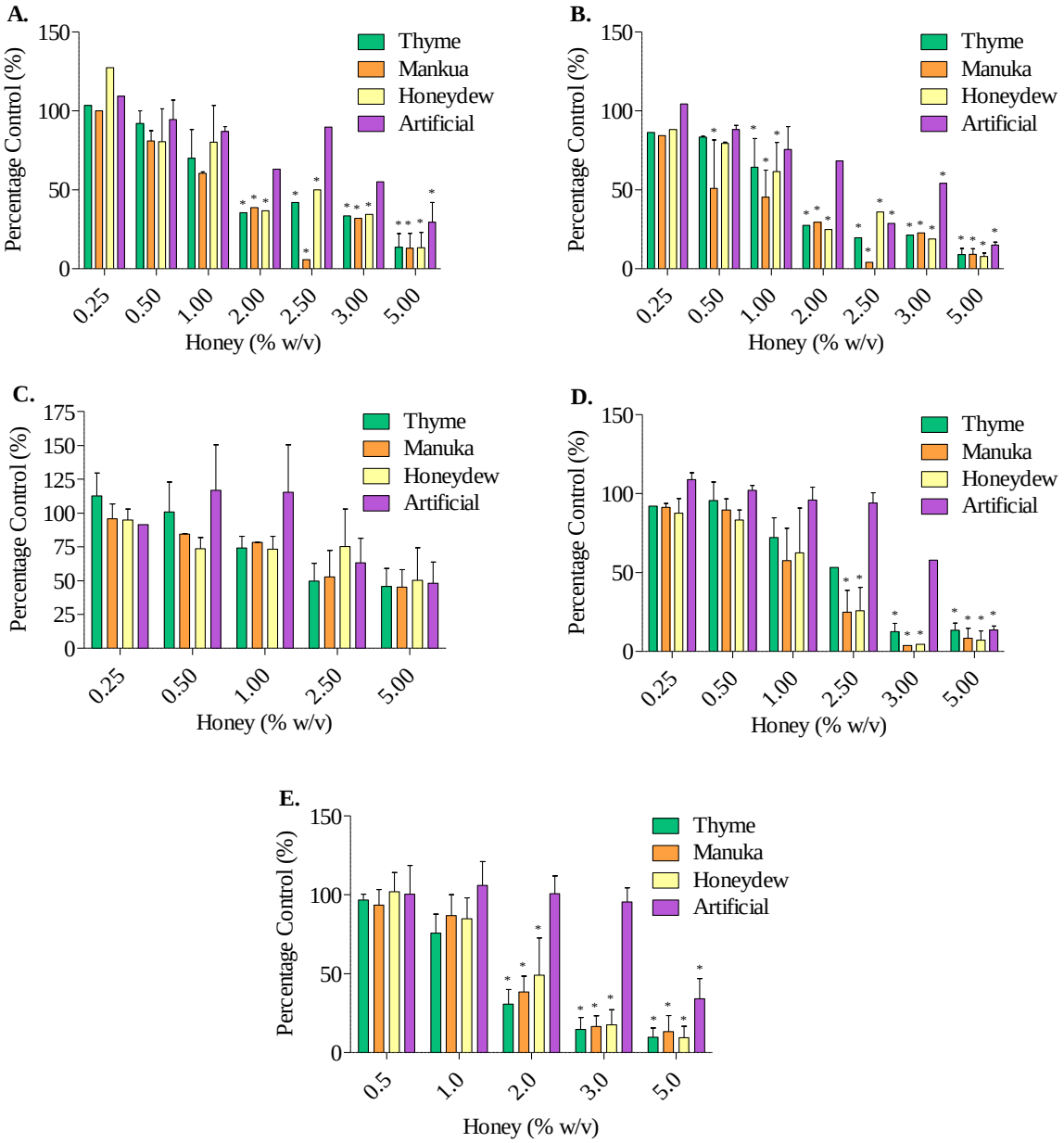


Figure 19. Effect of Honey on PC3 and DU145 Cell Adhesion to Collagen I. (A, B) PC3 and (**C, D, E**) DU145 cells were seeded in 96-well plates coated with collagen I (5 µg/mL), at a density of $1x10^4$ cells/well. Immediately after seeding, cells were treated with 0 - 5% (w/v) thyme, manuka, honeydew or artificial honey. PC3 cells were left to adhere for (**A**) 30 or (**B**) 60 min. DU145 cells were left to adhere for (**C**) 30, (**D**) 60 or (**E**) 90 min. Adhesion was measured via the MTT assay, as adhered cells were assumed viable. Experiments were completed in triplicate, with results expressed as mean percentage of control ± S.E.M (n = 3). Individual data were analysed using a two-way ANOVA followed by a Bonferroni post-hoc test, where $p<0.05$ was required for a statistically significant difference. * represents a significant difference between control (0% w/v) and individual treatment.

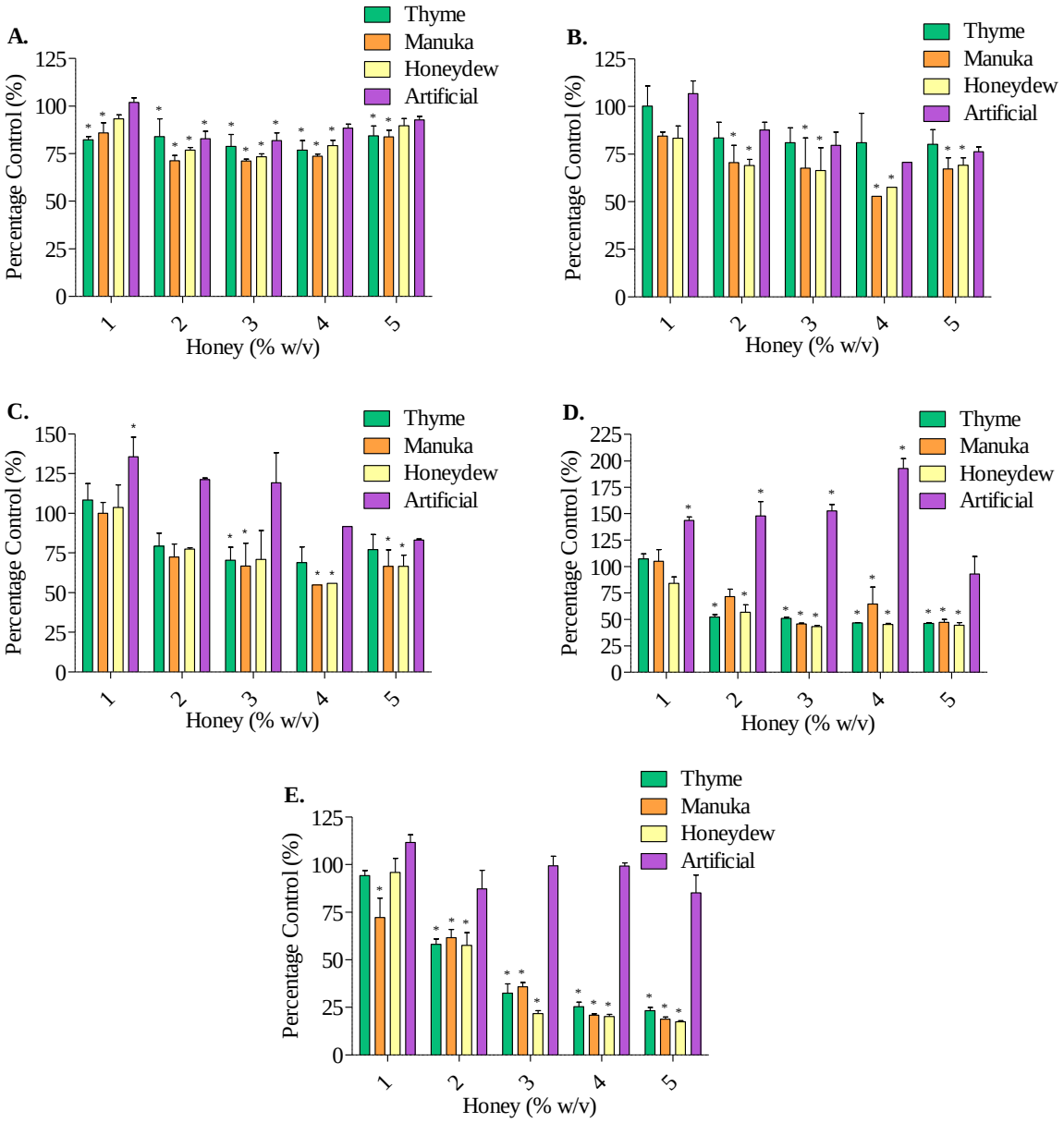


Figure 20. Effect of Honey on PC3 and DU145 Cell Adhesion to Fibronectin. (A, B) PC3 and (**C, D, E**) DU145 cells were seeded in 96-well plates coated with fibronectin (5 µg/mL), at a density of $1x10^4$ cells/well. Immediately after seeding, cells were treated with 0 - 5% (w/v) thyme, manuka, honeydew or artificial honey. PC3 cells were left to adhere for (**A**) 30 or (**B**) 60 min. DU145 cells were left to adhere for (**C**) 60, (**D**) 90 or (**E**) 120 min. Adhesion was measured via the MTT assay, as adhered cells were assumed viable. Experiments were completed in triplicate, with results expressed as mean percentage of control ± S.E.M (n = 3). Data were analysed using a two-way ANOVA followed by a Bonferroni post-hoc test, where $p<0.05$ was required for a statistically significant difference. * represents a significant difference between control (0% w/v) and individual treatment.

Overall, honey caused a greater loss of PC3 cell adhesion towards collagen I compared to fibronectin. In contrast, honey caused a similar loss of DU145 cell adhesion towards both collagen I and fibronectin, which suggests that the two cell types express differing integrin profiles. The expression of β1 integrin on many cancer cell types may be used for collagen I or fibronectin attachment (Zhang *et al.*, 1993; Jokinen *et al.*, 2004). Administration of honey reduced the adhesion of DU145 cells to fibronectin more than PC3 cells, suggesting that DU145 cells 'preferred' attachment to fibronectin. This was supported by Witkowski *et al*. (1993) who showed that DU145 cells preferentially attached to fibronectin, and that 50 - 60% of the binding was due to the expression of α5β1 integrins. An overexpression of some integrins may prevent cell detachment. For example, the high expression of α5β1 in DU145 cells may reduce migration and result in a less invasive phenotype compared to PC3 cells (Ruoslahti *et al.*, 1989; Witkowski *et al.*, 1993). Nevertheless, the invasiveness of PC3 cells has been attributed to the expression of α6 and β1 integrins, which show a greater affinity for laminin (Witkowski *et al.*, 1993; Suyin *et al.*, 2013). The affinity for laminin of the highly expressed α6β1 integrins in PC3 cells suggests that future attachment assays should be performed towards laminin for the treatment of honey.

This is the first time that the inhibition of cancer cell adhesion by honey has been reported. Maddocks *et al*. (2013) reported that manuka honey (16 - 50% w/v) could inhibit the adhesion and invasion of 8 bacteria strains, all of which were identified to be important in wound healing. The authors concluded that the inhibition of bacterial adhesion was due to a reduction in fibronectin binding proteins, as well as the inhibition of biofilm production - a protein rich matrix excreted by bacteria to facilitate adhesion (Maddocks *et al.*, 2012; Maddocks *et al.*, 2013). Further, they showed that manuka honey could reduce the adhesion of bacteria to fibronectin (6 strains), fibrinogen (4 strains) and collagen (5 strains) ($p<0.05$).

As with most effects of honey, the reduction in prostate cancer cell adhesion is often assumed to be due to the presence of phenolic compounds. Lo *et al*., (2007) found that tea polyphenols, also present in honey, could reduce the adhesion and migration of rat vascular smooth muscle cells (SMC) to collagen and laminin. They demonstrated that the catechins (-)-Epigallocatechin-3-gallate and (-)-epicatechin-3-gallate could inhibit SMC adhesion to extracellular proteins through the down-regulation of β1-integrins (Lo *et al.*, 2007). Baicalein, also a flavonoid found in honey, was shown to inhibit the adhesion of human hepatoma cells (HA22T/VGH and SK-Hep1) towards Matrigel®, collagen I and gelatin (Chiu *et al.*, 2011; Campillo *et al.*, 2015). Reductions in cell adhesion by flavonoids found in honey may be further

explained by the reduction in expression and activity of MMPs, especially -2 and -9 (Liu *et al.*, 2011; Chen *et al.*, 2013a). With the exception of the present study, no studies have investigated the effects of the sugar or other honey components on adhesion.

It is unknown whether the loss of adhesion seen with honey was a result of interaction with cells, or whether it was due to an interaction with the extracellular matrix proteins. To investigate this, the adhesion assay should be performed after matrix-protein containing wells are exposed to honey for a period of time and then washed. Assuming that all honey was removed before cells were allowed to adhere, a decrease in adherence might suggest that honey causes the degradation or loss of adherence properties of ECM proteins, independent of cellular interaction. To further characterise the anti-adhesion effects of honey, the timing of honey administration should also be investigated. As in the present study, honey was administered along with cells, therefore the reduction suggested that honey prevented adherence occurring. If honey was administered after cells were allowed to adhere, a loss of adhesion might indicate a different mechanism by which honey works. The full characterisation of honeys effect on adhesion should be completed to determine whether the mechanism either prevents attachment, causes the loss of cell adhesion, or a combination of both.

3.3.2 Anti-metastatic Activity of Honey Measured by the Scratch Wound Healing Assay

A scratch wound healing assay is a quick and cost effective method for observing cell migration, however without the appropriate imaging software, results can only be used to observe trends. The assay can study the effects of drugs on cell-cell and cell-matrix interactions, while providing live-cell imaging to observe morphological alterations associated with metastasis (Liang *et al.*, 2007). The scratch wound healing assay was used to assess the effect of thyme honey on the migration of prostate cancer cells, over 20 h. As *in vitro* cell proliferation often occurs 24 h after seeding, 20 h was selected for the scratch wound healing assay to determine the effects of honey on cell migration, without proliferation.

Compared to control, thyme honey caused a concentration-dependent reduction in the migration of both PC3 and DU145 cells at 20 h (**Figure 21** PC3, **22** PC3, and **23** DU145). It was observed that for both cell lines, 2, 3 and 4% (w/v) thyme honey caused a reduction in migration. Photographs made for the administration of 5% (w/v) honey were not suitable for the assessment of migration, as they showed confounding factors such as cell death and loss of

adhesion. Morphologically, untreated PC3 cell were polarised and elongated as a result of cytoskeletal rearrangement; a previously reported event in cell metastasis (Petrie *et al.*, 2012) (**Figure 21, 22**). These morphological characteristics of migration were relatively unchanged in PC3 cells treated with honey, despite an inhibition in migration. Cell rounding occurred at high concentrations of honey (3 - 5% w/v), however elongated cells were still observed. Moskwa *et al*. (2014) reported similar observations of cell shrinking and rounding after the administration of 1% and 2.5% (w/v) Polish honey in the human glioblastoma multiforme U87MG cell line. The morphological alterations suggested that the inhibition of cancer cell adhesion by honey may not be mediated through cytoskeletal rearrangement, and therefore may be receptor mediated. Untreated DU145 cells did not display any morphological characteristics of migration, and therefore it was difficult to propose a mechanism of action for the loss of migration seen by honey (**Figure 23**).

Pictures at 20 h post 4% and 5% (w/v) thyme honey treatment in DU145 cells included a large proportion of cells that had lost adhesion, and therefore had floated into the centre scratch (**Figure 23**). Nevertheless, when observed under the microscope, adhered cells were distinct from those that had lost adherence, and therefore the inhibition of migration was apparent. As no quantification was made of migration, no confounding variables existed; however, in the future, all wells should be washed carefully to remove all non-adherent cells. Further criticism of the assay includes that concentrations used were demonstrated to produce substantial cell death in both cell lines. For this reason, the inhibition of cell migration may have been due to the induction of cell death, rather than the anti-migratory activity of honey. For this reason, Boyden chamber migration assays were used which employed maximal non-toxic concentrations of each honey, to ensure that cell death could not confound the inhibition of migration.

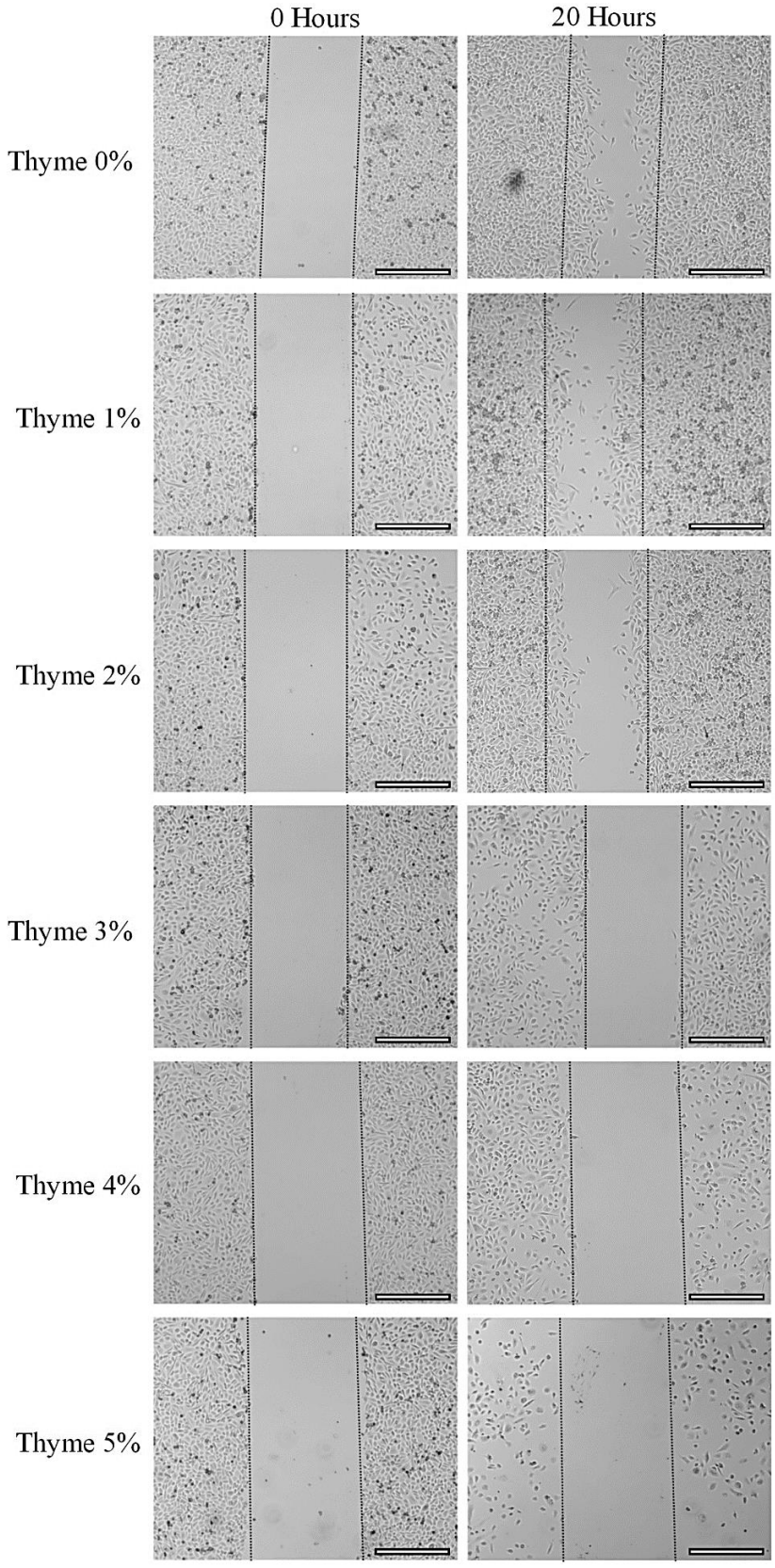


Figure 21. Effect of Thyme Honey on PC3 Cell Migration via the Scratch Wound Healing Assay. PC3 cells were seeded at a density of $3x10^5$ cells /well. Scratches were made pre-treatment (0 h). Cells were treated with 0 - 5% (w/v) thyme honey for 20 h. Visualisations were made using an inverted microscope at 40 x magnification, and were captured using a digital camera. All conditions were completed in triplicate, and representative images were presented (n = 3). Scale bars (white) represent 500 µm.

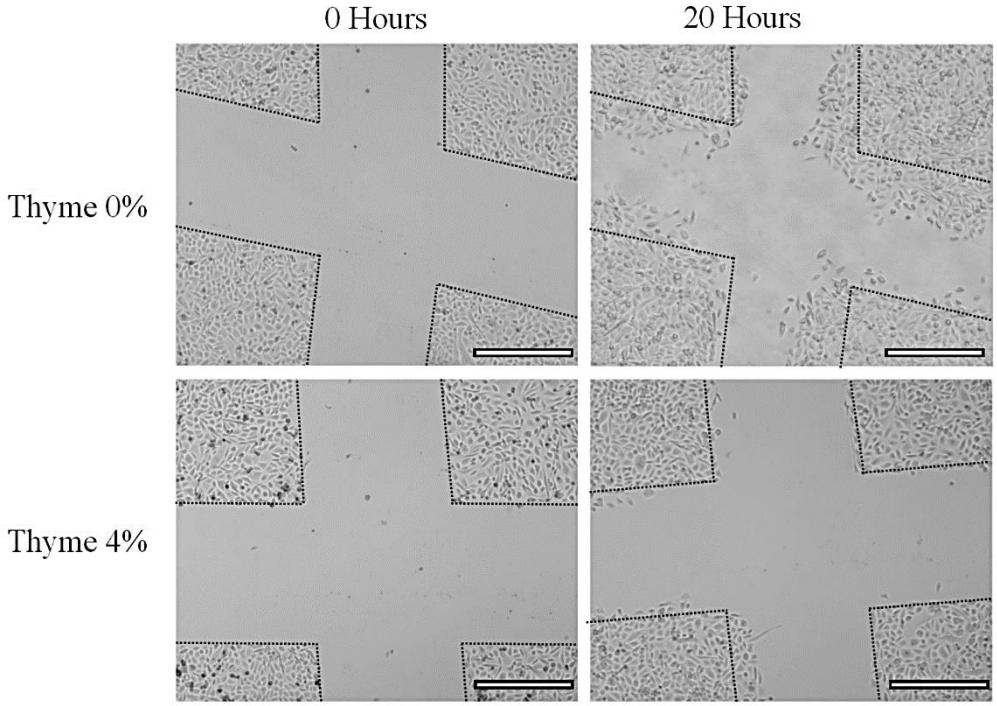


Figure 22. Effect of Thyme Honey on PC3 Cell Migration via the Scratch Wound Healing Assay. PC3 cells were seeded at a density of $3x10^5$ cells /well. Cross-scratches were made pre-treatment (0 h). Cells were treated with 0 and 4% (w/v) thyme honey for 20 h. Visualisations were made using an inverted microscope at 40 x magnification, and were captured using a digital camera. All conditions were completed in triplicate, and representative images were presented (n = 3). Scale bars (white) represent 500 µm.

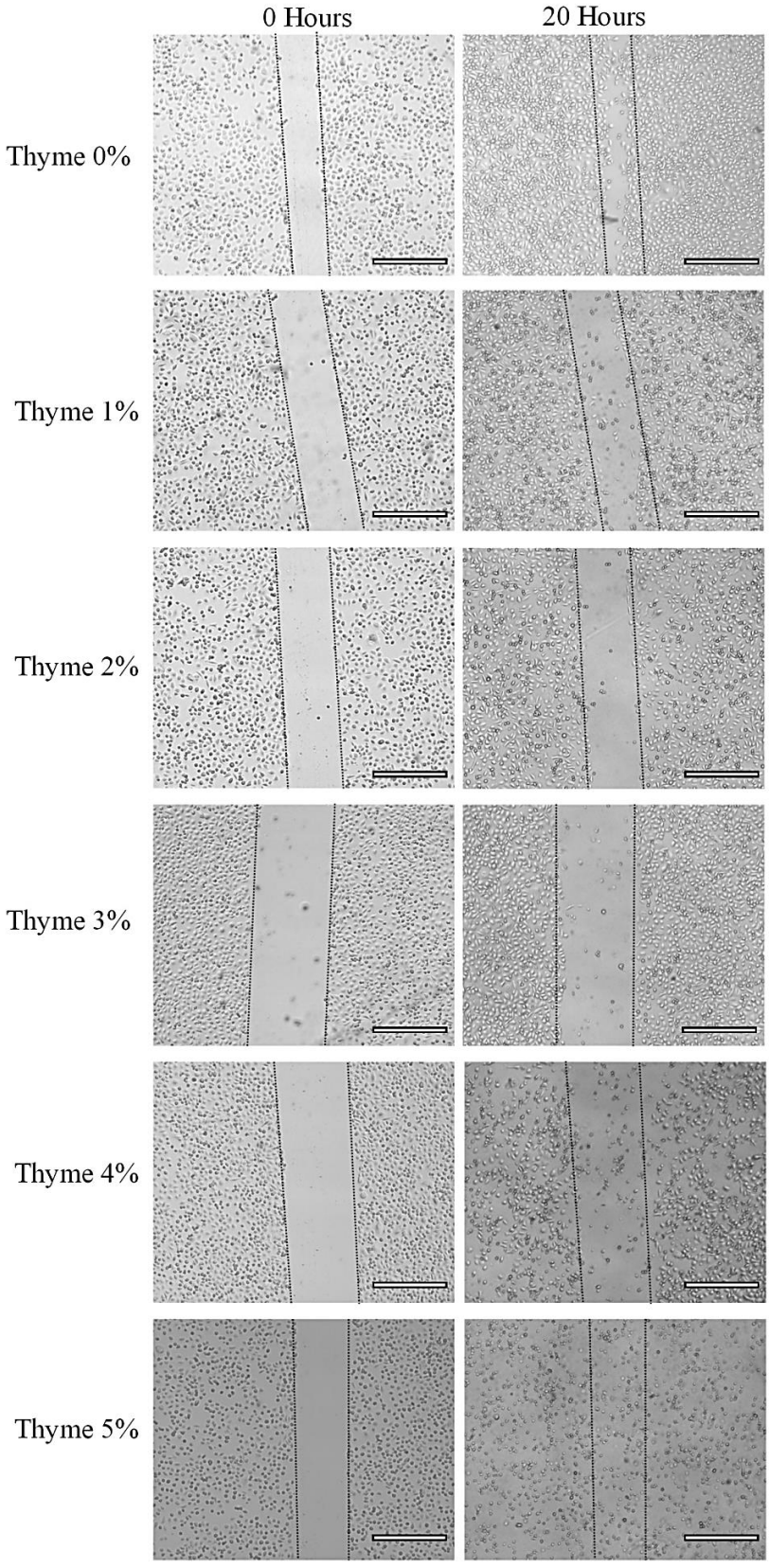


Figure 23. Effect of Thyme Honey on DU145 Cell Migration via the Scratch Wound Healing Assay. DU145 cells were seeded at a density of $3x10^5$ cells /well. Scratches were made pre-treatment (0 h). Cells were treated with 0-5% (w/v) thyme honey for 20 h. Visualisations were made using an inverted microscope at 40 x magnification, and were captured using a digital camera. All conditions were completed in triplicate, and representative images were presented (n = 3). Scale bars (white) represent 500 µm.

Some honey-related scratch wound healing assay results have been previously published, the majority used to assess the beneficial properties of honey with respect to topical wounds and dressings (Ranzato *et al.*, 2012; Sell *et al.*, 2012; Ranzato *et al.*, 2013). To date, there has been no assessment made for the anti-migratory effects of honey on cancer cells via the scratch wound healing assay. As no artificial honey control was used in the present study (**3.3.2**), it was not possible to conclude which component (sugar or non-sugar) of honey was responsible for the reduction in migration. However, as previous studies have shown that phenolic extracts, isolated from other substances, as well as single phenolic compounds can reduce the migration of cancer cells in a scratch wound healing assay, it could be assumed that phenolic compounds in honey may have been at least partially responsible (de Oliveira *et al.*, 2013; Dziedzic *et al.*, 2014).

Interestingly, previous reports suggest that the scope of the cancer cell selectivity of honey may extend beyond cytotoxicity. In contrast to the current findings that showed honey reduces the wound closure of cancer cells (**Figure 21**, **22**, **23**), honey has been shown to increase the wound closure of human dermal fibroblasts in a scratch wound healing assay (Ranzato *et al.*, 2013). The authors showed that three honeys accelerated fibroblast migration, through the induction of MMP-9 gelatinase activity. Their results were in agreement with previous studies that showed honey could increase re-epithelialization and wound repair by facilitating keratinocyte migration across a scratch wound healing assay (Majtan *et al.*, 2010; Ranzato *et al.*, 2012). These differences in migration may be context dependent; hence honey might promote the migration of cells involved in wound healing, while inhibiting the migration of cancerous cells. These differences may be attributed to the expression of cell- or phenotype-specific proteins during wound healing and cancer progression. During cancer progression, fibroblasts from the surrounding extracellular environment can be activated to become cancer-associated fibroblasts (CAF); a phenotypic alteration characterised by increased protease expression and tumourigenesis (Kharaishvili *et al.*, 2014). To further investigate this selectivity and possible duel-efficacy of honey, a comparison could be made between the expressions of proteases such as MMPs in honey-treated fibroblasts compared to their activated CAF counterpart.

3.3.3 Anti-metastatic Activity of Honey Measured by the Migration Assay

As it was shown that honey could inhibit prostate cancer cell migration in the scratch wound healing assay, Boyden chambers were employed to further assess the anti-migratory effects of honey. Only PC3 cells were investigated as these cells are a more invasive and aggressive

prostate cancer cell line, compared to the DU145 cells (Colella *et al.*, 2004; MuraliKrishna *et al.*, 2005; Kumar *et al.*, 2008). In order to better simulate an *in vivo* migration situation, the undersides of Boyden chambers were coated in collagen I (150 µg/mL). Lower wells contained 5% FBS medium and upper wells contained medium without FBS, in order to create a serum gradient to facilitate migration.

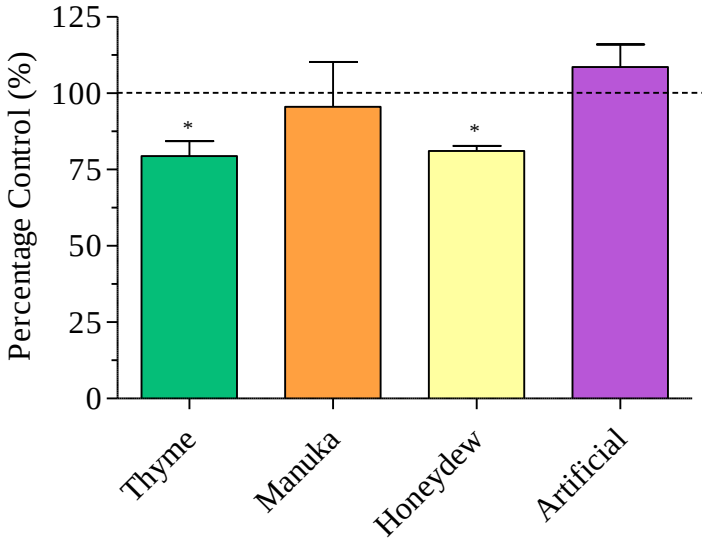


Figure 24. Effect of Honey on PC3 Cell Migration. The undersides of Boyden chamber inserts were coated in collagen I (150 µg/mL), and were placed in wells containing growth medium. PC3 cells suspended in serum-free growth medium were seeded at a density of $3x10^4$ cells/well. Cells were treated with 1% (w/v) thyme, manuka, honeydew or artificial honey for 48 h. After treatment, the MTT assay was used to quantify migrating cells. Experiments were completed in triplicate, and values were expressed as mean cell migration ± S.E.M (n = 3). Data were analysed using a two-tailed Student's t-test. * ($p<0.05$) represents a significant difference between individual treatment and untreated cell control.

After 48 h, the non-cytotoxic concentration of 1% (w/v) thyme or honeydew honey caused a significant reduction in PC3 cell migration compared to control of 20.59 ± 4.90 and 18.90 ± 1.62%, respectively ($p<0.05$) (**Figure 24**). These results were similar to those predicted from the scratch wound healing assay. Interestingly, manuka or artificial honey did not cause any reduction in cell migration. Despite the fact that manuka honey was not used to assess the migration of cells using the scratch wound healing assay, manuka honey was still expected to affect migration as it contains phenolic compounds similar to thyme honey. Artificial honey did not affect cell migration, suggesting that non-sugar related compounds in honey may be responsible.

Previously published data detailing the anti-migratory benefits of honey in cancer is limited. Gribel and Pashinskiĭ (1990) reported that honey may exhibit a greater *in vivo* anti-metastatic activity than anti-tumour activity in 5 strains of murine and rat tumours, and that the effects could be potentiated when given in combination with either cyclophosphamide or 5-fluorouracil. Unfortunately, the article was only distributed in Russian, and therefore no validation of their minimal abstract could be made. The anti-metastatic properties of manuka honey in cancer has however been reported, and have been related to the inhibition of cancer cell invasion and wide-spread metastasis through the down regulation of MMPs (Moskwa *et al.*, 2014). Therefore, despite no alteration in the migration of PC3 cells in the present study, manuka honey may still demonstrate anti-metastatic activity in PC3 cells via other mechanisms including inhibiting cell adhesion or invasion.

3.3.4 Anti-metastatic Activity of Honey Measured by the Invasion Assay

To further simulate the tumour microenvironment surrounding cancer cells, a migration Boyden chamber was used, with the addition of Matrigel® to the upper chamber. Matrigel® contains basement membrane proteins that resemble those through which cells must penetrate *in vivo* to invade the surrounding tissue. Compared to DU145 cells, PC3 cells have been demonstrated to express high levels of α6β1 integrins which favour adhesion to laminin (Witkowski *et al.*, 1993; Suyin *et al.*, 2013). The composition of Matrigel® includes laminin and collagen IV which may facilitate PC3 cell invasion (Hughes *et al.*, 2010).

After 72 h, the non-cytotoxic concentration of 0.5% (w/v) thyme, manuka or honeydew honey caused a significant reduction in PC3 cell invasion compared to control of 50.53 ± 4.10%, 60.44 ± 9.71 and 75.32 ± 0.19%, respectively ($p<0.05$) (**Figure 25**). Artificial honey caused a 46.77 ± 17.01% reduction in cell invasion, however, as experiments were only completed in duplicate, it was not statistically significant. The low sample size provided little power to detect a significant difference, and therefore it may true that artificial honey can reduce PC3 cell invasion, if a greater sample size was used. The findings were in agreement with the proposed hypothesis which predicted that honey would cause a reduction in the metastatic activity of PC3 cells.

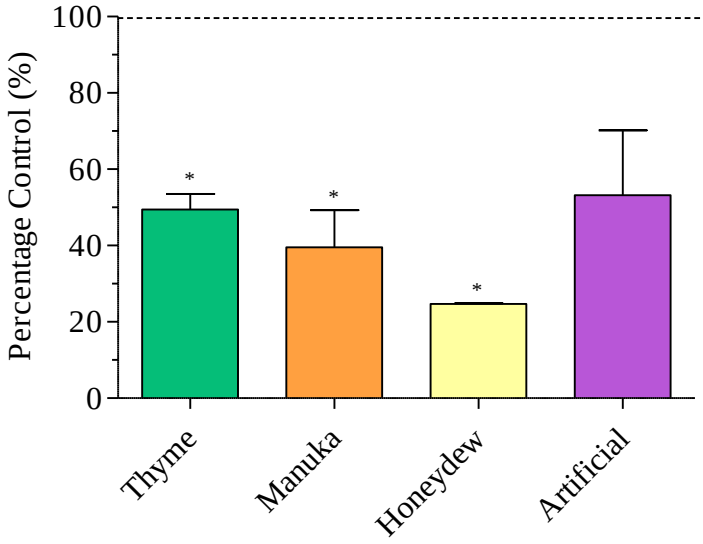


Figure 25. Effect of Honey on PC3 Cell Invasion. The underside of Boyden chamber inserts were coated in collagen I (150 µg/mL), and were placed in wells containing growth medium. The inside of each Boyden chamber was loaded with Matrigel®. PC3 cells suspended in serum-free growth medium were seeded at a density of $3x10^4$ cells/well. Cells were treated with 0.5% (w/v) thyme, manuka, honeydew or artificial honey for 72 h. After treatment, the MTT assay was used to quantify invading cells. Experiments were completed in duplicate, and values were expressed as mean cell migration ± S.E.M (n = 2). Data were analysed using a two-tailed Student's t-test. * ($p<0.05$) represents a significant difference between individual treatment and untreated cell control.

Previous reports have suggested that honey may inhibit migration and invasion in a range of cell types through changes in MMP expression. Fir honey, which is produced in a similar way to honeydew honey, was demonstrated to inhibit migration of human keratinocytes through the reduced expression of MMP-9 (Majtan *et al.*, 2013). Further, high phenolic-containing Polish honey (0.5% w/v) were demonstrated to inhibit the metastasis of human glioblastoma multiforme U87MG cells through the inhibition of MMP-2 (20 - 56%) and -9 (5 - 58%) (Moskwa *et al.*, 2014). Similarities between the findings in U87MG cells to those provided in the present report, suggested that the anti-metastatic activity of honey in PC3 and DU145 cells may be attributed a reduced expression of MMP-2 and -9. Other proteases such as uPA, serine proteases and cathepsins, along with tissue inhibitor metalloproteinase proteins (TIMPs) have been implicated in the migration and metastatic activity of cancer cells (Basset *et al.*, 1997; Reunanen *et al.*, 2000; Rao, 2003). Also, the inhibition of the pro-inflammatory marker NF-κB can reduce the expression of MMP-1, -2, -3 and -9 (Bond *et al.*, 2001; Lin *et al.*, 2010) Honey can reduce the expression and nuclear translocation of NF-κB both *in vivo* and *in vitro* (Batumalaie *et al.*, 2013; Hussein *et al.*, 2013). Therefore, it would be of interest to investigate

these in future studies through the use of gelatin zymography and Western blot. Further, it would be of interest to see whether an inhibition of MMP expression was due to the inhibition of NF-κB in both cell lines, if there were independent.

Other studies have shown that signalling and expression of Wnt and β-catenin may also be important in prostate cancer cell adhesion and growth, whereby β-catenin can facilitate cell adhesion through interactions with E-cadherin (Kypta *et al.*, 2012). In addition, it has been shown that activation of E-cadherin by small activating RNA can inhibit β-catenin, and therefore inhibit PC3 migration and invasion (Mao *et al.*, 2010). Luteolin, a dietary flavonoid also found in honey, was found to inhibit the *in vivo* lung invasion of PC3 cells in mice through the increased expression of E-cadherin, further highlighting the possible effects of phenolic compounds in cancer metastasis (Zhou *et al.*, 2009). It has been shown that flavonoids can modulate both Wnt and β-catenin, and therefore may explain the anti-metastatic activity of the high-flavonoid containing honeys in the present study (Surh, 2003; Amado *et al.*, 2011).

In order for cells to invade, proteases are expressed with integrins at the leading edge, where they facilitate the breakdown of the ECM (Friedl *et al.*, 2003). The upregulation of integrins for migration is modulated by focal adhesion kinase (FAK); a protein that has been previously implicated in cancer cell motility (Chan *et al.*, 2009). Due to the interaction between honey and integrin expression (mentioned above), the anti-migratory and anti-invasive activity of honey may be attributed to interactions of honey-components with FAK. Lee *et al*. (2004) demonstrated that 20 μM quercetin or luteolin, both flavonoids found in honey, could inhibit the activation of FAK and secretion of MMPs in the MIA PaCa-2 human pancreatic carcinoma cell line. Following this, Huang *et al*. (2005) reported that FAK was a key regulator in the metastatic potential of MIA PaCa-2 cells, and that both quercetin and luteolin could inhibit the tumour cell invasiveness.

The epithelial-mesenchymal transition (EMT) describes a process involved in the initiation of metastasis, in which cells lose their adhesive properties and gain a migratory phenotype, promoting metastasis (Kalluri *et al.*, 2009). Androgen depletion during treatment of metastatic prostate cancer may therefore induce EMT, and so a reduction in EMT alongside the androgen depletion therapy may be beneficial for prostate cancer treatment (Nouri *et al.*, 2014). Caffeic acid phenethyl ester (CAPE), found in honey, was shown to inhibit human pancreatic cancer EMT through the attenuation of vimentin overexpression, and therefore, may suggest a role for the inhibition of EMT by honey (Chen *et al.*, 2013b).

A reduction in E-cadherin expression and up-regulation of fibronectin have both been linked to the EMT, as well as the migration and invasion of mesenchymal stem cells (MSC) (Adhikary *et al.*, 2014). Further, MSC migration may be facilitated through the interaction between α5β1 integrins and fibronectin surrounding tumours (Veevers-Lowe *et al.*, 2011). Due to the effect of honey on the expression and activity of integrins, honey may also reduce the migration and invasion of MSCs towards cancer cells. The microenvironment infiltration of MSCs has recently been reported to increase prostate cancer growth and metastasis due to an increased secretion of cytokines and proteases (Luo *et al.*, 2014). As MSC migration and invasion has been reported to advance tumourigenesis, the inhibition of these proteases by honey may further reduce the growth and metastasis of the primary prostate tumour. Therefore, the anti-metastatic activity of honey in MSCs should be investigated, as the duel inhibition may provide *in vivo* benefit for the inhibition of cancer cell metastasis.

3.4 High Performance Liquid Chromatography (HPLC)

It has been reported that phenolic compounds may be responsible for a majority of the therapeutic benefits attributed to honey (Kassim *et al.*, 2010; Alvarez-Suarez *et al.*, 2013; Erejuwa *et al.*, 2014). Therefore, the different levels of anti-tumour and anti-metastatic activity between the three honeys in the present study may be due to differences in the concentrations of phenolic compounds. For this reason, the identification and quantification of phenolic compounds within thyme, manuka and honeydew honey was made.

Reverse-phase High Performance Liquid Chromatography (HPLC) is a technique that can be used to identify and quantify individual compounds. A mixture of compounds is passed through a column, and individual compounds will stay bound to the column for different periods of time (retention time). By previously determining the retention time and wavelength at which a peak absorbance is reached for each compound standard, comparisons can be made against the mixture to quantify individual compounds.

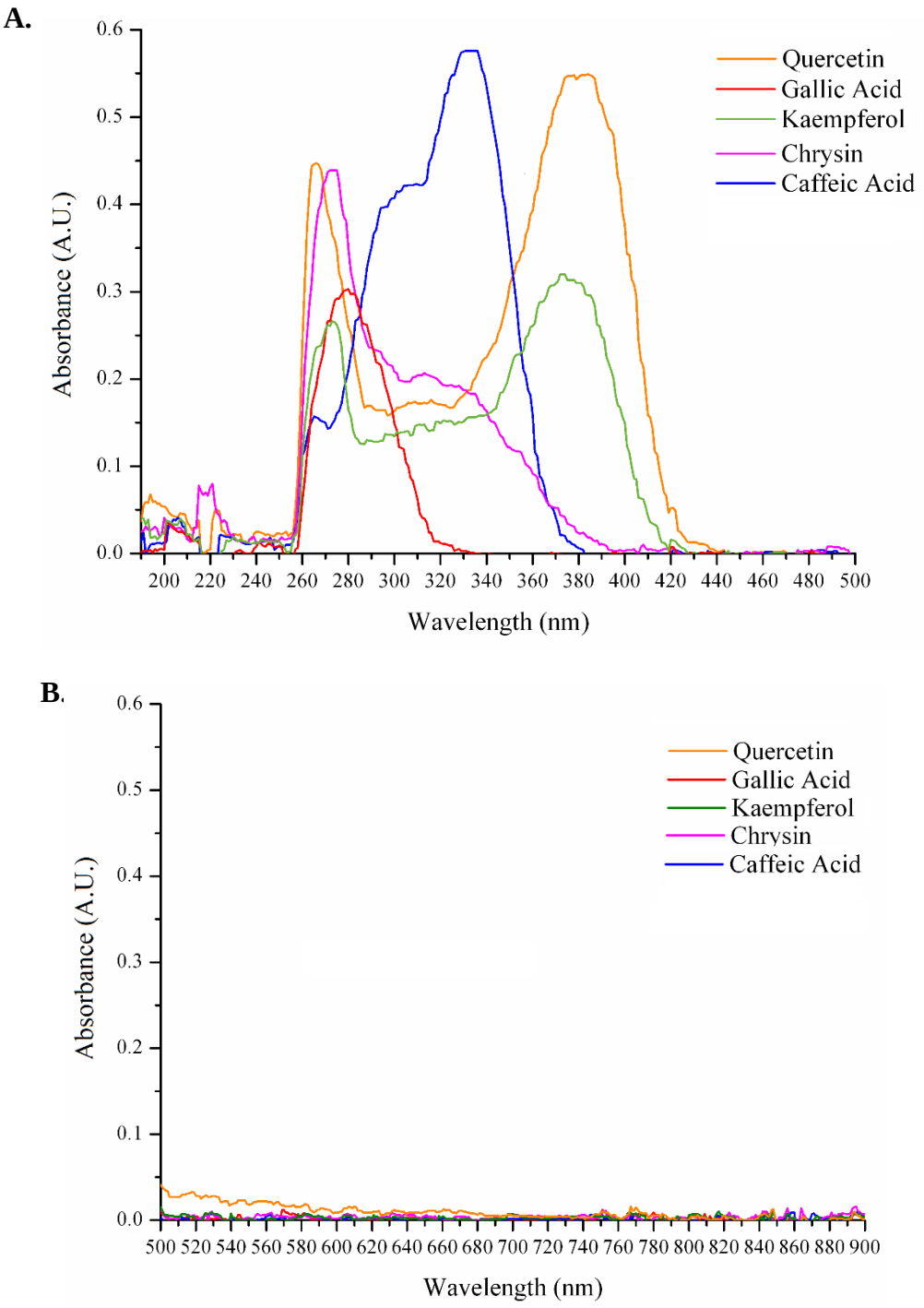


Figure 26. Absorbance Spectra of Honey-derived Phenolic Compound Standards. Absorbance spectrum for honey-derived phenolic compounds by spectrophotometry. (**A**) UV-lower visible spectrum (200 - 500 nm) shows absorbance profiles at low concentration. (**B**) Visible-infrared spectrum (500 - 900) demonstrates compound standard purity at high concentration. Profiles were used to determine optimal wavelengths of each compound for HPLC analysis. Wavelengths were selected as follows: quercetin 370 nm, gallic acid 270 nm, kaempferol 370 nm, chrysin 270 nm and caffeic acid 325 nm.

Using a spectrophotometer, absorbance spectrum profiles were constructed for a selection of 5 honey-derived phenolic compounds (2 phenolic acids and 3 flavonoids) (**Appendix 4**). These phenolic compounds were selected as they have been found at high concentrations in many honey types, and have consistently demonstrated anti-cancer activity in many cell types. Optimal wavelengths for each were selected as: 370 nm (quercetin and kaempferol), 325 nm (caffeic acid) and 270 nm (gallic acid and chrysin) (**Figure 26**). The choices of peak wavelengths were confirmed through comparisons between the UV absorbance spectra (**Figure 26**) and the wavelengths observed via HPLC using photodiode array detection. Wavelengths selected for each compound were different to those in most published literature. Conventionally, honey-derived phenols in HPLC are measured at 290 (phenolic acids) and 340 nm (flavonoids and polyphenols) (Ferreres *et al.*, 1994; Martos *et al.*, 1997; Makawi *et al.*, 2009; Kassim *et al.*, 2010). In the present study, the ability to use multiple wavelength detectors was hugely beneficial, as wavelengths could be selected specifically for each compound to optimise detection and increase peak identification confidence.

In order to quantify the 5 selected phenolic compounds in each honey sample, standards were individually eluted through the column over a range of concentrations (0.5 - 100 µg/mL). Standard equations were constructed by plotting the area under the peak versus concentration. Strong linearity was observed for all compound equations derived by linear regression (R^2>0.996) (**Table 11**). Further, through the elution of compound standards, retention times for each compound were determined (**Table 12**).

Table 11. Linear Regression Analysis for Compound Calibration Curves. Using linear regression, compound standards (0.5 - 100 µg/mL) were eluted with HPLC. Concentrations were plotted against area under the peak, and were used to derive an equation in order to quantify phenolic compounds in honey. Equation values were given as mean ± S.D. (n = 1)

Compound Standard	Equation (± SD)	R^2
Gallic Acid	y=0.9331 ± 0.008739x + 0.1888 ± 0.5006	0.9998
Caffeic Acid	y=1.754 ± 0.07566x + 2.528 ± 4.334	0.9963
Quercetin	y=1.397 ± 0.01004x + 1.394 ± 0.6642	0.9999
Kaempferol	y=1.431 ± 0.01663x + -0.3614 ± 0.9524	0.9997
Chrysin	y=2.001 ± 0.03654x + 0.6887 ± 1.047	0.9993

In the present study, all three New Zealand honeys underwent extraction using ethyl acetate to purify the phenolic compounds. Free-phenolic extracts consisted of phenolic compounds that exist in honey unbound to sugars, as aglycones. Total-phenolic extracts consisted of a

combination of phenolic-aglycones, and phenolic compounds that are bound to sugars as glycosides, where the sugars were removed by hydrolysis beforehand. Absorption chromatograms were obtained for each honey sample (free and total phenolic) from their elution through the C_{18} column. Representative absorption chromatograms are displayed for both the free and total phenolic compounds present in thyme honey as detected using a diode array at 370, 325 and 270 nm (**Figure 27, 28**).

Gallic acid recorded the highest concentration among the phenolic compounds in all three honeys, with the highest concentration being 1082.88 ± 16.25 µg/100 g free-phenol extracted honeydew honey (**Table 12**). In support of this finding, others have also found gallic acid to be the predominant phenolic acid in both Australian and New Zealand honeys (Yao *et al.*, 2003; Yao *et al.*, 2004), however the amount found here was less than concentrations reported in those manuka honey samples (5.74 - 8.36 mg/100 g honey) (Yao *et al.*, 2003). In the manuka honey absorbance chromatographs published by Yao *et al*., gallic acid was eluted at the same time as the injection peak, and therefore quantification of gallic acid could not have been made. Further, as many compounds co-eluted, confident identification of gallic acid could not have been undertaken and would have required improved optimisation to their gradient method. In the present study, quercetin showed the lowest concentration among all phenolic compounds, and it was only detectable in free phenol-extracted thyme and manuka honey (**Table 12**). In contrast, quercetin was earlier found to be undetectable in thyme honey (Abd El-Hady *et al.*, 2013). Differences between honey phenolic content might be explained by differences in geographical origin.

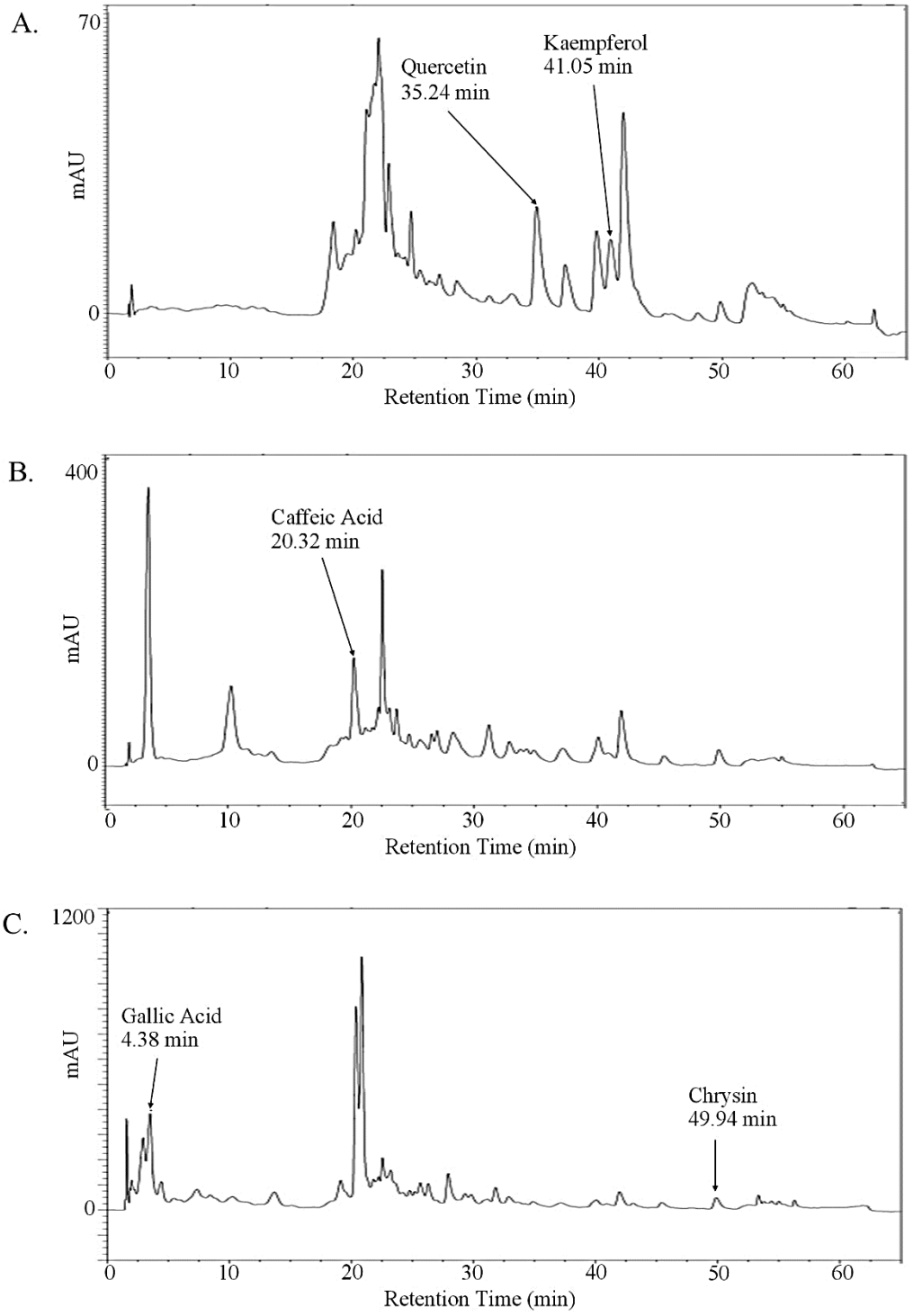


Figure 27. Absorption Chromatogram of Free Phenolic Compounds in Thyme Honey using HPLC. Representative chromatogram of free phenols extracted from thyme honey using ethyl acetate and eluted through a C_{18} column using HPLC. (**A**) Detectors were set to 370 nm for quercetin and kaempferol, (**B**) 325 nm for caffeic acid and (**C**) 270 nm for gallic acid and chrysin. Peak retention times were compared to standards to identify compounds.

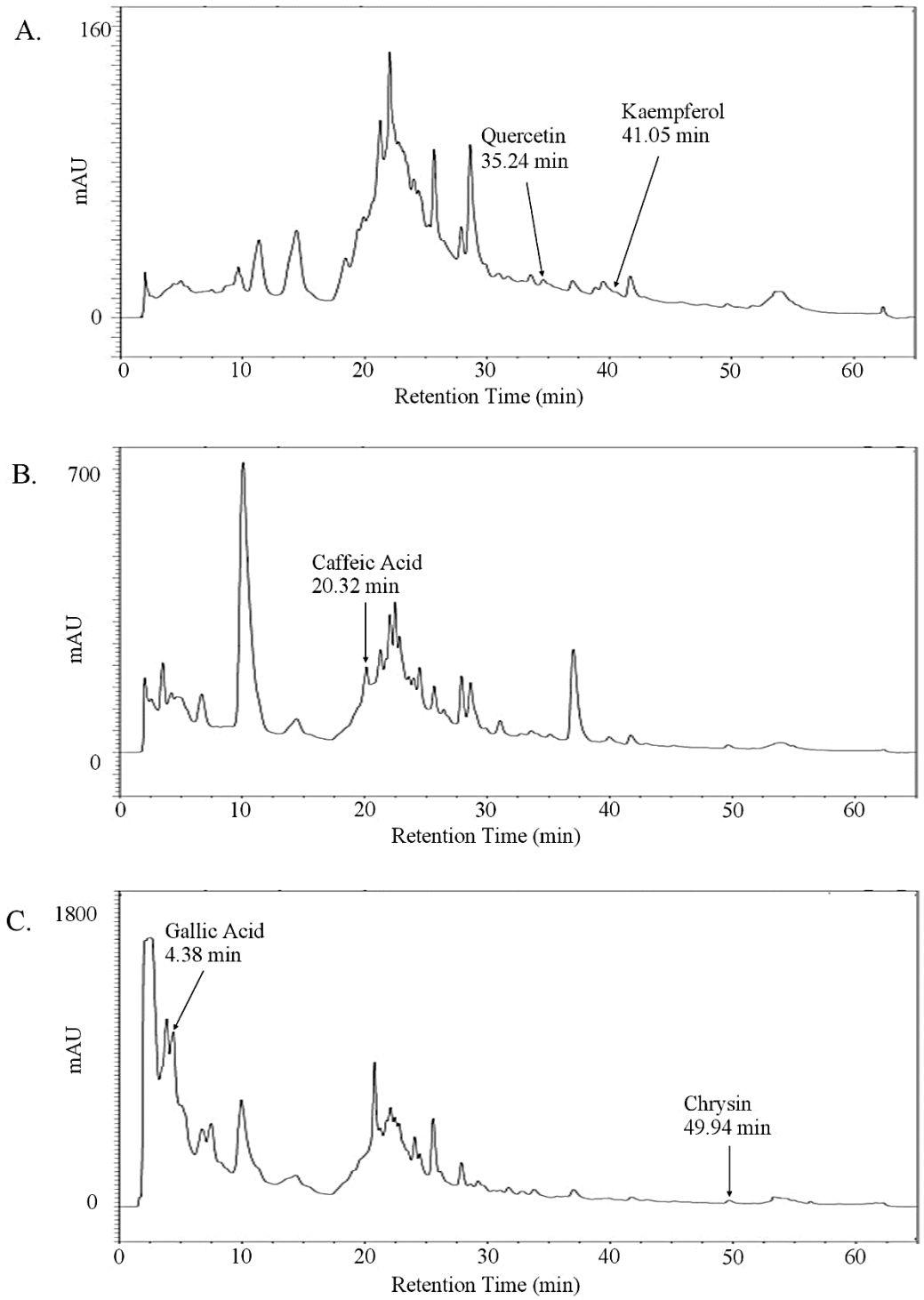


Figure 28. Absorption Chromatogram of Total Phenolic Compounds in Thyme Honey using HPLC. Representative chromatogram of total (bound and free) phenols extracted from thyme honey and ethyl acetate were eluted through a C_{18} column using HPLC.(**A**) Detectors were set to 370 nm for quercetin and kaempferol, (**B**) 325 nm for caffeic acid and (**C**) 270 nm for gallic acid and chrysin. Peak retention times were compared to standards to identify compounds.

Table 12. HPLC Analysis of Ethyl Acetate-Extracted Phenolic Compounds from Honey. Free-phenolic and total-phenolic extracts from each honey were analysed using HPLC with a photodiode array. Optimal compound wavelengths (λ) were determined using HPLC and spectrophotometry (**Figure 26**). Compound retention times were determined from standards, and expressed as mean min ± S.E.M (n = 18). Compound concentrations (µg/100 g honey) were calculated by comparing the area under the peak to standard calibration curves (**Table 11**), and were expressed as mean ± S.E.M (n = 6). Concentrations of each compound (nM), were also calculated from within a 5% (w/v) solution of honey.

Retention Time (min)	Compound	λ Maxima (nm)	Honey	[µg/100 g honey]		[5% (w/v) solution] (nM)	
				Free Phenols	Total Phenols	Free Phenols	Total Phenols
4.38 ± 0.02	Gallic Acid	270	Thyme	94.18 ± 2.54	685.97 ± 15.17	276.80 ± 7.47	2016.00 ± 44.57
			Manuka	741.45 ± 27.37	584.46 ± 25.86	2179.00 ± 80.43	1718.00 ± 76.02
			Honeydew	48.13 ± 6.174	1082.88 ± 16.25	141.50 ± 18.15	3183.00 ± 47.77
20.32 ± 0.02	Caffeic Acid	325	Thyme	90.22 ± 2.72	83.67 ± 2.69	250.40 ± 7.56	232.20 ± 7.45
			Manuka	44.80 ± 3.543	131.31 ± 17.05	124.30 ± 9.83	364.40 ± 47.31
			Honeydew	28.89 ± 2.52	96.57 ± 9.03	80.18 ± 6.99	268.00 ± 25.05
35.24 ± 0.12	Quercetin	370	Thyme	38.22 ± 3.20	–	63.23 ± 5.30	–
			Manuka	10.60 ± 7.40	–	17.54 ± 12.24	–
			Honeydew	–	–	–	–
41.05 ± 0.10	Kaempferol	370	Thyme	45.33 ± 13.78	33.12 ± 12.18	79.19 ± 24.07	57.86 ± 0.57
			Manuka	9.28 ± 2.64	12.18 ± 1.98	16.21 ± 4.61	21.29 ± 3.50
			Honeydew	11.21 ± 1.80	8.61 ± 0.15	19.59 ± 3.14	15.04 ± 0.26
49.94 ± 0.07	Chrysin	270	Thyme	53.00 ± 0.27	38.10 ± 0.35	104.3 ± 0.523	74.94 ± 0.69
			Manuka	77.60 ± 0.84	72.13 ± 1.30	152.60 ± 1.66	141.90 ± 2.54
			Honeydew	46.38 ± 0.46	26.82 ± 0.57	91.22 ± 0.90	52.75 ± 1.11

Many unidentified peaks were observed in chromatograms among all of the honey samples, most of which recorded a higher absorbance than sample compounds. Of the 180 - 200 constituents in honey, multiple phenolic compounds are present in a wide range of concentrations (White, 1978; Gheldof *et al.*, 2002; Alvarez-Suarez *et al.*, 2013). The scope of the present study included the identification of only five phenolic compounds, and therefore, expansion of the study could include the identification of further compounds.

A comparison can be made between phenolics compounds (µg/100 g honey) extracted from New Zealand honeys in the current study (**Table 12**), against Bangladeshi (Moniruzzaman *et al.*, 2014), Malaysian (Kassim *et al.*, 2010), Australian (Yaoa *et al.*, 2005), Sudanese (Makawi *et al.*, 2009) and Spanish (Ferreres *et al.*, 1991) honey. Of the phenolic acids present in New Zealand honey, gallic acid (584 - 1082 µg/100 g) was more abundant than caffeic acid (83 - 131 µg/100 g). Gallic acid concentration was similar to Australian (1580 µg/100 g) and Bangladeshi (610 µg/100 g) honey, but higher than in Malaysian honey (341 µg/100 g). Caffeic acid was similar to Malaysian honey (158 µg/100 g) but less than Bangladeshi honey (2660 µg/100 g). Of the flavonoids in New Zealand honey, chrysin (46 - 77 µg/100 g) was found at the highest concentration, followed by kaempferol (9 - 45 µg/100 g) and quercetin (0 - 38 µg/100 g). Chrysin was similar to Malaysian honey (69 µg/100 g) but greater than in Spanish honey (24 µg/100 g). Both quercetin and kaempferol had a similar concentration to Malaysian honey (66 µg/100 g and 16 µg/100 g, respectively). A comparison between Sudanese honey and the New Zealand honeys showed that the concentration of quercetin was similar to that found in sunflower honey (70 µg/100 g) but less than blue nile (320 µg/100 g); and kaempferol concentration was similar to that found in blue nile (40 µg/100 g) but less than sunflower (535 µg/100 g). In study by Yao *et al*. (2003), New Zealand manuka honey was reported to contain a higher total flavonoid content (3.06 mg/100 g honey) compared to Australian jelly bush honey (2.22 mg/100 g honey). Quercetin (13.8%) isorhamnetin (12.9%) chrysin (12.7%) and luteolin (12.6%) represented 52% of the total flavonoids. Of the remaining 48% total flavonoids, compounds including kaempferol, quercetin 3-methyl ether, pinocembrin, myricetin and many others were found at low concentrations (Yao *et al.*, 2003). Weston *et al*. (1999) found some of the same flavonoids as Yao *et al.* such as chrysin in manuka honey, however, at much lower levels (0.01 vs 0.38 mg/100 g honey). Yao *et al*. explained that the difference in flavonoid content could be due to differences in extraction methods. This further highlighted the importance of sensitive separation and extraction methods, and illustrated the difficulty in providing a standardised quantification of phenolic compounds in honey.

The phenolic composition of honey varies greatly, with geographical location, floral origin and inter-seasonal variables being likely to be responsible for the majority of differences (Pyrzynska *et al.*, 2009; Moniruzzaman *et al.*, 2014). It is difficult to compare the quantity of a compound in the present study, against those recorded in the literature, as there are a wide range of methods involved in extraction, purification and HPLC. Further, limitations to each process including method sensitivity and degradation of phenolics by chemicals, mean it is not possible to recover 100% of a compound from a honey sample, and therefore, the exact quantity of a compound may not be known. Nevertheless, the greater the extraction and purification of phenolic compounds from the honey matrix is, the more accurate the quantification of each compound can be.

3.4.1 Glycosides and Aglycones

Phenolic compounds in honey may exist as either glycosides bound to sugars, or as aglycones that have been released from sugars. In honey, flavonoids often exist as aglycones, however, phenolic acids predominantly exist as glycosides that originate from pollen (Ferreres *et al.*, 1991). Due to an increased water solubility, phenolic acids bound to sugars as glycosides may have a lower yield when extracted by organic solvents such as ethyl acetate, therefore, it was necessary to hydrolyse the sugar bonds for HPLC analysis (D'Arcy, 2005; Kassim *et al.*, 2010).

In the present study, free-phenolic extract samples contained unbound phenolic compounds (aglycones) from within honey, which were extracted readily by ethyl acetate alone. Total-phenolic extract samples contained all phenolics, including those present in honey as free phenolic-aglycones, and those originally found bound as phenolic-glycosides, which required the hydrolysis of the glyosidic ester bond to release the phenolics as aglycones (Wahdan, 1998). It was therefore expected that total-phenolic extract samples from honey would contain a higher concentration of phenolic compounds compared to the free-phenolic extract samples, as the total-phenolic extracts would contain phenolics that originated from both aglycone and glycoside form. It was therefore important to quantify not only the phenolic-aglycones, but also the phenolic-glycosides, as the quantification of aglycones alone would have underestimated the total phenolics present. The proportion of phenolic compounds that existed as aglycones compared to glycosides could be measured using HPLC, by comparing the free-phenolic extracts to the total-phenolic extracts.

In contrast to the phenolic acids investigated, the flavonoids, quercetin, kaempferol and chrysin, showed a greater recovery in free-phenolic extracts. These findings suggested that, in New Zealand honey, phenolic acids are bound to sugars as glycosides, and flavonoids exist as free aglycones. Interestingly, the flavonoids showed a reduced recovery in the total-phenolic extracts, compared to the free-phenolic extracts, suggesting that the total-phenolic extraction method may have been responsible. This decrease may have been due to the degradation of flavonoids by hydrolysis, as previously shown by Merken *et al.* (2000). The authors showed that extended hydrolysis can degrade quercetin, which therefore, may explain the reduced concentration of flavonoids in total-phenolic extract samples in the present study. In addition, the optimal time for a hydrolysis reaction has been suggested to be 1 - 2 h (Pyrzynska *et al.*, 2009), however, 4 h was used in the current study, adapted from the extraction of phenolic acids from honey by Wadhan (1998). Therefore, the duration of glycoside hydrolysis during extraction should be reduced in all future experiments, to prevent flavonoid degradation.

Due to the hydrolysis of phenolic acid-glycosides resulting in the increased release of phenolic acid-aglycones, a higher recovery of caffeic acid and gallic acid were observed in total-phenolic extracts from all honey types, with the exception of gallic acid in manuka honey. Gallic acid in manuka honey was found to be 20% lower in the total-phenolic manuka extract of honey, compared to the free-phenolic extract (**Table 12**). It may be possible that gallic acid exists as an aglycone in manuka honey, and therefore, is subjected to degradation by hydrolysis similar to that seen in flavonoid aglycones. The existence as an aglycone may be due to the fact that manuka honey contains high concentrations of the organic pyruvaldehyde compound, methyglyoxal (MGO), as well as an enzyme, glucose oxidase, that produces the ROS hydrogen peroxide (White *et al.*, 1980). In honey, reactions between MGO and hydrogen peroxide can lead to the formation of free radicals (Nakayama *et al.*, 2007). Radicals, along with hydrogen peroxide, have been reported to be partially responsible for the hydrolysis of glycoside bonds between phenolic compounds and sugars in honey (Brudzynski *et al.*, 2011b; Hussein *et al.*, 2011). This hydrogen peroxide-mediated hydrolysis may have increased the conversion of gallic acid from a glycoside to an aglycone, allowing for the degradation of gallic acid by the additional hydrolysis step during the total-phenolic extraction of manuka honey. However, contrary to this proposed mechanism, MGO in manuka honey has also been reported to reduce the accumulation of hydrogen peroxide by inhibiting glucose oxidase (Majtan *et al.*, 2014). Nevertheless, no reports have been made suggesting this lowers the total free radical activity in manuka honey, and therefore, free radical reactions may still be part of the explanation.

3.4.2 Critical Evaluation of HPLC Methods

3.4.2.1 Sample Preparation

Before attempting ethyl acetate extraction to concentrate the phenolic compounds, methanol extraction was tested, however, it resulted in a poor column elution and a low compound recovery. These results were similar to those found by Alvarez (2010), who showed that alcohol extraction of phenolics could only extract 40% of the protein-bound phenolics in honey. In contrast, Kassim *et al*. (2010) showed that when compared to ethyl acetate, methanol extraction of honey recorded a 2 - 4 fold higher recovery of phenolic compounds. Differences between Kassim *et al*. and the current study could be explained due to their use of an XAD-2 resin filter, which can allow for a greater absorption and extraction of organic compounds (Rosler *et al*., 1984). The increased recovery of phenolic compounds by use of XAD-2 resin, when compared to a C_{18} column as used in the present study, was also shown by Pyrzunska *et al*. (2009). In the present study, due to the reduced recovery seen for methanol with a C_{18} column and a lack of XAD-2 resin, ethyl acetate extraction together with the C_{18} column was used.

3.4.2.2 The use of a Gradient Elution

Preliminary experiments, involving an isocratic elution with methanol and acetic acid, were used for the separation and quantification of extracted phenolics compounds, as adapted from Makawi *et al*. (2009). The isocratic elution of phenolic acids using methanol and acetic acid in water has also been performed previously (Zgórka *et al.*, 2001), however, due to the hydrophilicity and small size of phenolic acids, their retention time is often short (Moniruzzaman *et al.*, 2014). In contrast, as flavonoids are more hydrophobic compared to phenolic acids, their retention time may be longer. Preliminary results found that the isocratic elution of honey resulted in a poor separation of caffeic acid and gallic acid which coeluted early, while the flavonoid chrysin had a retention time that exceeded 90 min (data not shown). It has been suggested that when multiple phenolic compounds, with variations in polarities and chemical structure, are present such as phenolic acids and flavonoids, a gradient elution is necessary (Stalikas, 2007; Moniruzzaman *et al.*, 2014). Due to the inferior power of the isocratic system with regard to honey, a gradient elution was used.

3.4.2.3 Conclusion of Phenolic Compound Quantification by HPLC

Taken together, the phenolic content of honey may be extremely variable due to differences between floral sources, geographical locations, and harvesting seasons. Further, the quantification of phenolic compounds from honey may be variable due to differences in methods between HPLC, extraction and purification. For these reasons, it may be difficult to know the true concentration of a phenolic compound within honey. If honey were to be administered clinically, these differences may translate to an altered efficacy, therefore, the standardisation of methods for the quantification of honey-derived phenolics, along with the standardisation of phenolic content by supplementation, may be required before honey can be accepted as a medicine.

Chapter 4: Honey-derived Phenolic Compounds Results and Discussion

It has frequently been suggested that phenolic compounds may be responsible for the majority of honey-related benefits, and therefore, the present study aimed to investigate the independent anti-metastatic activity of five phenolic compounds. The phenolic acids (gallic acid and caffeic acid) and flavonoids (quercetin, kaempferol, and chrysin) were detected in all honey samples, with the exception of quercetin in honeydew honey (**Table 12**). As these compounds have often been identified at high concentrations in many honey types (Makawi *et al.*, 2009; Kassim *et al.*, 2010; Moniruzzaman *et al.*, 2014), they were selected for further investigation.

4.1 Cytotoxicity of Honey-derived Phenolic Compounds

4.1.1 MTT Assay Optimisation

During assay optimisation, it was found that high concentrations of some phenolic compounds in medium only, recorded a change in absorbances at 560 nm, despite no colour change being visible (**Figure 29A**, **D**). It was found that at high concentrations, kaempferol (>50 µM), chrysin (>100 µM) and quercetin (>300 µM) had precipitated into growth medium (**Figure 29A**, **D**).

Next, when MTT was added to medium with compounds, all compounds caused an increase in absorbance at 560 nm (**Figure 29B**, **E**). Of these compounds, quercetin caused the largest colour change, which became maximal at 100 µM. Chrysin caused the least colour change, which could only be measured with 500 µM chrysin.

Finally, formazan crystals from the reduction of MTT by the phenolic compounds were solubilised in DMSO (10:90 v/v) (**Figure 29C**, **F**). With the exception of chrysin that displayed no change in absorbance, all compounds plateaued at a maximal absorbance between 100 - 200 µM. Interestingly, high concentrations of kaempferol caused a colour change from dark purple, to purple/red, suggesting a shift in the formazan spectrum peak maxima.

At all stages throughout the assay, a DMSO control caused no change in absorbance at 560 nm. Interestingly, when MTT was directly added to compound stocks (DMSO) in the absence of growth medium, no colour change was observed, which suggested that growth medium may contain cofactors required for the reduction of MTT.

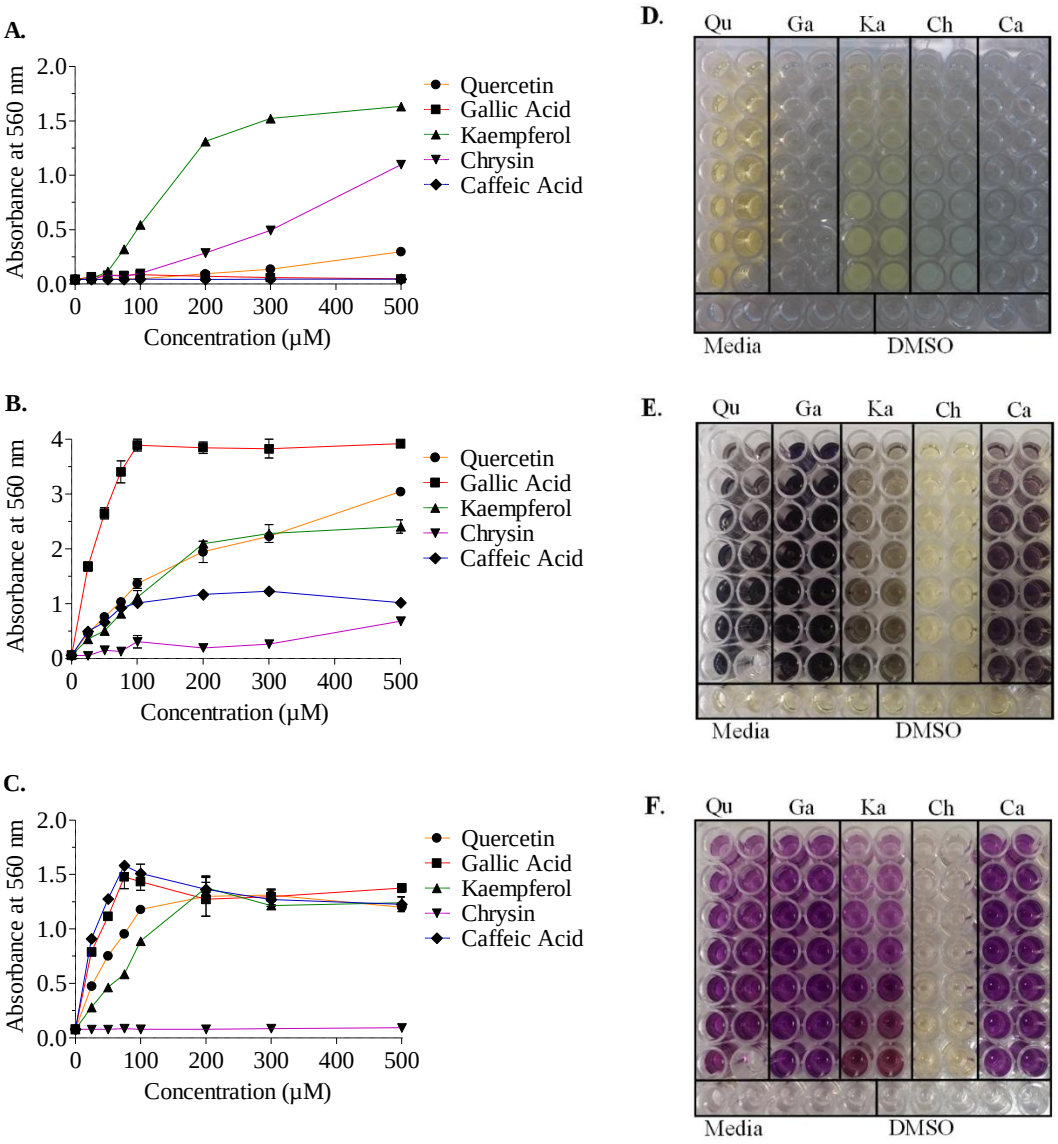


Figure 29. Intrinsic Reduction of MTT from Honey-derived Phenolic Compounds. A wide concentration range of honey-derived phenolic compounds (25, 50, 70, 100, 200, 300 and 500 µM) dissolved in growth medium, were examined at different stages throughout the MTT assay. Absorbances were measured at 560 nm using a spectrophotometer and photographs were taken at each stage: (**A**, **D**) compounds dissolved in DMSO, (**B**, **E**) Compounds with MTT (5 mg/mL) for 30 min, (**C**, **F**) Formazan crystals from the reduction of MTT by compounds, solubilised in DMSO (10:90 v/v).

In a similar manner to the observations made here, Pagliacci *et al*. (1993) identified a potential limitation to the MTT assay in which genistein, an isoflavone found in many foods and plants, possessed the ability to reduce MTT to formazan, resulting in cell death being underestimated. Since 1993, many groups have identified, in the absence of cells, the ability of both plant extracts (Bruggisser *et al*., 2002; Shoemaker *et al*., 2004; Muraina *et al*., 2009) and phenolic compounds such as quercetin, caffeic acid, luteolin and apigen, to reduce MTT to formazan (Habtemariam, 1995; Peng *et al*., 2005; Talorete *et al*., 2006). Reduction of MTT by both anti-

oxidants and chemotherapeutic agents, independent of their effect on cell death, has been linked to the presence of free thiol groups, again resulting in an underestimated cytotoxicity (Natarajan *et al.*, 1999). Further, the polyphenol resveratrol was shown to paradoxically increase MTT reduction while decreasing cell proliferation, suggesting that cytotoxicity from compounds that possess anti-oxidant activity should not be measured using the MTT assay (Bernhard *et al.*, 2003). Despite an abundance of evidence for MTT reduction in the absence of cells, papers are still published in which the MTT assay is used to assess cell death or proliferation caused by compounds containing many phenolic groups and anti-oxidant properties (Danihelova *et al.*, 2013; Jaramillo-Carmona *et al.*, 2014; Liao *et al.*, 2014).

The extensive reduction of MTT by honey-derived phenolic compounds at low concentrations demonstrated that the MTT assay may not suitable for the assessment of cytotoxicity of phenolic compounds. However, it was important to assess its usage, since this assay is part of the anti-metastatic assays which follow.

4.1.2 Cytotoxicity of Honey-derived Phenolic Compounds Measured by the SRB Assay

A maximal non-lethal concentration for each honey-derived phenolic compound for the use in the anti-metastatic assays, was determined using the SRB assay. No interaction between the compounds and the SRB assay was detected (**Appendix 3**).

Concentration-dependent effects in both cell lines were found for quercetin, gallic acid and caffeic acid ($p<0.05$) (**Figure 30**, **31**). All compounds demonstrated a time-dependent decrease in cell viability, however, only quercetin and gallic acid has a significant effect in both cell lines ($p<0.05$). In PC3 cells, gallic acid was the most cytotoxic compound, followed by quercetin and caffeic acid. No significant cytotoxicity was recorded for kaempferol and chrysin up to 150 µM, however, a maximal non-lethal concentration of 100 µM was selected for chrysin in the anti-metastatic assays, due to large variances in results.

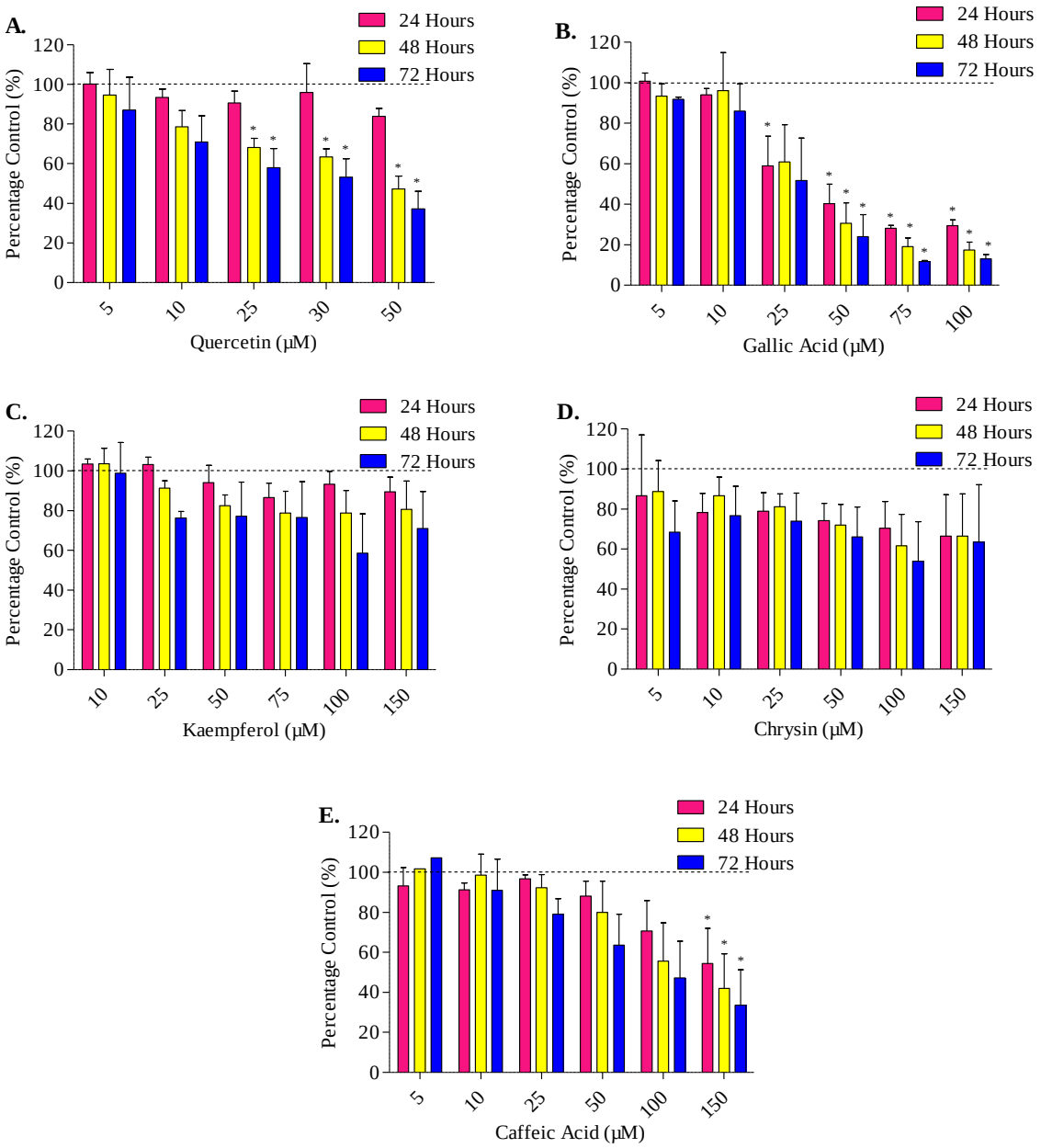


Figure 30. Honey-derived Phenolic Compound Cytotoxicity Quantified by the SRB Assay in PC3 Cells. PC3 cells were seeded at a density of $3x10^3$ cells/well and treated with (**A**) quercetin (0 - 50 µM), (**B**) gallic acid (0 - 100 µM), (**C**) kaempferol (0 - 150 µM), (**D**) chrysin (0 - 150 µM) or (**E**) caffeic acid (0 - 150 µM) for 24, 48 and 72 h. After incubation time, compounds were removed and viability was established via the SRB assay. Experiments were completed in triplicate, and values were expressed as mean percentage cell viability ± S.E.M (n = 3). Data were analysed using a two-way ANOVA followed by a Bonferroni post-hoc test, where $p<0.05$ was required for a statistically significant difference. * represents a significant difference between control and individual treatment.

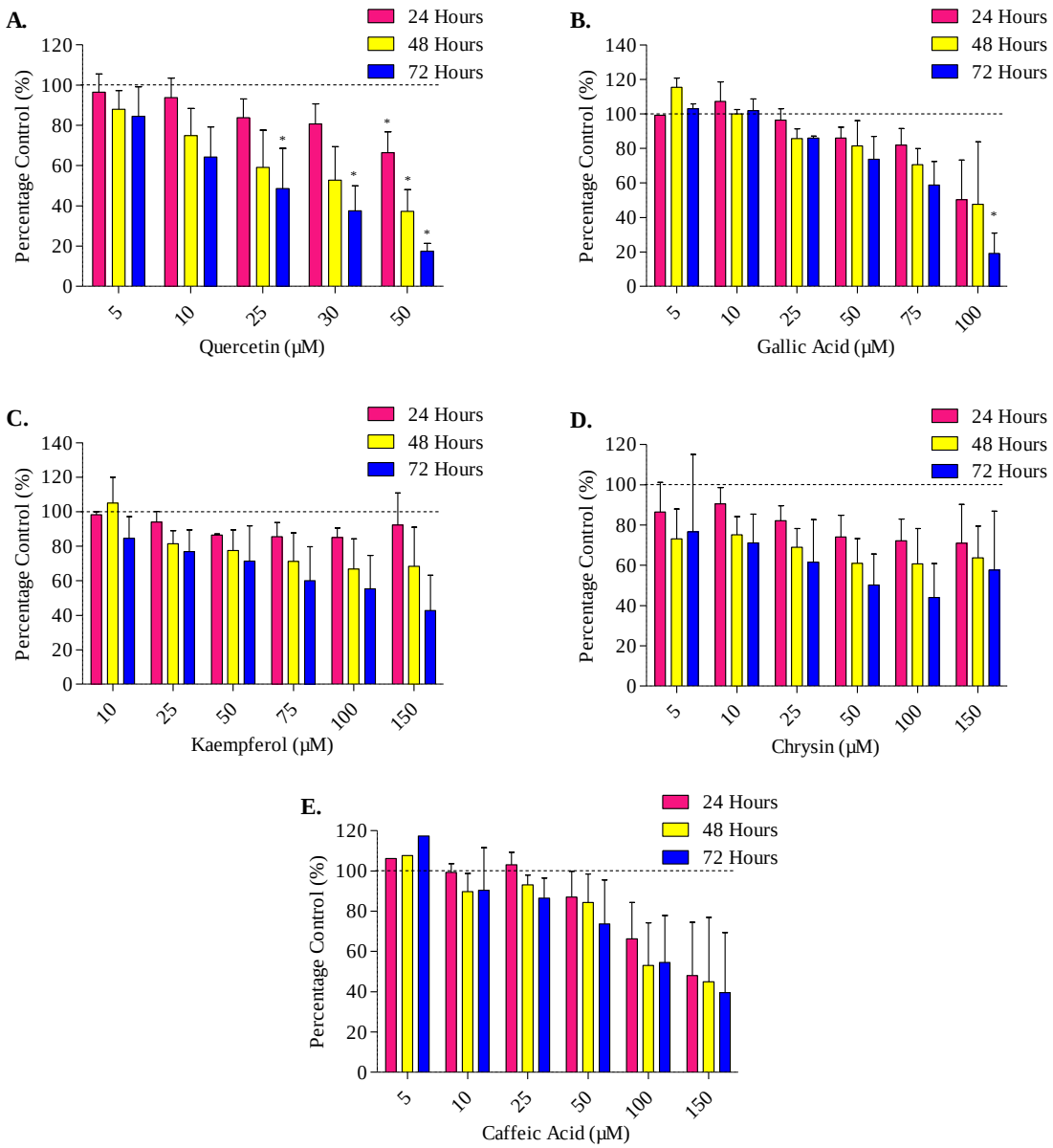


Figure 31. Honey-derived Phenolic Compound Cytotoxicity Quantified by the SRB Assay in DU145 Cells. DU145 cells were seeded at a density of $5x10^3$ cells/well and treated with (**A**) quercetin (0 - 50 µM), (**B**) gallic acid (0 - 100 µM), (**C**) kaempferol (0-150 µM), (**D**) chrysin (0 - 150 µM) or (**E**) caffeic acid (0 - 150 µM) for 24, 48 and 72 h. After incubation time, compounds were removed and viability was established via the SRB assay. Experiments were completed in triplicate, and values were expressed as mean percentage cell viability ± S.E.M (n = 3). Data were analysed using a two-way ANOVA followed by a Bonferroni post-hoc test, where $p<0.05$ was required for a statistically significant difference. * represents a significant difference between control and individual treatment.

Results from the SRB assay were not similar to those reported in literature. At 72 h, 50 µM chrysin inhibited proliferation of C6 glioma cells by 90%, attributed to an increase in G1 phase arrest and inhibition of cyclin-dependent kinase 2 and 4, where we found no significant cytotoxicity at this concentration and time point (Weng *et al.*, 2005). In another study, gallic acid (150 and 300 µM) caused 55% and 93% DU145 cell death in 24 h, in a concentration but

not time-dependent manner, however, here we showed that gallic acid (100 µM) caused 50% DU145 cell death at 24 h, in both a concentration- and time-dependent manner (Veluri *et al.*, 2006). Agarwal *et al*. (2006) demonstrated that gallic acid caused apoptotic cell death in DU145 cells in a concentration- but not time-dependent manner through the inhibition of cyclin-dependent kinases, and cleavage of caspase-3 and -9. The authors reported that 50 µM gallic acid resulted in 40% cell death at 24 h, almost 3-fold more potent than in the present study. Further, they found that the pan-caspase inhibitor, ZVAD, did not suppress the cytotoxic effect of gallic acid, and therefore suggested the concurrent involvement of caspase-independent mechanisms (Agarwal *et al.*, 2006).

Wang *et al*. (2013) reported that quercetin had a 48 h IC_{50} of 22.12 µM in PC3 cells, which was 2-fold lower than that found in the present study. Differences between values shown here and the literature were not further investigated, but might have been attributed to differences between cell and compound stocks. The calculation of IC_{50} values for these compounds was not required for the present study, as the aim was to determine a maximal non-lethal concentration to be used for migration and invasion assays (**Table 13**). It was however hypothesised in the present study, that phenolic compounds would exhibit cytotoxicity towards both PC3 and DU145 cell lines, which was demonstrated.

Table 13. Maximal Non-cytotoxic Concentrations of Honey-derived Phenolic Compounds

		Maximal Non-cytotoxic Concentration (µM)		
Cell Line	Compound	24 h	48 h	72 h
PC3	Quercetin	50	10	10
	Gallic acid	10	10	10
	Kaempferol	150	150	150
	Chrysin	150	100	100
	Caffeic Acid	50	50	50
DU145	Quercetin	30	10	10
	Gallic acid	25	10	10
	Kaempferol	150	50	50
	Chrysin	50	25	10
	Caffeic Acid	25	25	25

4.2 Anti-metastatic Activity of Honey-derived Phenolic Compounds

4.2.1 Anti-metastatic Activity of Honey-derived Phenolic Compounds Measured by the Adhesion Assay

Adhesion for both cell lines to collagen I and fibronectin was used to determine whether any of the 5 honey-derived phenolic compounds were responsible for the loss of cell attachment seen from honey (**3.1.3.1**). At 60 min, no compound had any effect on either PC3 or DU145 cell adhesion to fibronectin, at any concentration (**Figure 32**). Only quercetin and kaempferol (150 µM) caused any significant change to cell adhesion to collagen I. Quercetin caused a reduction in PC3 cell adhesion to collagen I of 47.14 ± 10.27% ($p<0.05$). By contrast, kaempferol caused an increase in PC3 and DU145 cell adhesion to collagen I of 117.20 ± 11.10% and 104.10 ± 10.09%, respectively ($p<0.05$).

The increase in adhesion of both cell lines to collagen I ($p<0.05$) when incubated with high concentrations of kaempferol (**Figure 32C**), was likely to be related to precipitation of kaempferol, as was mentioned earlier (**Figure 29**). It was concluded that kaempferol precipitants were left on the bottom of the well, and therefore reduced the MTT reagent, resulting in an apparent increased adhesion. Interestingly, 150 µM kaempferol did not affect the adhesion of cells to fibronectin (**Figure 32C**). Kaempferol may have decreased cell adhesion to fibronectin, however, the interaction between the precipitation and the MTT assay may have appeared to restore the loss of adhesion. Unlike when it was used to confirm the findings of the MTT assay for cytotoxicity, the SRB assay could not be used for the adhesion assay, as it is a protein determination assay, and would therefore bind to the collagen I and fibronectin on the bottom of the plates. Kim *et al*. (2015) demonstrated that kaempferol could inhibit the adhesion of human lymphoma U937 cells to fibronectin, using an adhesion assay similar to that used in the present study, however, using crystal violet for cell quantification. It is not known whether kaempferol can inhibit the adhesion of PC3 or DU145 cells to fibronectin, and therefore, the experiment should be repeated with alterations to the method by using a different cell viability assay, while also preventing compound precipitation.

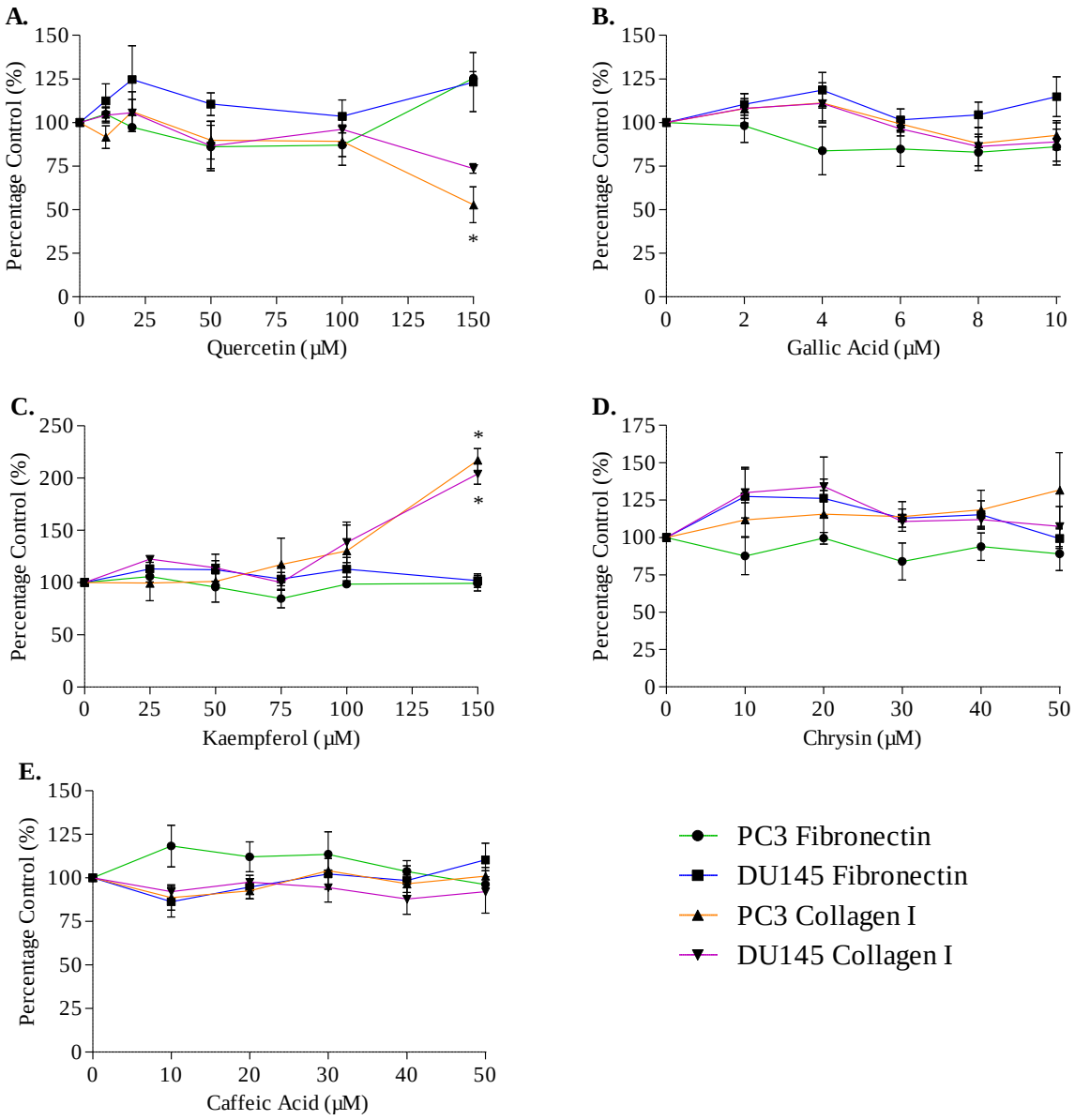


Figure 32. Effect of Honey-derived Phenolic Compounds on PC3 and DU145 Cell Adhesion. PC3 and DU145 cells were seeded at a density of $1x10^4$ cells/well and treated with (**A**) quercetin (0 - 150 µM), (**B**) gallic acid (0 - 10 µM), (**C**) kaempferol (0 - 150 µM), (**D**) chrysin (0 - 50 µM) or (**E**) caffeic acid (0 - 50 µM) for 60 min. Adhesion was measured via the MTT assay, as adhered cells were assumed viable. Experiments were completed in triplicate, and values were expressed as mean percentage cell viability ± S.E.M (n = 3). Data were analysed using a two-way ANOVA followed by a Bonferroni post-hoc test, where $p<0.05$ was required for a statistically significant difference. * represents a significant difference between control and individual treatment.

An explanation for the reduction in collagen I adhesion of both cell lines by quercetin, and possibly kaempferol, may be due to the expression of receptors on the cell surface. The expression of epidermal growth factor receptor (EGFR) has been reported to be over expressed in PC3 and DU145 cells, and may contribute to the metastatic potential of both cell lines (Huang *et al.*, 2002; Pignon *et al.*, 2009). DU145 cell adhesion to collagen I is mediated through the expression of EGFR as well as by integrins (Lamb *et al.*, 2011). Flavonoids and phytoestrogens such as quercetin have been shown to decrease the activity of the EGFR, which may explain part of the decrease in adhesion seen in **Figure 32** (Kumar *et al.*, 2008; Bhat *et al.*, 2014).

Apart from this, previous studies have only examined prostate cancer interactions with laminin, rather than other ECM proteins. Prostate cancer can overexpress integrins such as α6β1 that may bind to laminin 1 (Lamb *et al.*, 2011). Further, the activity of the androgen receptor (AR) in prostate cancer cells can increase the expression of α6β1 integrins that bind laminin. Polyphenols including quercetin and caffeic acid may inhibit the AR, therefore may decrease AR activity, and decrease α6β1 expression (Ren *et al.*, 2000; Xing *et al.*, 2001; Lall *et al.*, 2015). Overall, the administration of polyphenols may decrease the adhesion of cells to extracellular matrix proteins such as laminin through the decreased integrin expression. Therefore, it would be of interest to investigate the effect of honey-derived phenolics on PC3 and DU145 cells adhesion to laminin.

4.2.2 Anti-metastatic Activity of Honey-derived Phenolic Compounds Measured by the Migration Assay

Due to the nature of the Boyden chamber assay, it is not possible to use the SRB assay to quantify migration, and therefore, the MTT assay was used. The MTT assay was previously demonstrated to be affected by phenolic compounds, however, because concentrations of each compound were low, it was assumed there would be minimal interaction. Despite the fact that no compound, with the exception of quercetin (150 µM), caused any effect on cell adhesion, their effect on migration was examined.

Using 48 h non-cytotoxic concentrations, all compounds with the exception of caffeic acid caused a decrease in PC3 cell migration ($p<0.05$) (**Figure 33**). Gallic acid (10 µM) caused the greatest reduction in migration of 49.10 ± 8.33% compared to the untreated control ($p<0.05$). Together, these results demonstrated that phenolic compounds found in honey may possess anti-metastatic activity against PC3 cells, however that not all compounds are similar.

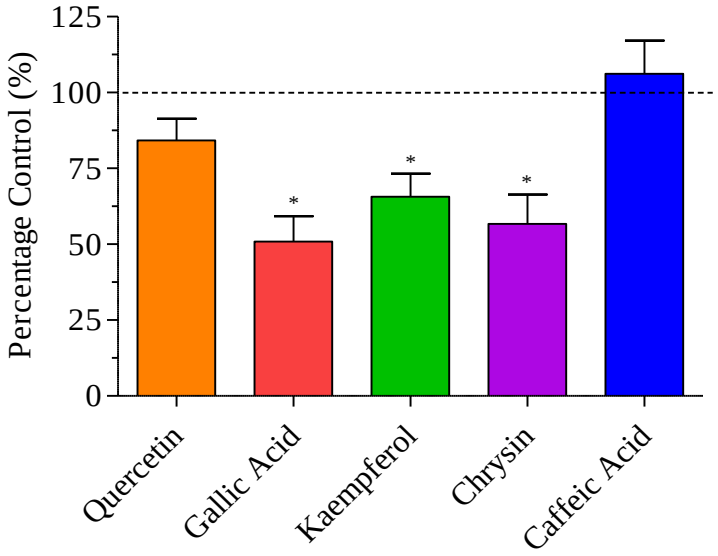


Figure 33. Effect of Honey-derived Phenolic Compounds on PC3 Cell Migration. The undersides of Boyden chamber inserts were coated in collagen I (150 µg/mL), and were placed in wells containing growth medium. PC3 cells suspended in serum-free growth medium were seeded at a density of $3x10^4$ cells/well. Cells were treated with quercetin (10 µM), gallic acid (10 µM), kaempferol (150 µM), chrysin (100 µM) or caffeic acid (50 µM) for 48 h. After treatment, the MTT assay was used to quantify migrating cells. Experiments were completed in triplicate, and values were expressed as mean cell migration ± S.E.M (n = 3). Data were analysed using a two-tailed Student's t-test. * ($p<0.05$) represents a significant difference between individual treatment and control.

Previously, Ho *et al*. (2010) reported that gallic acid (2 - 3.5 µM) could inhibit the migration of gastric cancer AGS cells by 60%, investigated using Boyden chambers. Further, they demonstrated that gallic acid inhibited the expression of MMP-2 and -9 600-times more than caffeic acid, which may explain the differences between gallic acid and caffeic acid seen in the present study. The authors proposed that the anti-metastatic mechanism of action of gallic acid was due to a decrease in RhoA expression, a decrease in MMP expression via inhibition of NF-κB, and actin filament rearrangement through the Ras/PI3K/AKT signalling pathway (Ho *et al.*, 2010).

4.2.3 Anti-metastatic Activity of Honey-derived Phenolic Compounds Measured by the Invasion Assay

Due to a reduction in PC3 migration found by the administration of honey-derived compounds, their effect on invasion was investigated. As a result of the inhibitory effects of polyphenols on cell adhesion to laminin, as discussed earlier, it was predicted that phenolic compounds would reduce cell invasion through the laminin-containing Matrigel®. Using non-cytotoxic concentrations at 72 h, all compounds with the exception of kaempferol caused a reduction in

PC3 cell invasion, however, not all were significant (**Figure 34**). This was due to the low sample size (n = 2), therefore an increase in sample size may be needed to reveal significance.

Compared a DMSO control, quercetin (10 µM) and caffeic acid (50 µM) resulted in a reduction in PC3 cell invasion of 45.28 ± 0.64% and 14.44 ± 1.17% , respectively ($p<0.05$). Further, chrysin (100 µM) and gallic acid (10 µM) reduced invasion by 39.57 ± 14.57% and 29.03 ± 15.86%, respectively. The reductions in invasion by gallic acid and chrysin were not statistically significant, as the sample size of 2 repeats (3 pseudo-repeats each) did not provide enough power. Finally, kaempferol only recorded a reduction in invasion of 5.04 ± 2.11 %. Overall, the reductions in invasion were comparable to those in migration, with the exception of kaempferol and caffeic acid. These differences may be explained by to the addition of Matrigel®. The various phenolic compounds may display anti-metastatic activity through different mechanisms, for example since Matrigel® contains laminin, interactions may be inhibited by some phytochemicals but not others.

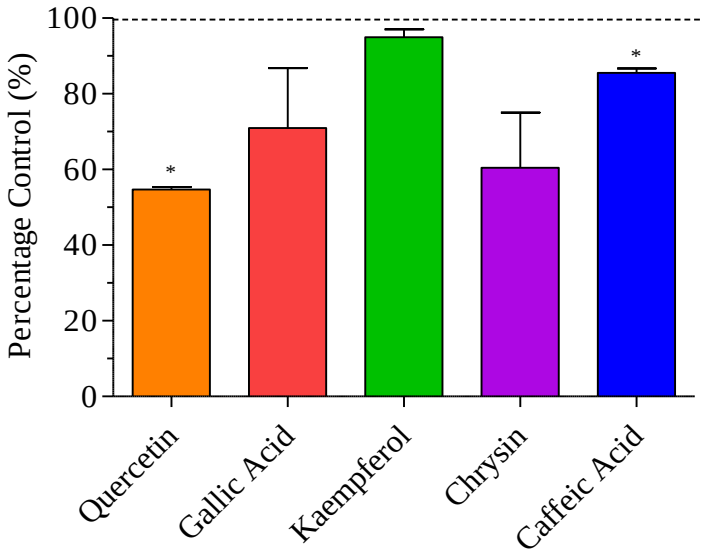


Figure 34. Effect of Honey-derived Phenolic Compounds on PC3 Cell Invasion. The undersides of Boyden chamber inserts were coated in collagen I (150 µg/mL), and were placed in wells containing growth medium. The inside of each Boyden chamber was loaded with Matrigel®. PC3 cells suspended in serum-free growth medium were seeded at a density of $3x10^4$ cells/well. Cells were treated with quercetin (10 µM), gallic acid (10 µM), kaempferol (150 µM), chrysin (100 µM) or caffeic acid (50 µM) for 72 h. After treatment, the MTT assay was used to quantify migrating cells. Experiments were completed in duplicate, and values were expressed as mean cell migration ± S.E.M (n = 2). Data were analysed using a two-tailed Student's t-test. * ($p<0.05$) represents a significant difference between individual treatment and control.

Various theories have been proposed for the anti-metastatic effect of quercetin, as an example of flavonoids. Concentrations of 50 - 100 µM has been reported to down-regulate the expression of both MMP-2 and -9 in PC3 prostate cancer cells (Vijayababu *et al.*, 2006), as well as to reduce the effects of EGFR in PC3 cells (Kumar *et al.*, 2008), which may explain the reduction in adhesion, migration and invasion reported in the present study. Further, a reduction in ROS activity in these cell types can reduce MMP-9 and membrane-type 1 MMP activity, and therefore may provide a mechanism of action for the inhibition of metastasis by anti-oxidant phenolic compounds (Kumar *et al.*, 2008; Nguyen *et al.*, 2011).

An alternative mechanism for the anti-metastatic benefit of quercetin may be dependent on the increase in the tumour microenvironment pH. It is known that a reduction in pH can promote an invasive cell phenotype and the production of proteases, which may aid in the metastasis of cancer (Kato *et al.*, 2013). Lactate production from cellular anaerobic respiration is largely responsible for the acidic environment surrounding a tumour (Kato *et al.*, 2013). As well, the binding of lactate to the lactate transporter has been reported to increase the motility and invasiveness of tumour cells (Goetze *et al.*, 2011). Since quercetin is known to be an inhibitor of the lactate transporter, it may reduce the metastasis of cancer through the amelioration of hypoxia- and lactate-induced motility (Kim *et al.*, 1984).

Anti-metastatic effects have also been reported for caffeic acid, as an example of phenolic acids. Chung *et al*. (2004) showed that caffeic acid could inhibit both MMP-2 and -9 in HepG2 cells through the inhibition of NF-κB activity. Later, Chung *et al*. (2009) showed that oral administration of 20 mg/kg caffeic acid could reduce liver metastasis of HepG2 tumour xenografts in nude mice due to the reduction in MMP-2 and -9 expression. The pre-metabolite of caffeic acid, caffeic acid phenethyl ester (CAPE) was also demonstrated possess anti-metastatic activity against the human HT1080 fibrosarcoma cell line. The authors showed that CAPE inhibited cell invasion, migration and motility through the reduced expression of MMP-2 and -9 (Hwang *et al.*, 2006). In the present study, caffeic acid had no effect on the adhesion or migration of PC3 or DU145 cells, however caused a reduction in the invasion of PC3 cells. These results may demonstrate that not all polyphenols demonstrate similar properties, and that caffeic acid present in honey at high concentrations may be partially responsible for the anti-metastatic activity of honey.

Taken together, honey-derived phenolic compounds in the present study demonstrated anti-metastatic activity towards PC3 cells. However, the activity was mechanism dependent, as

some compounds only affected some metastatic-related mechanisms. Nevertheless, these results were in agreement with the proposed hypothesis that honey-derived phenolic compounds could exhibit anti-metastatic activity in PC3 and DU145 cells. This again may demonstrate the benefit of whole honey administration over compounds alone, since the anti-metastatic mechanisms would be combined. To further investigate these findings, combination studies should be used to determine the additive or synergistic benefits of phenolic compounds in honey.

4.2.4 The Potential Benefit of Phenolic Compounds in Combination

As honey contains multiple polyphenols, the effect of combination treatment *in vivo* and *in vitro* is of interest. In the present study, phenolic compounds were found in honey within nM ranges (**Table 12**), however when individually used *in vitro* at µM ranges, they still demonstrated minimal toxic and anti-adhesion properties. This suggests that the benefit of honey may be due to a combination of many phenolic compounds, and not individual activities. The redundancy within many biological systems, such as within the protease degradome, may further highlight the ineffectiveness of individual phenolic treatment, and the benefit of combination (Edwards *et al.*, 2008). To better understand what compounds make honey an effective anti-cancer agent, a stepwise artificial honey could be created, in which combinations of phenolic compounds are added to sugars in their respective naturally-occurring concentrations. Following this, comparative analysis could be made to determine compound synergism, and whether the effects seen in the present study were due to a few compounds, or many.

The synergistic potential of phenolic compounds from pomegranate extracts (*Punica granatum*) have been reported in a similar but less structured manner to that suggested above. Albrecht *et al*. (2004) demonstrated that a combination of polyphenol-rich extracts from pomegranate seeds, fermented juice and precarp could not only decrease LNCaP, PC3 and DU145 cell viability, but also decrease the invasion of PC3 cells through Matrigel®. Following this, Lansky *et al*. (2005) investigated the effects of four phenolic compounds from pomegranate extracts (ellagic acid, caffeic acid, luteolin and punicic acid) alone and in combination against the invasion of PC3 cells through Matrigel®. Their findings were of interest to the present study as ellagic acid, caffeic acid and luteolin are found in honey at 3300, 160 and 33 µg/100 g honey, respectively (Kassim *et al.*, 2010). The authors concluded that all four compounds inhibited PC3 cell invasion. Further, they concluded that combinations were supra-additive, and possibly

synergistic, where an increase in combinations from 2 to 4 resulted in further reductions in PC3 cell invasion (Lansky *et al.*, 2005a). This was the first time that combinations of individual phenolic compounds have been investigated in cancer cell invasion. Combinations of honey-derived phenolic compounds from the present study may also decrease PC3 cells invasion in an additive or synergistic manner, and may support an increased benefit of honey-derived phenolic and sugar ratios.

Chapter 5: Bee Venom and Melittin Results and Discussion

5.1 Bee Venom and Melittin Cytotoxicity

5.1.1 MTT Assay Optimisation

In order to determine whether the MTT assay was appropriate for measuring the cytotoxicity of bee venom and melittin, their interaction with MTT in the absence of cells was measured. Melittin (5 µg/mL) caused an increase in the reduction of MTT at 3 h (**Figure 35**). This was the first time that an interaction between melittin and MTT has been reported. Nevertheless, because the interaction was not significant and was minimal, the MTT assay was still used.

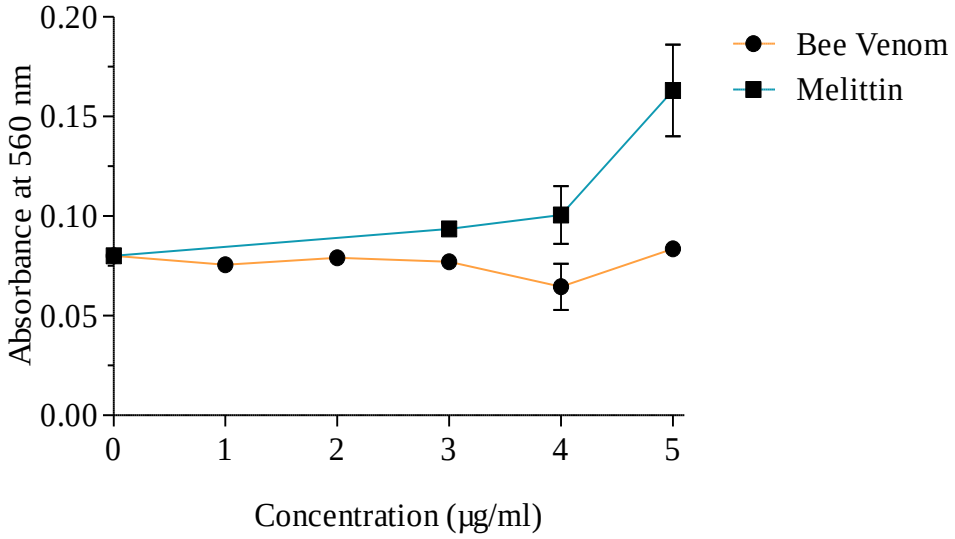


Figure 35. Intrinsic Reduction of MTT from Bee Venom and Melittin. MTT (5 mg/mL) in growth medium was treated with 0 - 5 µg/mL bee venom and melittin for 3 h. Formazan crystals were solubilised using DMSO. Absorbances were measured at 560 nm. Data were expressed as mean absorbance at 560 nm ± S.E.M (n = 2).

5.1.2 Cytotoxicity of Bee Venom and Melittin by the MTT Assay

In order to find a maximal non-cytotoxic concentration for bee venom and melittin for use in the metastatic assays, the MTT assay was used. Concentrations were based on cytotoxicity established by Jeong *et al*. (2014). Bee venom (0 - 5 µg/mL) had no effect on cell viability in either cell line over 72 h (**Figure 36**). Melittin (0 - 5 µg/mL) was also non-cytotoxic in PC3 cells over 72 h. Melittin did, however, cause a concentration-dependent decrease in cell viability in DU145 cells ($p<0.05$), with a maximal non-cytotoxic concentration of 2 µg/mL at 24, 48 and 72 h.

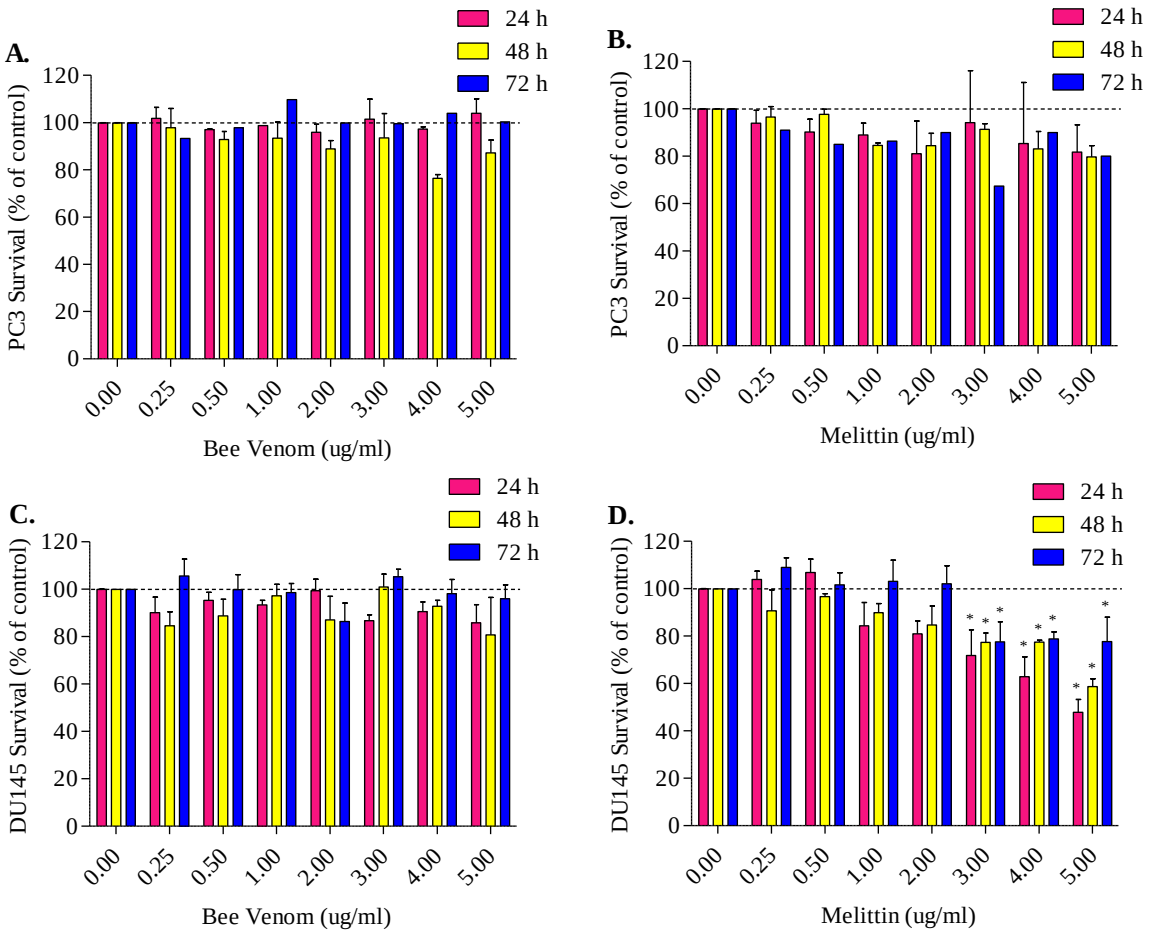


Figure 36. Bee Venom and Melittin Cytotoxicity Quantified by the MTT Assay. (**A**, **B**) PC3 cells and (**C**, **D**) DU145 cells were seeded at a density of $3x10^3$ and $5x10^3$ cells/well, respectively. Cells were treated with (**A**, **C**) bee venom (0 - 5 µg/mL) or (**B**, **D**) melittin (0 - 5 µg/mL) for 24, 48 and 72 h. After treatment, compounds were removed and viability was established via the MTT assay. Experiments were completed in triplicate, and values were expressed as mean percentage cell viability ± S.E.M (n = 3). Data were analysed using a two-way ANOVA followed by a Bonferroni post-hoc test, where $p<0.05$ was required for a statistically significant difference. * represents a significant difference between untreated control and individual treatment.

5.1.3 Cytotoxicity of Bee Venom and Melittin by the SRB Assay

The use of the MTT assay reported that melittin may be selectively cytotoxic towards DU145 cells. As PC3 cells were used for migration and invasion assays, it was important to confirm the differences in melittin cytotoxicity between PC3 and DU145 cells.

Using the SRB assay, it was found that bee venom was non-cytotoxic at 5 µg/mL in both cell line (**Figure 37**). In contrast to the MTT assay, the SRB assay showed that 5 µg/mL melittin was non-cytotoxic to DU145 cells at 24, 48 and 72 h. In further contrast, it was found that melittin caused a reduction in PC3 cell viability ($p<0.05$), with a maximal non-cytotoxic

concentration of 3 µg/mL for 24 h, and 2 µg/mL for 48 and 72 h. These results were not expected, and did not agree with the proposed hypothesis that bee venom and melittin would be cytotoxic to both PC3 and DU145 cells.

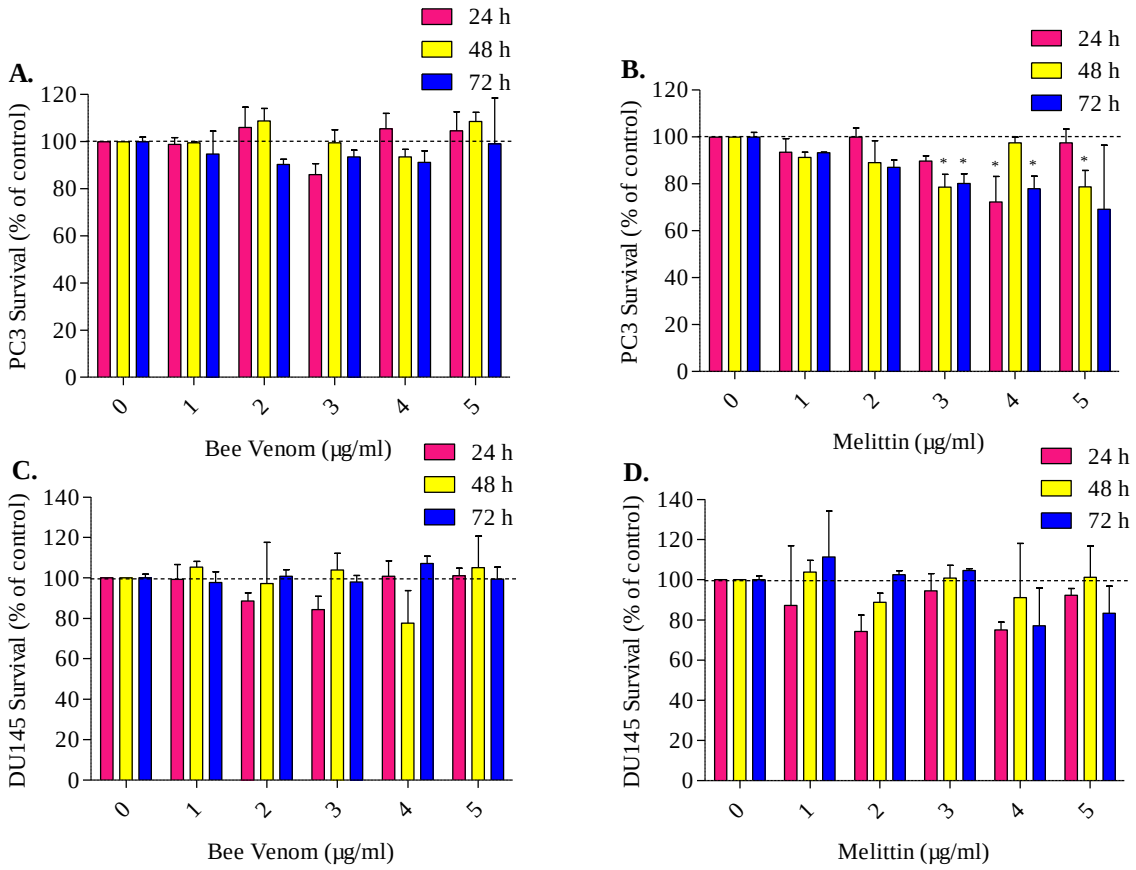


Figure 37. Bee Venom and Melittin Cytotoxicity Quantified by the SRB Assay. (A, B) PC3 cells and (C, D) DU145 cells were seeded at a density of $3x10^3$ and $5x10^3$ cells/well, respectively. Cells were treated with (A, C) bee venom (0 - 5 µg/mL) or (B, D) melittin (0 - 5 µg/mL) for 24, 48 and 72 h. After treatment, compounds were removed and viability was established via the SRB assay. Experiments were completed in triplicate, and values were expressed as mean percentage cell viability ± S.E.M (n = 3). Data were analysed using a two-way ANOVA followed by a Bonferroni post-hoc test, where $p<0.05$ was required for a statistically significant difference. * represents a significant difference between control and individual treatment.

Two other studies have examined the effect of these compounds in prostate cancer cells. Park *et al*. (2011) reported that bee venom and melittin inhibited prostate cancer cell proliferation in a concentration- and time-dependent manner. At 24 h, melittin (2.5 µg/mL) reduced PC3 and DU145 cell number by 90% and 75%, respectively. At 24 h, bee venom (5 µg/mL) reduced PC3 and DU145 cell number by 25% and 10%, respectively. As bee venom is a natural product, there may have existed differences in the authors' Korean bee venom compared to the Chinese bee venom used in the present study. The melittin used between both studies was the same, purchased from Sigma-Aldrich at 85% purity (determined by HPLC). Other differences could

be Park *et al*. (2011) determined cytotoxicity using the direct TB exclusion assay, compared to the indirect colourimetric assays used here. Further, genetic differences in cell stocks may account for the variations seen between the two studies, as cross-contamination of stocks may alter drug sensitivity (Reid *et al.*, 2004). Nevertheless, the maximal non-cytotoxic concentrations obtained in the present study (**Table 14**), were used for the assessment of anti-metastatic activity, as they were relevant to the stocks of cell lines used.

Table 14. Maximal Non-cytotoxic Concentrations of Bee Venom and Melittin

			Maximal Non-cytotoxic Concentration (µg/mL)		
Cell Line	Honey	Assay	24 h	48 h	72 h
PC3	Bee Venom	MTT	5	5	5
		SRB	5	5	5
	Melittin	MTT	5	5	5
		SRB	3	2	2
DU145	Bee Venom	MTT	5	5	5
		SRB	5	5	5
	Melittin	MTT	2	2	2
		SRB	5	5	5

5.2 Anti-metastatic Activity of Bee Venom and Melittin

Bee venom and melittin have been suggested to possess anti-metastatic activity in cancer through the inhibition of MMPs and FAK-associated pathways, and therefore, may be beneficial for the treatment of metastatic prostate cancer (Son *et al.*, 2007; Oršolić, 2012; Jeong *et al.*, 2014). The FAK-associated Rac-1 is a guanosine triphosphate (GTP)-binding protein responsible for cell migration, adhesion and invasion (Kuroda *et al.*, 1998). Rac-1 has been linked to the androgen-independence and metastasis of prostate cancer (Kobayashi *et al.*, 2010; Lin *et al.*, 2012). Furthermore, the inhibition of Rac-1 has been shown to inhibit the migration of prostate cancer (Dirat *et al.*, 2014). Lui *et al*. (2008) reported that melittin could decrease liver cancer cell metastasis through the inhibition of Rac-1 associated pathways, which may therefore provide a mechanism for the inhibition of prostate cancer metastasis.

5.2.1 Anti-metastatic Activity of Bee Venom and Melittin Measured by the Adhesion Assay

The effect of bee venom and melittin on cell attachment was investigated using the adhesion assay. Bee venom (0 - 5 µg/mL) caused no change in either PC3 or DU145 adhesion to collagen I or fibronectin (**Figure 38**). Further, melittin (0 - 5 µg/mL) caused no change in PC3 or DU145 adhesion to fibronectin. 5 µg/mL melittin caused a 26.02 ± 16.56% reduction in PC3 adhesion to collagen I, however this was not significant. 5 µg/mL melittin caused a significant 36.93 ± 14.95 % reduction in DU145 cell adhesion to collagen I ($p<0.05$) (**Figure 38**).

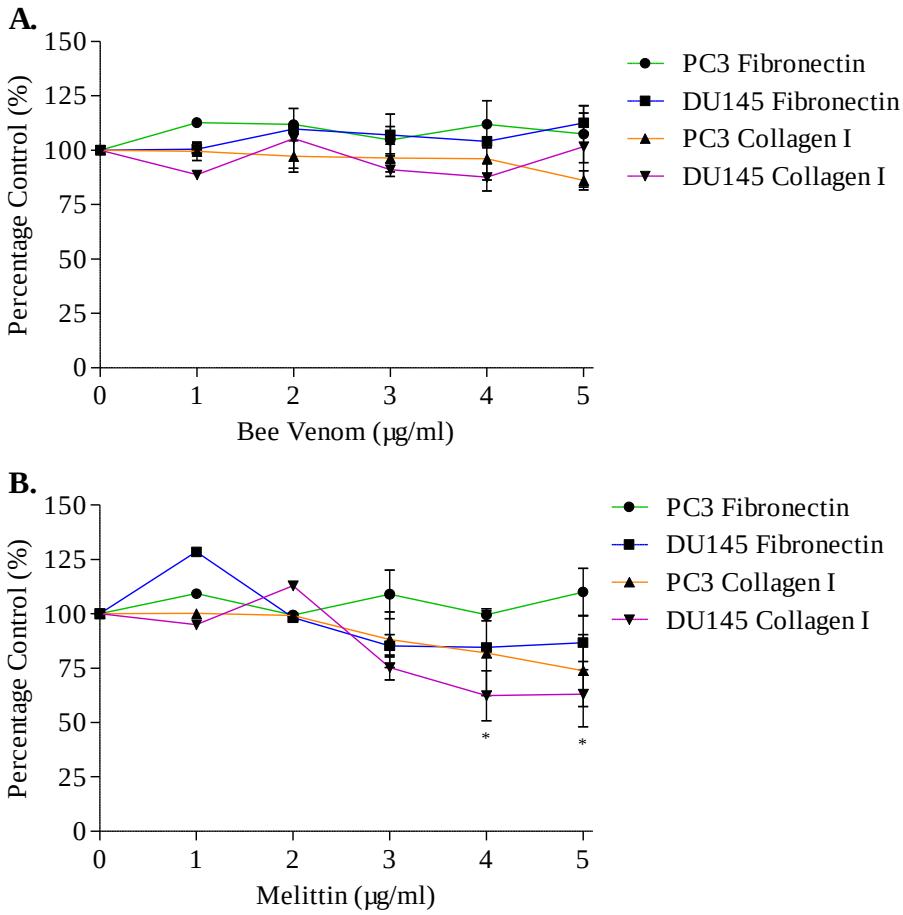


Figure 38. Effect of Bee Venom and Melittin on PC3 and DU145 Cell Adhesion. PC3 and DU145 cells were seeded at a density of $1x10^4$ cells/well and treated with (**A**) bee venom (0 - 5 µg/mL) or (**B**) melittin (0 - 5 µg/mL) for 60 min. Adhesion was measured via the MTT assay, as adhered cells were assumed viable. Experiments were completed in triplicate, and values were expressed as mean percentage cell viability ± S.E.M (n = 3). Data were analysed using a two-way ANOVA followed by a Bonferroni post-hoc test, where $p<0.05$ was required for a statistically significant difference. * represents a significant difference between control and individual treatment.

5.2.2 Anti-metastatic Activity of Bee Venom and Melittin Measured by the Migration Assay

To further investigate the possible anti-metastatic properties of bee venom and melittin, a migration assay was completed. The non-cytotoxic concentration at 48 h for bee venom and melittin of 5 µg/ml, as determined using the MTT assay, were used (**Table 14**). Compared to untreated control, melittin (5 µg/mL) cause a reduction in PC3 cell migration of 63.31 ± 7.57% ($p<0.05$). Bee venom (5 µg/mL) caused a 24.61 ± 14.84% reduction in PC3 migration, however this was not significant (**Figure 39**). Compared to bee venom, melittin caused a further 36.69 ± 16.66% reduction in PC3 migration ($p<0.05$).

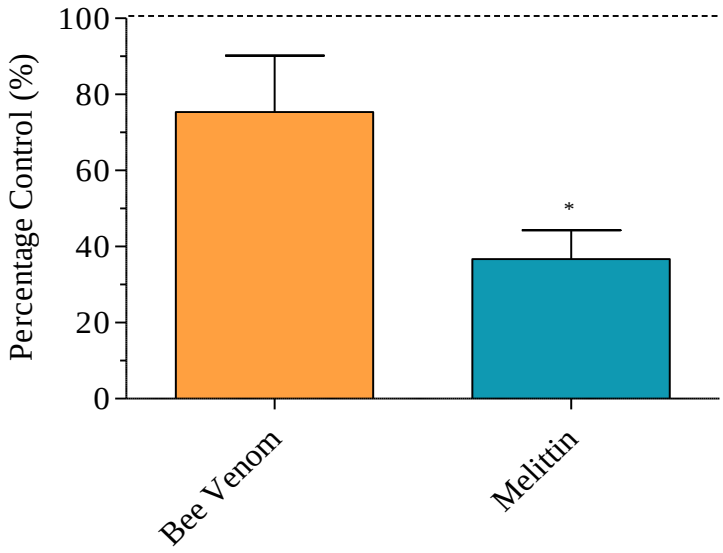


Figure 39. Effect of Bee Venom and Melittin on PC3 Cell Migration. The undersides of Boyden chamber inserts were coated in collagen I (150 µg/mL), and were placed in wells containing growth medium. PC3 cells suspended in serum-free growth medium were seeded at a density of $3x10^4$ cells/well. Cells were treated with bee venom (5 µg/mL) or melittin (5 µg/mL) for 48 h. After treatment, the MTT assay was used to quantify migrating cells. Experiments were completed in triplicate, and values were expressed as mean cell migration ± S.E.M (n = 3). Data were analysed using a two-tailed Student's t-test. * ($p<0.05$) represents a significant difference between melittin treatment and control.

Melittin has previously been shown to inhibit the migration and motility of cancer cells. Liu *et al*. (2008) reported that melittin (8 µg/mL) could reduce the migration of four hepatocarcinoma cell lines by 60% - 75%, attributed to a reduction in Rac1 activity and an increased organisation of microfilament. Furthermore, the authors reported that melittin could decrease the lung metastasis in mice inoculated with hepatocarcinoma cells, however, no mention of overall survival rate was mentioned (Liu *et al.*, 2008). In contrast to the benefits observed, high concentrations of melittin caused liver injury, and therefore creation of melittin structural

analogues with decreased toxicity may be beneficial. Jeong *et al*. (2014) investigated the effects of bee venom and melittin on epidermal growth factor (EGF)-induced motility and invasion of breast cancer cells. They reported that either 2 µg/mL bee venom or melittin could inhibit the motility of two breast cancer cell lines, assessed using the scratch wound healing assay. In contrast to Lui *et al*. (2008), the authors showed that the reduction in cell motility was due to a loss of filamentous actin (F-actin) reorganisation.

5.2.3 Anti-metastatic Activity of Bee Venom and Melittin Measured by the Invasion Assay

To investigate the potential of bee venom and melittin to inhibit PC3 cell movement through the ECM, an invasion assay was carried out. As the MTT assay was used for the measurement of invasion, the non-cytotoxic concentrations at 72 h for bee venom and melittin were used (**Table 14**, 5 µg/mL). Compared to untreated control, bee venom and melittin (5 µg/mL) cause a reduction in PC3 cell invasion of 25.63 ± 13.39% and 43.78 ± 10.36%, respectively (**Figure 40**). Neither reduction was statistically significant, as the sample size of 2 repeats (3 pseudo-repeats each), did not provide enough power. Compared to bee venom, melittin caused a further 18.15 ± 16.93% reduction in PC3 migration.

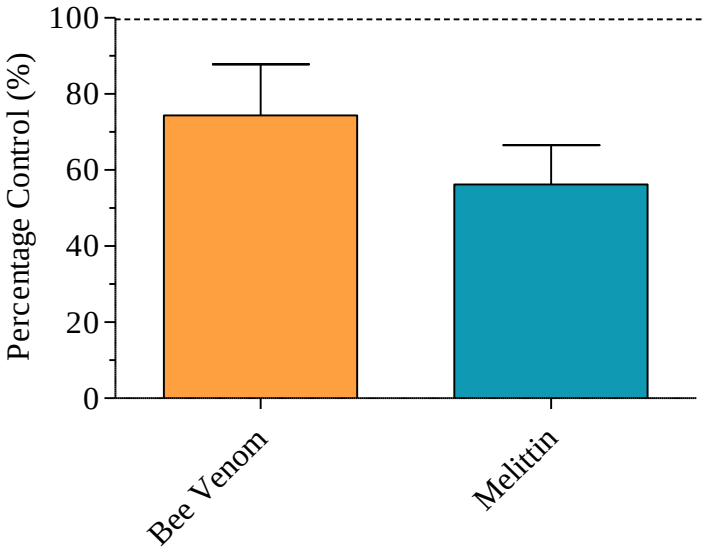


Figure 40. Effect of Bee Venom and Melittin on PC3 Cell Invasion. The undersides of Boyden chamber inserts were coated in collagen I (150 µg/mL), and were placed in wells containing growth medium. The inside of each Boyden chamber was loaded with Matrigel® and PC3 cells suspended in serum-free growth medium were seeded at a density of $3x10^4$ cells/well. Cells were treated with bee venom (5 µg/mL) or melittin (5 µg/mL) for 72 h. After this time, the MTT assay was used to quantify invading cells. Experiments were completed in duplicate, and values were expressed as mean cell migration ± S.E.M (n = 2).

The present study hypothesised that bee venom and melittin would exhibit anti-metastatic activity towards prostate cancer cells. The findings regarding melittin support the hypothesis, however, this is not the case for bee venom. Bee venom did cause a reduction in both PC3 migration and invasion, however this was not significant. A greater sample size in the future may reduce variance and demonstrate significance. As melittin caused a greater inhibition of adhesion, migration and invasion, it may be the case that melittin is responsible for the anti-metastatic activity of bee venom, but was too dilute in the bee venom sample used here.

Other studies have shown that bee venom and melittin (2 µg/mL) both reduce the invasion of EGF-induced breast cancer cells by 50 - 60% (Jeong *et al.*, 2014). The reduced invasion was correlated to a lower expression of both MMP-9 and FAK. Further, melittin increased E-cadherin expression while reducing vimentin expression, suggesting that the decrease in invasion may be attributed to an inhibition of the epithelial-mesenchymal transition (EMT) in breast cancer cells. These recently proposed mechanisms showed that melittin may act via the mTOR signalling pathway, independent of calcium calmodulin or PLA_2 activity. Oršolić *et al*. (2003b) reported that intravenous administration of bee venom could reduce the lung metastasis of transplantable mammary carcinoma cells in mice, however subcutaneous, administration could not. Further, the authors proposed that bee venom *in vivo* worked via an indirect anti-cancer mechanism which modulated the lymph node immune system, not by the induction of apoptosis and necrosis in tumour cells as shown *in vitro* (Oršolić *et al.*, 2003b). In agreement, McDonald *et al*. (1979) reported that bee venom provided no protection against the incidence of cancer among beekeepers, who were assumed to be chronically exposed to intradermal bee venom (bee stings). These reports suggest that the administration route of bee venom may be important to its anti-cancer effects. Overall, bee venom may provide *in vivo* anti-cancer benefits through the modulation of the immune system when administered intravenously, and that regular administration of bee venom by beestings may provide little benefit, regardless of its *in vitro* effects.

These reports, along with the results presented in **Figure 38, 39** and **40**, demonstrated that melittin might possess anti-metastatic activity, and therefore, could be further investigated for the prevention or treatment of metastatic prostate cancer.

Chapter 6: Overall Discussion and Conclusion

6.1 Honey

Measuring the cytotoxicity of honey was more complicated than expected. Firstly, interactions of the honey with the assay components were found, as discussed earlier. As well, it was observed that the cytotoxicity of honey in these assays might be largely be attributed to the loss of cell adhesion. It was not known whether honey causes cell death which results in the loss of adhesion, or whether honey causes the loss of cell adhesion causing cells to die. It was previously demonstrated that PC3 cell adhesion to matrix proteins via interactions between integrins and laminin 1 might increase the survival of cells, measured by an increase in the pro-survival protein BCL-xL (Lamb *et al.*, 2011).

As adhesion is required for the survival of cells, and honey has been shown to inhibit cell adhesion, honey may demonstrate cytotoxicity via an indirect mechanism not previously described in the literature, through the loss of cell adhesion. To investigate this, an assessment of viability on cells that have lost adherence should be made. The detection of viable non-adherent cells may suggest an indirect cytotoxic effect of honey. Further, non-adhered cells should be rescued and washed to see whether the loss of attachment is permanent. This was attempted once using the TB assay, where a large proportion of non-adherent cells were found to still be viable, however fewer than in the untreated control. Further, treated cells were rescued and washed to allow for re-adherence which did not occur, however, future optimisation of this assay is needed.

The inhibition of invasion and migration of PC3 cells in the present study may be due to phenolic compounds not selected for investigation. Pitchakaran *et al.* (2013) demonstrated that via proteolytic inhibition, the invasion and migration of PC3 cells could be inhibited by ellagic acid; a polyphenol found in high concentrations throughout honey (Kassim *et al.*, 2010; Pitchakarn *et al.*, 2013). Further, a review by Weng *et al*. (2012) highlighted the anti-metastatic activity of dietary phytochemicals, many of which can be found in honey. These reasons may explain why high concentrations of the honey-derived phenolic compounds in the present study could not explain the loss of adhesion caused by honey.

In the present study, honeydew honey was shown to cause a greater reduction in both migration, invasion and viability, compared to thyme and manuka honey. This suggests that the mixture

of compounds in New Zealand honeydew honey may have a greater potential for the treatment of prostate cancer than other honey types. It has previously been reported that phenolic content may correlate to the beneficial activity of honey. Similar to other studies, the present study showed that honeydew honey contains high quantities of all phenolic compounds tested, with the exception of quercetin (White *et al.*, 1980; Astwood *et al.*, 1998). Pine and fir honey, similar to honeydew, have been reported to be superior to other monofloral honeys due to the increase phenolic, enzyme and mineral content (Majtan *et al.*, 2011). In contrast, Greek pine and fir honey was demonstrated to be less effective in both prostate and breast cancer compared to thyme honey, which again highlighted the variability in honey content based on geographical origin (Tsiapara *et al.*, 2009).

Honey may prevent carcinogenesis through a multi-staged process. The present study has provided evidence for the cytotoxic effect of honey in two prostate cancer cell lines. Further, evidence was provided for the reduction of metastasis, with honey inhibiting adhesion, migration and invasion of prostate cancer cells *in vitro*. A recent review by Erejuwa *et al*. (2014) summarises studies that show that honey may inhibit the initiation of tumourigenesis, proliferation and metastatic progression throughout a large range of cancer cell lines. In addition, studies have shown that the prophylactic consumption of honey may reduce carcinogenesis and metastasis in mouse models (Orsolić *et al.*, 2003a; Oršolić *et al.*, 2005). Due to the profound effect of honey on cancer-related processes in breast (MCF-7) and prostate (PC3) cancer cell lines, the daily consumption of honey in humans has previously been recommended for cancer prevention by several authors in spite of the lack of animal and human trials (Tsiapara *et al.*, 2009; Alvarez-Suarez *et al.*, 2013). Further, the daily consumption of polyphenols and dietary phytochemicals has been suggested due to their anti-metastatic activity in numerous *in vitro* and *in vivo* tumour models (Weng *et al.*, 2012). In the present study, evidence was provided for the cytotoxic and anti-metastatic activity of three New Zealand honeys in PC3 and DU145 prostate cancer cell lines, and therefore, further research may show a diet supplemented with honey may also be recommended in patients susceptible or suffering from prostate cancer.

The present study provided evidence to suggest that saccharides in honey as in the artificial honey control may hold partial responsibility for some of the effect of honey including the decrease in cell viability, decrease in adhesion to collagen I, and decrease in PC3 cell invasion. Chua *et al* (2013) attributed the total anti-oxidant activity of honey solely to the presence of anti-oxidant compounds such as flavonoids ($r = 0.9276$) and vitamins ($r = 0.8226$).

Nevertheless, artificial honey containing only sugars might increase the total anti-oxidant capacity of the blood without increasing serum phenolic content. A single oral administration of honey (1.5 g/kg) in humans was shown to increase the total-phenolic, anti-oxidant and reducing capacity of the plasma ($p<0.05$) (Schramm *et al.*, 2003). Administration of corn syrup as a sugar control in the same study did not increase the total plasma phenolic levels, however, did increase plasma anti-oxidant capacity ($p<0.05$). These findings further support the idea that carbohydrates may demonstrate anti-oxidant activity through the formation of Maillard products, or by acting as reducing sugars. This suggests that the activity of honey is due to presence of both phenolics and sugars, and may further support its benefit over other natural phenol sources.

Nevertheless, it has been repeatedly reported that the beneficial effects of honey are entirely due to its high phenolic content. As honey contains many phenolic acids and flavonoids, it is difficult to determine the individual effect of every single compound. It is more likely that the benefit of honey is due to the combination of phenolic compounds, with individual compounds providing little or no benefit. As an example, Pichichero *et al*. (2010) suggested that chrysin may be responsible for the cytotoxic effect of acacia honey on human (A375) and murine (B16-F1) melanoma cells lines. However, concentrations of chrysin used by the authors (150 µM) were not representative of the concentration of chrysin in our honey (**Table 12**). The present study demonstrated that phenolic compounds may have cytotoxic effects *in vitro* at concentrations between 10 - 150 µM. Quercetin (150 µM) was the only phenolic compound to effect cell adhesion. Further, phenolic compounds were shown to reduce the migration (quercetin, gallic acid, kaempferol and chrysin) and invasion (quercetin, chrysin, caffeic acid) of prostate cancer cells *in vitro*. However, similar to Pichichero *et al*. (2010), the concentration of each phenolic compound was not representative of those found in honey, which were shown here to exist at concentrations between 0.06 at 2 µM (**Table 12**). These results suggested that although some phenolic compounds may display cytotoxic and anti-metastatic activity alone, the effects of honey may not be attributed to individual compounds, but through combination. Therefore, the *in vitro* effects of honey may be due to the high concentration of both saccharides and phenolic compounds, and that combination may be essential to their efficacy.

6.1.1 A Possible Alternative Mechanism of Action for Honey

Despite the androgen-insensitive status of advanced metastatic prostate cancer cells, oestrogens may still play a role in the regulation of growth and metastasis (Carruba *et al.*, 1994). Phenolic

constituents of honey may have oestrogen-related effects, in several different ways. For example, quercetin and kaempferol are phytoestrogens, mimicking the effects of oestrogen in cells (Yildiz, 2010). The compounds were suggested to be responsible for the effects seen in MCF-7 breast and PC3 prostate cancer cells, in which Greek honey was shown to have anti-oestrogenic effects at low concentrations but oestrogenic effects at high concentrations (Tsiapara *et al.*, 2009). Other polyphenols such as caffeic acid have been suggested to reduce oestrogen receptor (ER) expression in prostate cancer cells, and thereby decrease cell viability (Chuu *et al.*, 2012).

Previous studies have shown that estradiol can inhibit the growth of PC3 cells through interactions with oestrogen receptors (ER), which may provide further evidence for the inhibitory action of phytoestrogens in PC3 cells (Carruba *et al.*, 1994). To determine the different effects of oestrogen in prostate cancer, Lau *et al*., (2000) investigated the expression of ER-α and ER-β in four prostate cell lines. They reported that PC3 cells expressed both ER-α and ER-β, and therefore both oestrogens and antioestrogens could inhibit cell growth. In contrast, the DU145 cell line only expressed ER-β, and therefore only antioestrogens could inhibit cell growth (Lau *et al.*, 2000). Chuu *et al*. (2012) demonstrated that CAPE could suppress the phosphorylation of ER-α to a greater extent than ER-β, which suggested it may have a greater effect in ER-α expressing cells such as PC3. Therefore, honey may have a greater effect on cell growth in the more aggressive PC3 prostate cancer cell line, due the presence of phytoestrogens in high concentrations (Tsiapara *et al.*, 2009).

6.1.2 Other Possible uses of Honey during Cancer Treatment

Evidence has been provided for the possible benefit of honey in prostate cancer, through the direct *in vitro* cytotoxic and anti-metastatic activity in prostate cancer cells. However, honey may be indirectly beneficial in prostate cancer through the reduction of side-effects associated with chemotherapy.

Currently, treatment following the early diagnosis of prostate cancer involves either radical prostatectomy or hormone therapy including luteinising or gonadotropin releasing hormone blockers and anti-androgens (Messing *et al.*, 1999; Weckermann *et al.*, 2004). If prostate cancer status has become hormone refractory, non-androgen related therapy may be provided by inhibiting microtubule activity in mitosis with docetaxel, cabazitaxel or estramustine, inhibiting topoisomerase II with mitoxantrone or epirubicin, or inhibiting mitosis with paclitaxel (Lester

et al.; Hong, 2007). Chemotherapeutics including paclitaxel, decetaxel, epirubicin and mitoxantrone have been reported to cause cardiotoxicity in patients, which may occur in more than 20% of cases (Pai *et al.*, 2000). Further, cardiac events such as blood pressure alterations, myocardial infarction, arrhythmias, pericarditis and electrocardiographic variations have also been reported (Pai *et al.*, 2000). Since a cardio-protective activity of honey has been reported previously, honey may prevent side effects when co-administered during chemotherapy (Rakha *et al.*, 2008; Alvarez-Suarez *et al.*, 2013).

Alongside chemotherapeutics, prednisone is often given to assist in the reduction of inflammation side effects, prevention of nausea, increase of appetite, and to lower white blood cell migration and accumulation around the tumour (Tannock *et al.*, 1996). Fernandez-Cabezudo *et al*. (2013) demonstrated that the co-administration of intravenous manuka honey and paclitaxel improved host survival and reduced toxicity associated with chemotherapy in a melanoma mouse model. These findings further support the use of honey as an anti-cancer adjuvant, and highlight the importance of further investigation. A study by Sharma *et al*. (2008) demonstrated the benefits of honey towards the adverse side effects of anti-tuberculosis drugs. The authors reported that 1 teaspoon of honey (5 mL), taken 3 times a week for 2 months alongside 4 chemotherapeutics used for the treatment of tuberculosis, could reduce gastrointestinal disorders and inflammation, while increasing appetite (Sharma *et al.*, 2008). Since honey may provide benefits to prednisone, and has the added benefits of low cost and a likely high tolerance, the potential use of honey as a replacement for prednisone, as well as a direct anti-cancer agent, should be further investigated.

The surgical implantation of Ehrlich ascites mammary adenocarcinoma ascites mammary adenocarcinoma was inhibited by the administration of honey to the tumour site, both pre- and post-operatively, therefore, authors suggested that honey may be beneficial in tumour surgery (Hamzaoglu *et al.*, 2000). Comments from Facino (2004), towards the study by Hamzaoglu *et al*., (2000), were similar to those discussed in the present study with regards to the clinical administration of honey in cancer treatment. Facino (2004) suggested that results could be attributed to the viscosity or osmolality of honey. Further comment was made that the authors did not account for differences in the constituents of honey, and that honey was a complex mixture of many phytochemicals. In addition, the inability of Hamzaoglu *et al*. (2000) to account for the viscosity of honey highlighted the need to include an artificial honey control in all future experiments.

Honey consumption may be beneficial for prostate cancer patients, independent of the direct interaction between phenolics and cancer cells. Akinloye *et al*. (2009) found that out of 120 men diagnosed with prostate cancer, the levels of prostate specific antigen (PSA) inversely correlated to their serum anti-oxidant status, with men with high PSA levels recording low anti-oxidant levels (Akinloye *et al.*, 2009). This was not the first time that cancer progression has been correlated to a reduction in serum anti-oxidant status, which suggests that an increase in endogenous and exogenous anti-oxidants may provide treatment benefit (Upadhya *et al.*, 2004; Sener *et al.*, 2007; Kasapovic *et al.*, 2008; Nathan *et al.*, 2011). The decrease in anti-oxidants may be due to their turnover in preventing cancer-driven oxidative damage, and therefore may further support the idea of increasing serum anti-oxidant status for the treatment of cancer (Upadhya *et al.*, 2004). The previously discussed serum anti-oxidant effect of both phenolic compounds and sugars may further highlight the benefit of honey consumption during prostate cancer treatment.

Honey consumption could also be recommended in cancer patients for its neuropharmacological benefits. Depression and anxiety are common in patients diagnosed with cancer, both of which may be present in up to 50% of new cases, and can persist throughout the duration of treatment (Burgess *et al.*, 2005). Further, depression and anxiety have independently been linked to a worsened prognosis and poor adherence to cancer treatment (Arrieta *et al.*, 2013). Systemic and local inflammation during cancer treatment has been linked to increased nociception in patients with bone metastatic prostate cancer; which may further increase depression and anxiety (Olson *et al.*, 1999; Falk *et al.*, 2014). Studies in both mice and rats have demonstrated that honey may possess anxiolytic, anti-depressive and anti-nociceptive activity through the modulation of inflammation, or via dopaminergic and serotonergic systems (Akanmu *et al.*, 2011; Oyekunle *et al.*, 2011; Al-Rahbi *et al.*, 2014). No current research has been conducted in human patients regarding honey consumption for the treatment of neurological symptoms associated with cancer, however, polyphenol administration for the treatment of anxiety and depression has been previously recommended (Bouayed, 2010).

6.1.3 Bioavailability of Honey Phenolics

Due to the extensive metabolism and conjugation of polyphenols during absorption, only a small proportion of the original compound may exist in the plasma, and even less at the site of action (Del Rio *et al.*, 2013). Despite this, the metabolites of flavonoids and phenolic acids may still retain some of the anti-oxidant activity of their original aglycone form, and therefore,

metabolism may not affect the biological activity of all polyphenols (Manach *et al.*, 1998). Individual analysis of each polyphenol metabolite should be conducted, as there is contradicting evidence suggesting that metabolites may have a reduced activity, due to their elimination (Manach *et al.*, 1998; Manach *et al.*, 2004b; Del Rio *et al.*, 2013). As an example, Stalmach *et al.* (2009) demonstrated the complexity of chlorogenic acid metabolism to caffeic acid; a polyphenol found at high concentrations in both coffee and honey. After consumption, chlorogenic acid is converted to caffeic acid in the gut by esterase enzymes. The authors reported multiple conjugations to caffeic acid catalysed by catechol-O-methyl transferase, sulfotransferase, esterases, reductases, Co-enzyme A and glycuronosyltransferase (Stalmach *et al.*, 2009). This suggested that despite the high intake and initial plasma concentration of chlorogenic acid and the metabolite caffeic acid, the extensive metabolism of phenolic compounds might result in very low circulating concentrations of the original compound. This might further suggest that *in vitro* analysis of phenolics for the treatment of cancer may have little clinical significance due to their extensive metabolism *in vivo*. For the use of dietary polyphenols in cancer treatment, the *in vitro* investigation of conjugates or metabolites may provide a more powerful clinical significance than investigation of the original compounds themselves.

Little evidence has been generated so far to show that honey consumption increases the tissue or plasma accumulation of phenolic compounds. As an example of this, the poor bioavailability of chrysin, along with its low concentration in honey, suggests a limited benefit of orally administered honey. Chrysin is a potent aromatase inhibitor with an IC_{50} of 4.2 µM. Due to its poor absorption, an orally administered dose of 27.6 g chrysin is required to reach a plasma concentration of 4.2 µM in humans (Walle *et al.*, 2001). To achieve a plasma concentration of 100 µM (25.42 µg/mL) as used in the current study for the inhibition of invasion and migration of prostate cancer cells, an orally administered dose of 635.5 g would be required. Based off calculations which determined the quantity of chrysin in honey (**Table 12**), to reach a plasma concentration of 100 µM would require the consumption of 81.89×10^6 kg honey; 6.5 times greater than the annual NZ honey production volume (Coriolis, 2012). Nevertheless, studies have examined the accumulation of other polyphenols in healthy human prostate biopsy tissue after the consumption of polyphenol rich foods. Consumption of green tea over 3 to 6 weeks led to the identification of polyphenols and their metabolites in the prostate tissue of 9 healthy men, at concentrations between 18 - 38 pmol/g (Wang *et al.*, 2010). Consumption of 35 g walnuts or 200 mL pomegranate juice per day for 3 days or 6 weeks allowed for the detection of the metabolite ellagic acid at a maximum concentration of 2 ng/g of human prostate tissue

(Gonzalez-Sarrias *et al.*, 2010). These results suggest that consumption of phenolic rich foods over 3 days to 6 weeks could lead to an accumulation of polyphenols or their metabolites, including, from honey. Interestingly, it has been suggested that plasma concentrations may not be an accurate biomarker for exposure to drugs, since concentrations of compounds in tissues do not always directly correlate with their plasma concentrations (Manach *et al.*, 2004b). Hong *et al*. (2002) showed that after oral administration, the polyphenol genistein was less concentrated in the prostate tissue of men with benign prostatic hyperplasia, compared to men with normal prostates. Further, they showed that the relative accumulation of polyphenols differed between the plasma and prostate tissue of the same men (Hong *et al.*, 2002). As well, Mauback *et al*. (2003) found that some isoflavones from soybeans could accumulate at low µM concentrations in breast tissue compared to plasma, however others were much lower in breast tissue than in plasma. This suggests that flavonoid accumulation may be tissue specific, and therefore the administration of certain dietary polyphenols may be more beneficial in different cancer types. Currently, there has been no full-body accumulation study of dietary phenolics. Unfortunately, this may only be completed by inter-study comparisons, as it may prove difficult to recruit subjects willing to undergo multiple invasive biopsies.

To compensate for the poor bioavailability of phenolics from honey, intravenous administration may be possible. Quercetin administered intravenously to men during a phase I clinical trial to establish pharmacokinetics, demonstrated safety at high plasma concentrations (Ferry *et al.*, 1996). These findings, along with those discussed in the present study, may suggest that the clinical anti-cancer activity of honey may only be possible through intravenous administration of a standardised medicinal honey.

The intravenous infusion of honey in humans has been previously used for the treatment of rheumatoid arthritis, however, the tolerated dose may be less than that in animals. One example includes a case study involving a 33 year-old woman treated for rheumatoid factor-positive rheumatoid arthritis over 5 years with non-steroidal anti-inflammatories, steroids, and methotrexate (Abdulrhman, 2007). Throughout the duration of her treatment, she suffered side effects including alopecia, joint pain, fatigue and stiffness. Upon stopping all medications, honey therapy was used as an alternative treatment. The patient was orally administered 100 mL honey a day along with intravenous honey beginning at 5% (w/v), which was increased to 20% (w/v) over 4 weeks. Initially, symptoms of joint pain, back pain and stiffness increased, however after 10 days, the patient reported improvement in all areas greater than that from her original medications. Two months into the honey therapy, she was found to be 8 weeks

pregnant, however treatment continued until just before full term. Interestingly, when the patient stopped bee honey therapy for two months surrounding her delivery, her symptoms recurred. She delivered a baby of normal weight with no abnormalities, which suggested honey may have no teratogenic activity. Her immediate tolerance to the intravenous administration of honey (5% - 20% w/v) was variable, where she often experienced transient episodes of fever, chills, and joint pain for no more than 60 min following infusion. Throughout her course, no anaphylaxis was reported. These results were not similar to those found in mice or sheep, where intravenous infusion of honey (50% or 5% w/v, respectively) caused no systemic side effects over 14 - 50 days (Al-Waili, 2003; Fernandez-Cabezudo *et al.*, 2013). Results from the case study suggest that intravenous honey may be tolerated at low concentrations in humans.

The amount of *in vivo* evidence for intravenous honey administration in humans is extremely low. Due to the possibility of transient systemic toxicity from honey, and a decreased tolerance compared to other animals, the safety and pharmacokinetics of intravenous honey in humans for the treatment of cancer must be further investigated. Nevertheless, before honey may be considered for investigation in clinical trials, *in vitro* data must provide impressive results that show promise. The effects of honey on cancer progression and metastasis still require extensive examination through the use of multiple *in vitro* assays, as well as *in vivo* models that use multiple routes of administration.

6.2 Bee Venom

6.2.1 Potential use of Bee Venom in Cancer

Currently, human clinical trials for the use of bee venom in cancer treatment are yet to be carried out. Nevertheless, an epidemiological study by McDonald *et al.* (1979) investigated the prevalence of cancer among 580 beekeepers, assumed to be regularly exposed to bee venom through bee stings. The authors reported that the incidence of cancer among beekeepers was lower than the expected, where only lung cancer was significantly lower ($p<0.05$), however, this could have been attributed to a lower smoking incidence, and therefore they concluded that there were neither any benefit nor any adverse effect of being chronically exposed to bee venom. Due to the lack of human clinical data, further investigation is needed.

Bee venom *in vitro* was demonstrated to be cytotoxic to Lewis lung carcinoma cells (LLC) but not peripheral blood mononuclear lymphocytes (Huh *et al.*, 2010). Further, it was reported to

inhibit the proliferation of and migration of human umbilical vein endothelial cells (HUVECs), suggesting it may have anti-angiogenic activity. *In vivo*, the administration of 1 or 10 µg/mL bee venom in mice inoculated with LLC decreased primary tumour volume by 49% and 62%, respectively (Huh *et al.*, 2010). Further, bee venom treatment reduced the spontaneous lung metastasis in mice post-tumour excision, as well as extended the median survival time from 27 to 58 days. These results demonstrated that bee venom therapy in mice may provide benefit when combined with surgery of the primary tumour.

Bee venom may also be beneficial when given adjunctively with conventional chemotherapeutics. In A2780cp human ovarian cells, the combination of bee venom (4 µg/mL) and cisplatin (10 mg/mL) exhibited a synergistic cytotoxicity of 50% at 24 h, compared to compounds alone (8 µg/mL and 25 mg/mL, respectively) (Alizadehnohi *et al.*, 2012). The lung metastasis of injected MCa mammary carcinoma cells following administration of bee venom was investigated in mice (Oršolić *et al.*, 2003b). The authors reported that intravenous, but not subcutaneous, bee venom caused a 65% reduction in lung metastasis, further suggesting the route of administration was important. Further, they demonstrated that the subcutaneous orthotopic administration of bee venom could increase overall survival of mice, since compared to control mice who died after 20 days, some mice did not develop tumours, and all treated mice survived the course of the experiment (50 days). After examination of the lymph nodes of mice treated with intravenous bee venom, they concluded that the anti-cancer activity of bee venom was due to the stimulation of cellular immune responses in local lymph nodes. Oršolić *et al*. (2003b) also exposed MCa cells in culture to 2.85 µg/mL bee venom and found that cell death occurred within 3 h, and via both necrotic and apoptotic mechanisms. They concluded that despite the direct cytotoxic effect bee venom had on MCa cells, the benefit of bee venom therapy *in vivo* may be due to the stimulation of immune responses and the activation of local lymph nodes, overall resulting in a reduced metastasis and tumourigenesis.

Bee venom has been previously reported to be well tolerated in patients that are non-allergic (Wesselius *et al.*, 2005). Kim *et al*. (2004) demonstrated the safety of subcutaneously injected bee venom in rats, where bee venom caused no side effects at concentrations 200 times higher than those used clinically in conventional apitherapy; the administration of honey bee products for medical use.

Together, the use of bee venom in cancer therapy may be beneficial, not only for the direct cytotoxic effects, but also for its anti-metastatic activity and lack of adverse effects, as shown

by the beekeeper study (McDonald *et al*., 1979). Results from the present study however suggested that bee venom may only provide minimal benefit for prostate cancer, and therefore further justification for its use may be needed.

6.2.2 Potential use of Melittin in Cancer

The anti-cancer activity of bee venom has been largely attributed to melittin, through a range of mechanisms (Son *et al.*, 2007; Oršolić, 2012). Melittin has been shown to destroy cell membranes and cause necrotic cell death by integrating into the lipid bilayer, or by increasing PLA_2 activity (Lad *et al.*, 1979; Ownby *et al.*, 1997; Pratt *et al.*, 2005). The selectivity of melittin towards cancer cells has been reported. As cancer cells display a greater membrane potential compared to normal cells, membrane targeting peptides such as melittin may selectively disrupt cancer cells (Son *et al.*, 2007). The interaction between melittin and hormone receptors may suggest a targeted effect of melittin towards both breast and prostate cancer (Son *et al.*, 2007). Melittin was shown to inhibit the activation of NF-κB, a transcription factor important in the regulation of metastatic and inflammatory genes (Park *et al.*, 2011).

Further to this, melittin may be protective to some normal cells. Park *et al*. (2014) recently demonstrated that melittin could suppress apoptotic cell death and NF-κB activation in normal hepatocytes that were induced with tumour necrosis factor-α. In contrast, melittin has been shown to be toxic towards red blood cells, lymphocytes, and skeletal myocytes (Ownby *et al.*, 1997; Pratt *et al.*, 2005). Since cell specificity is unlikely, it may be important to develop a selective targeting mechanism for the delivery of melittin. For example, Holle *et al*. (2003) demonstrated that melittin could cause selective tumour cell death through targeted therapy. Melittin was conjugated to avidin via a bond which required proteolysis to release melittin. Having found that MMP-2 was overexpressed at tumour sites, the authors reported that melittin was more cytotoxic towards cell lines that expressed high MMP-2 levels such as DU145 prostate cancer and SK-OV-3 ovarian cancer cells, compared to normal enteroendocrine L-cells (Holle *et al.*, 2003). Later, Holle *et al*. (2009) demonstrated that melittin released from a conjugate of two MMP-2-dependent latency-associated peptides could decrease B16 tumour volume by 70% in mice that expressed high MMP-2 activity around the primary tumour site. Both of these examples suggested that melittin may display high anti-proliferative and cytotoxic activity when delivery was optimised using targeted therapy. The present study demonstrated the anti-metastatic effect of melittin, which suggests that melittin may also provide benefit in the treatment of metastatic prostate cancer.

Melittin has been reported to exhibit selectivity for cell lines that overexpress the RAS oncogene: a gene family responsible for proliferation, differentiation and survival (Sharma, 1992; Fernández-Medarde *et al.*, 2011). Prostate cancer can switch from the RAS/ERK pathway to the PI3K/AKT, and therefore may not always be sensitive to melittin (Selvaraj *et al.*, 2014). In contrast, von Lintig *et al.* (2000) demonstrated that out of 20 breast cancers, RAS was overexpressed in the most aggressive cancers *in vivo*, and that RAS could be a potential target for therapy. Melittin may therefore demonstrate greater activity towards tumour types that often overexpress the RAS oncogene, such as breast cancer, colon carcinoma, pancreatic ductal adenocarcinoma and non-small cell lung carcinoma (von Lintig *et al.*, 2000; Fernández-Medarde *et al.*, 2011).

6.3 Future Directions

The approach used in the present study could be expanded into many different areas. As discussed, melittin may have a greater effect in cell lines that overexpress the RAS oncogene, and honey may have a greater effect in cell lines that have a greater dependence on ROS signalling. Cancer types with these characteristics could be explored. It has further been discussed that honey-derived phenolic compounds may accumulate in breast tissue at a greater amount, compared to prostate tissue. In addition to the possible anti-oestrogenic effects of phytoestrogens in honey, honey and melittin may exhibit a greater anti-cancer effect in metastatic breast cancer. For these reasons, future studies could include the use of both the MDA-MB-231 triple-negative and MCF-7 ER-positive breast cancer cells.

Honey may have an effect on the tumour stroma. Mesenchymal stem cells (MSCs) and cancer-associated fibroblasts (CAFs) have been implicated in the progression of cancer cell metastasis, through the secretion of proteases and cytokines. In addition, a reduction in MSC and CAF activity has been shown to inhibit cancer cell metastasis. As honey, and honey-derived phenolic compounds, have been reported to inhibit the migration of cancer cells through the inhibition of proteases, changes in the expression of proteases by MSCs and CAFs co-cultured with cancer cells could be investigated using gelatin zymography and Western blot. The inhibition of MSC- and CAF-mediated cancer cell metastasis would provide an additional mechanism for the anti-metastatic activity of honey *in vivo*, and therefore, provide further justification for its use in cancer treatment.

The anti-cancer activity of honey may be different when comparing cells cultured at conventional *in vitro* pH (7.4 - 7.6), compared to the *in vivo* acidic microenvironment surrounding a tumour (pH 5.8 - 7.6) (Wike-Hooley *et al.*, 1984). The surrounding tumour acidity is attributed to changes in the metabolic function of cancer cells, in which anaerobic respiration results in the production of lactate acid (Kato *et al.*, 2013). *In vitro*, a reduction in extracellular pH is mimicked by a reduction in intracellular pH, which can result in a switch from aerobic glycolysis to anaerobic ATP production (Mazzio *et al.*, 2012). Acidic conditions surrounding cancer cells may be indicative of hypoxia, and have been shown to promote and support a more invasive and less adherent cell phenotype (Kato *et al.*, 2005; Goetze *et al.*, 2011; Estrella *et al.*, 2013). In the present study, it was shown that thyme and honeydew honey have the ability to reduce both the invasiveness and migration of PC3 cells, cultured at pH 7.5. Because a reduction in pH can increase the metastatic phenotype of cells, it may be important to see whether honey has the same anti-metastatic ability in cells cultured at pH 6.5. Previously, the anti-cancer effects of honey have been attributed to the anti-oxidant characteristics of phenolic compounds that may interfere with cancer cell mitochondria (Alvarez-Suarez *et al.*, 2013). As changes in the extracellular pH, leading to changes in the intracellular pH, may alter the function of the mitochondria, it would be interesting to see whether honey has a similar or increased efficacy over a range of culture medium pH.

As well, it could be investigated whether honey may reduce cancer cell metastasis via the inhibition of angiogenesis. A tumour requires new blood vessels once it reaches a diameter of a few millimetres, around $1x10^6$ cells (Weidner *et al.*, 1991). Therefore, angiogenesis is essential for the expansion and growth of a tumour (Folkman, 1990; Weidner *et al.*, 1991). The process of angiogenesis is not dissimilar to cancer cell migration, in that endothelial cells respond to cytokines that originate from both cancerous and migratory cells. Honey has been demonstrated to be beneficial in wound healing by facilitating angiogenesis, often attributed to hydrogen peroxide formation (Molan, 2002; Barui *et al.*, 2014). In contrast, both crude honey and honey-derived phenolics may demonstrate anti-angiogenic activity in cancer situations (Igura *et al.*, 2001; Abdel Aziz *et al.*, 2009; Wang *et al.*, 2012; Zhao *et al.*, 2013). Due to the context-dependent inhibition of angiogenesis, it would be interesting to see whether the three New Zealand honeys could inhibit angiogenesis through the inhibition of migration, proliferation and matrix degradation of HUVEC, as assessed using zymogen assays, Boyden chambers, and the MTT assay (Goodwin, 2007).

6.4 Overall Conclusion

Evidence presented here, along with reports from the literature, suggest that honey may possess anti-cancer activity both *in vitro* and *in vivo* among many cell lines. Specifically, the anti-cancer properties of honey may be due to a combination of cytotoxicity and anti-metastatic activity. In the present study, honey was shown to rapidly reduce the viability of PC3 and DU145 cells. It was suggested that the loss of viability was largely due to a loss of adhesion. Following on from this, honey was shown to exhibit anti-metastatic activity through the reduction in adhesion, migration and invasion of PC3 cells. Of the three New Zealand honeys, honeydew honey was shown to have the greatest cytotoxic and anti-metastatic activity across both cell lines.

As phenolic compounds have been suggested to be responsible for the beneficial activity of honey, the quantities of two phenolic acids and three flavonoids were determined in the three honeys, by HPLC. The results showed that gallic acid was present in the highest amount, followed by caffeic acid, chrysin, kaempferol and then quercetin. Honeydew honey was shown to contain the highest quantity of gallic acid; a phenolic compound previously reported to possess anti-cancer activity across many cancer cell lines, including prostate. The high quantity of gallic acid may explain the increased benefit of honeydew honey, when compared to thyme and manuka honey.

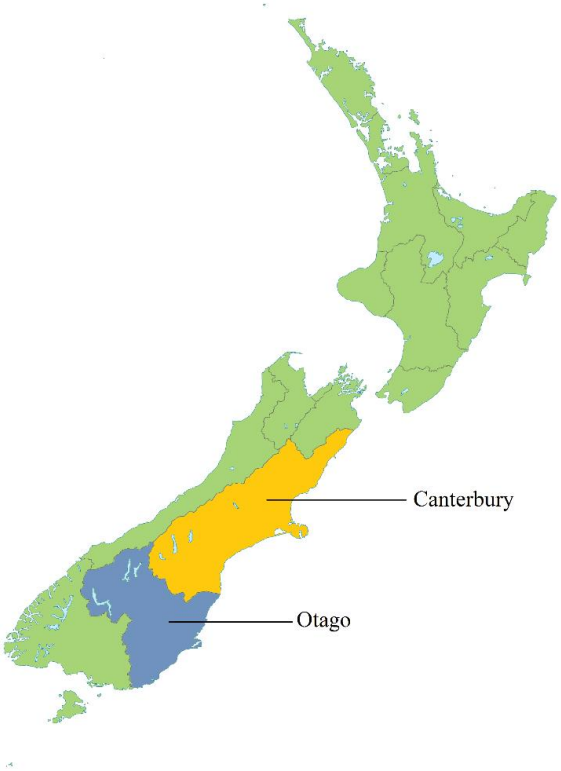
The cytotoxicity of the five phenolic compounds was reported across both of the prostate cancer cell lines, with gallic acid being the most cytotoxic. However, the rapid loss of cell viability and adhesion by honey could not be explained by the presence of the five phenolic compounds alone, which suggested the activity of honey may be due to either the presence of other compounds, or a combination of compounds that includes sugars. Gallic acid, kaempferol and chrysin were shown to inhibit PC3 cell migration by 40% - 50%, while all compounds, with the exception of kaempferol, were shown to inhibit PC3 cell invasion by 15% - 55%. Interestingly, only a high concentration of quercetin was shown to reduce prostate cancer cell adhesion to collagen. This suggested that the anti-metastatic activity of the honey-derived phenolic compounds may be due to mechanisms that were not adhesion mediated, despite their effect on cell viability.

Contrary to previous reports, bee venom and melittin were found to be non-cytotoxic towards prostate cancer cells at concentrations up to 5 µg/ml over 72 h. Bee venom caused a reduction in the migration and invasion of PC3 cells by 25%, however, this was not statistically

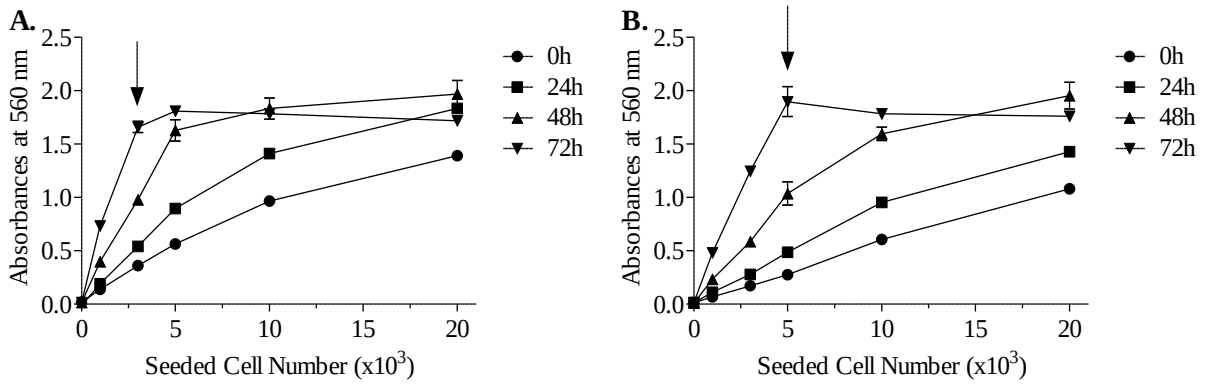
significant. Melittin caused a reduction in the migration and invasion of PC3 cells of 60% and 45%, respectively. These findings suggest that the presence of melittin may explain the anti-metastatic activity of bee venom, and that further investigation should be made regarding the benefits of melittin in metastatic prostate cancer.

Overall, New Zealand honey may be beneficial for the treatment or prevention of metastatic prostate cancer, with honeydew honey displaying a greater benefit compared to thyme and manuka hone. However, before its use in patients with prostate cancer, further *in vitro* and *in vivo* investigation is required. The anti-cancer activity of honey could not be explained by the presence of phenolic compounds alone, and therefore combinations of phenolic compounds with sugars should be investigated. Further, the poor oral bioavailability of phenolic compounds, as previously reported by other groups, suggest that the oral consumption of honey may provide little benefit towards the treatment of cancer as concentrations used *in vitro* may not be achievable in the plasma. In addition, melittin, the major constituent of bee venom, may possess anti-metastatic activity in prostate cancer, independent of its effects on cell viability. Nevertheless, intravenous administration of all honey bee products may negate their poor absorption and, therefore further investigation should be carried out.

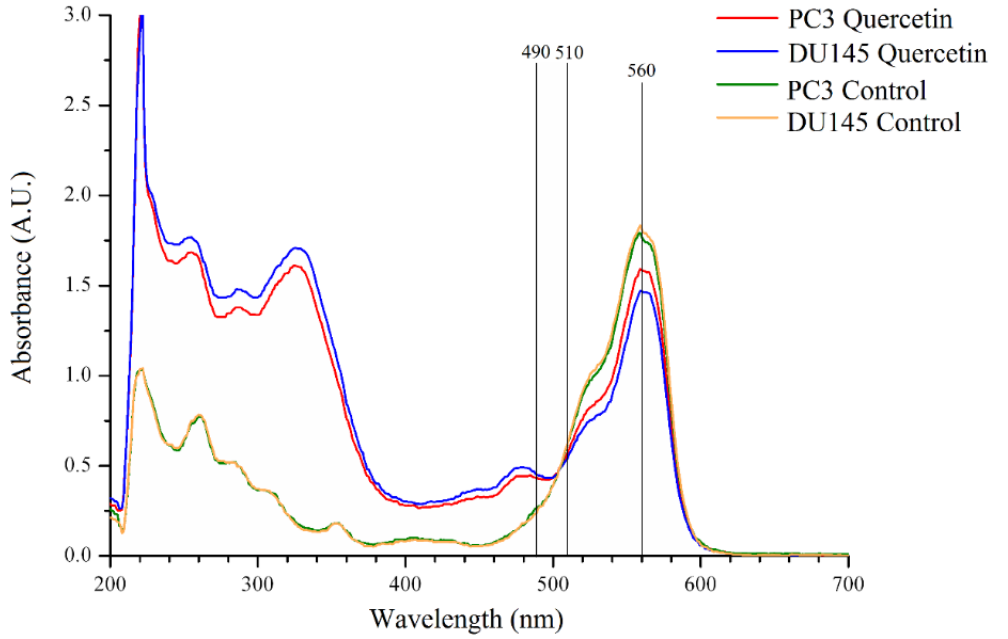
In conclusion, all honey bee products in the present study demonstrated potential for the treatment of metastatic prostate cancer, however, further examination that produces impressive *in vitro* results must be conducted before being trialled in animal models or patients.



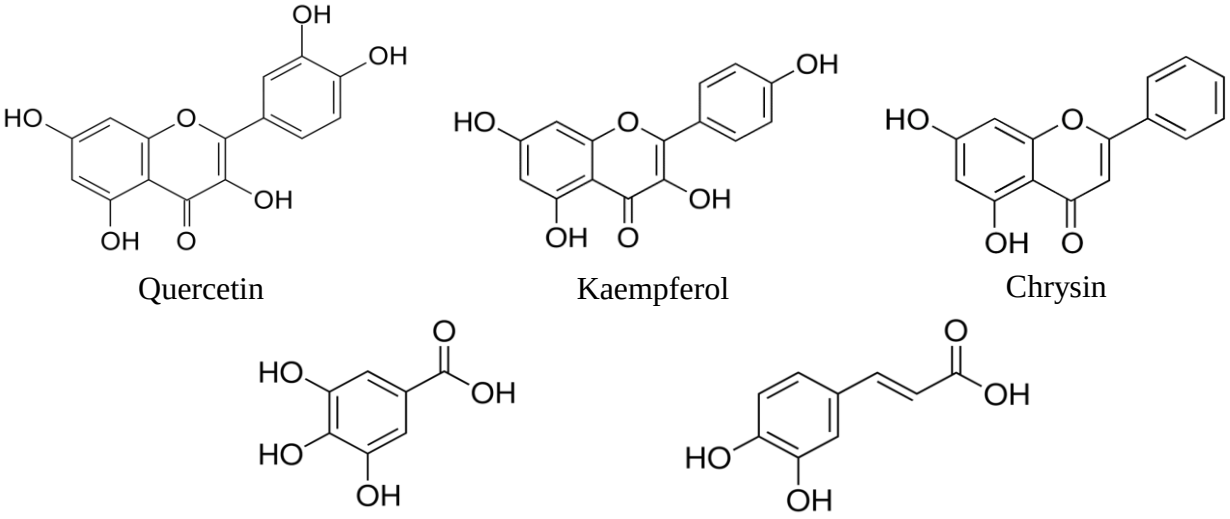
Appendix 1. Honey Origins within New Zealand. Both Thyme (*Thymus vulgaris*) and Manuka (*Leptoseprmum scoparium*) honey originated from the Otago region, whereas Black/Red Beechforest Honeydew (*Nothofagus solandri, N. fusca*) originated from the Canterbury region of the South Island, New Zealand. Picture was adapted from NZ Power Stations, Wikipedia (2015).



Appendix 2. Determination of Optimal Seeding Density in a 96-well Plate. (**A**) PC3 and (**B**) DU145 cells were seeded in 96-well plates over a range of densities from 0 - 2x10^3 cells/well. Following 24 h adherence (0 h), viability was established using the MTT assay. Wells were completed in quadruplicate, and values were expressed as mean percentage cell viability ± S.E.M. Optimal seeding density was determined as when cells could reach maximal absorbance without losing linearity at 72 h (represented by →). Densities selected were 3x10^3 (PC3) and 5x10^3 (DU145) /well.



Appendix 3. Interaction between Quercetin and the SRB Assay. PC3 and DU145 cells seeded at a density of $3x10^4$ cells/well (24-well plate) were treated with 100 µM quercetin, or a medium only control for 24 h. After treatment, cells were fixed in TCA and stained with SRB. After drying, SRB was solubilised using Tris base. An absorbance spectrum was constructed for the solubilised SRB from each cell line using a spectrophotometer between 200 and 700 nm. The wavelength of 510 nm was selected for the measurement of cytotoxicity using the SRB assay, as no interaction between quercetin and the SRB assay was found.



Appendix 4. Chemical Structures of Honey-derived Phenolic Compounds. The following honey-derived phenolic compounds were investigated in the present study. The flavonoids quercetin, kaempferol and chrysin are displayed in the upper half of the diagram. Their basic structure is comprised of two phenolic rings, connected by a heterocyclic ring (containing oxygen). The phenolic acids gallic acid and caffeic acid are displayed in the bottom half of the diagram. Their basic structure is comprised of a phenolic ring coupled to an organic carboxylic acid.

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