The Antioxidant and Anticancer Potentials of New Zealand Manuka Honey and Thyme Honey

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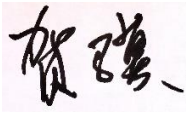
Attestation of Authorship

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person (except where explicitly defined in the acknowledgements), nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a university or other institution of higher learning.

Signed 

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Abbreviations

ABAP: 2, 2'- azo-bis (2-aminopropane) dihydrochloride
ABTS/ TEAC: 2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid/ Trolox equivalent antioxidant capacity
AD: Anno Domini
ADP: Adenosine diphosphate
AIDS: Human immunodeficiency virus infection and acquired immune deficiency syndrome
APAF-1: Apoptotic protease activating factor 1
ATP: Adenosine triphosphate
BC: Before Christ
BID: BH3 interacting-domain death agonist
Caco-2 cell line: Human epithelial colorectal adenocarcinoma cells
CIN: Chromosomal instability
CRC: Colorectal Cancer
CUPRAC: Cupric ion reducing antioxidant capacity
CYP1A: Cytochrome P450 1A
Cyt. c: Cytochrome complex
DMSO: Dimethyl sulfoxide
DNA: Deoxyribonucleic acid
DPPH: 2, 2-di (4-tert-octylphenyl)-1-picrylhydrazyl
EREs: estrogen response elements
ET: Electron transfer reactions
FCR: Folin-Ciocalteu Reagent
FRAP: Ferric reducing antioxidant power
FUDR: 5-fluoro-2'-deoxyuridine
GI system: Gastrointestinal system
GSTs: Glutathione S-transferases
hr: Hour
HAT: Hydrogen atom transfer reactions
HDL-C: High-density lipoprotein-cholesterol
H_2O_2: Hydrogen peroxide
HTS: High throughput screening
IAP: Inhibitor of apoptosis
IC_{50}: The concentration of the experimental compounds generating 50% inhibition in cell growth
IL1: Interleukin-1
IL-6: Interlueken-6
LDL-C: Low-density lipoprotein-cholesterol
Log: Logarithm
M: Moles per liter (mol/L)
max.: Maximum
MDR: Multidrug-resistant
MeCCNU: Methyl-CCNU
MGO: Methylglyoxal

min: Minute
min.: Minimum
mM: Millimoles per liter (mmol/L)
MSI: Microsatellite instability
MTT: 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
NF-κB: Nuclear factor-kappaB
NK-cells: Natural killer cells
NO: Nitric oxide
NOS: Nigerooligosaccharides
OD value: UV absorbance
ORAC: Oxygen radical absorbance capacity
PARP: Poly (ADP-ribose) polymerase
PBS: Phosphate Buffered Saline
PGF2α: Prostaglandin F2α
PLA2: Phospholipase A2
R^2 value: Coefficient of determination
RIP: Receptor-interacting protein
RJ: Royal jelly proteins
ROS: Reactive oxygen species
SCFA: Short chain fatty acid
SULTs: Sulfotransferases
TEAC: Trolox equivalent antioxidant capacity
TG: Triglycerides
TNF: Tumor necrosis factor
TRADD: TNFR associated death domain protein
TRAF: TNF receptor-associated factor
Trp-p-1: 3-Amino-1,4-dimethyl-5H-pyridol [4,3-b] indole
UGTs: UDP-glucuronosyltransferases
UV: Ultraviolet
v/v: volume/ volume
5-FU: 5-fluorouracil
µM: Micromoles per liter

Abstract

This study was conducted to test the antioxidant and anticancer activities of New Zealand Manuka honey and Thyme honey. The antioxidant activity of these two types of honey were tested by using two antioxidant assays, 2, 2-diphenyl-1-picrylhydrazyl free radical assay (DPPH), and Cupric Ion Reducing Antioxidant Capacity assay (CUPRAC), to test the free radical scavenging capacity and the bis (neocuproine) copper (II) cation (Cu (II)-Nc) reducing ability of tested honeys respectively. The anticancer activity concerning these two kinds of honey against Caco-2 cells was evaluated by MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) cell proliferation assay.

The experiment results demonstrated that both antioxidant and anticancer activities of tested honeys are time- and concentration-dependent. Both the DPPH radical scavenging capacity and cupric ion reducing capacity of Thyme honey are greater than those of Manuka honey at the same concentrations, incubation time and solvents. Similarly, the anti-proliferative effect of Thyme honey is 1.5-2-fold more potent than that of Manuka honey in Caco-2 cells after treatment for 48 and 72 hours. Therefore, along with the anticancer activity, the strong antioxidant capacities of tested honeys make them promising sources for further extraction/development of anticancer and chemopreventive compounds. Further preclinical and clinical studies are warranted to elucidate the therapeutic values of any specific extracts of tested honeys.

Chapter 1: General Introduction and Literature Review

1.1 Introduction to Honey

Honey is a common beekeeping product worldwide, which contains more than 200 compounds (Doner, 1977). The honeybee produces honey by collecting the nectar from a variety of flowers (nectar honey), and secretions of living parts of plants or excretions of plant-sucking insects (honeydew honey), and then store them as a primary food source in wax honeycombs inside the beehive. They convert nectar into honey by regurgitation and evaporation. The relation between honey and human beings could be dated back to Stone Age (E. Crane, 1983). Application of honey as a drug and an ointment against ulcers, tracing back to 2100-2000 BC, was first recorded in a Sumerian tablet writing, and also recorded by Aristotle 384-322 BC as a salve for sore eyes and wounds (E. Crane, 1975; Mandal & Mandal, 2011). Followed by Celus, a Greek physician in Rome ca. 25 AD, revealed that honey is likely to act as a treatment for diarrhea (Bogdanov, Jurendic, Sieber, & Gallmann, 2008). Honey as an excellent ingredient for the preparation of epileptic medicines was recorded in the Materia Medica, and as a remedy for passing too much urine (diabetes mellitus) in the Papyrus Ebers (Eadie, 2004; Korczowski, 1985). The therapeutic properties of honey even occur in holy books. The Bible narrates that King Solomon suggested his son to eat honey as it is good, while the Holy Hadith depicts that Prophet Mohamed recommended honey to one of his followers as a remedy against diarrhea (Bogdanov et al., 2008). Indeed, in a large percentage of ancient cultures honey

was applied in both nutritional and medical purposes (Allsop & Miller, 1996; E. E. Crane, 2013). Apart from being an important food resource and an ambiguously medical supply, honey was employed as a specific treatment matching symptoms such as wound infections, burns, ocular infections, sore throat, digital dermatitis, gastroenteritis, peptic ulcers, and eye problems from around 350 BC (P. C. Molan, 1999b). Thus, honey, as a vital carbohydrate source and the only widely available sweetener before the appearance of industrial sugar production around 1800, was of major importance for the humanity for an extended period in history (E. Crane, 1975). At present, except for nutritional and culinary uses, honey still plays a role in the treatment of wounds, burns, infections, bed sores, and gastroenteritis in infants (León-Ruiz et al., 2013; P. C. Molan, 1999a; Oelschlaegel et al., 2012).

Honey is considered to have a number of well-documented therapeutic capacities such as antibacterial, anti-inflammatory, and antioxidant activities (Bogdanov et al., 2008; Hadagali & Chua, 2014; P. C. Molan, 1999b). However, the therapeutic effects of honey are contingent on both physical and chemical properties, which are caused by various botanical sources, honey bee's metabolism, environmental conditions, climatic conditions, and seasonal conditions (Basualdo, Sgroy, Finola, & Marioli, 2007). This section aims to introduce the research area of this thesis, illustrating the composition of honey, outlining the therapeutic properties of honey with respect to different physiological effects. The purpose of this study is revealed at the end of this part.

1.1.1 Composition of Honey

Honey contains at least 200 components such as sugars, proteins, minerals, vitamins, enzymes, peptides, polyphenols, flavonoids, phenolic acids, free amino acids, organic acids, and other phytochemicals (Gheldof, Wang, & Engeseth, 2002; Sato & Miyata, 2000; Terrab, González, Díez, & Heredia, 2003). In fact, the composition of honey may vary a lot due to the influences caused by botanical origin, environment, and geographical factors (Oddo et al., 2004). The average honey composition is shown in Table 1.

Table 1. The average composition of honey (%)

	Blossom honey		Honeydew honey	
	average	min.-max.	average	min.-max.
Water	17.2	15-20	16.3	15-20
Monosaccharides				
fructose	38.2	30-45	31.8	28-40
glucose	31.3	24-40	26.1	19-32
Disaccharides				
sucrose	0.7	0.1-4.8	0.5	0.1-4.7
others	0.5	0.5-1	3.0	0.1-6
Trisaccharides				
melezitose	<0.1		4.0	0.3-22.0
erlose	0.8	0.5-6	1.0	0.1-6
others	0.5	0.5-1	3.0	0.1-6
undetermined oligosaccharides	3.1		10.1	
Total sugars	79.7		80.5	
Minerals	0.2	0.1-0.5	0.9	0.6-2.0
Amino Acids, proteins	0.3	0.2-0.4	0.6	0.4-0.7
Acids	0.5	0.2-0.8	1.1	0.8-1.5
pH-value	3.9	3.5-4.5	5.2	4.5-6.5

- **Data are collected from Bogdanov et al. (2008)**

Normally, the major constituents of honey are carbohydrates, containing monosaccharides such as glucose averaging 38.2%, and fructose averaging 31.2%, as

well as around 25 different oligosaccharides (e.g. disaccharides sucrose, trehalose, maltose, and turanose), occupying roughly 95% of honey dry weight, and water representing roughly 17.2% (Alvarez-Suarez, Tulipani, Romandini, Bertoli, & Battino, 2010; Doner, 1977). As a sort of prebiotic, oligosaccharides in honey are beneficial to gastrointestinal health by nourishing good bacteria in the large bowel or colon (Raftaniamiri, Khandelwal, & Aruna, 2010). Aside from carbohydrate, honey contains abundant ingredients such as proteins, minerals, organic acids, amino acids, organic acids, vitamins, polyphenols and aroma compounds (Gheldof et al., 2002) as shown in Table 2.

Table 2. Main constituents of honey

Ingredient	Amount in 100g
Energy (kcal)	
Carbohydrates (kcal)	300
Proteins (g)	0.5
Fats (g)	0
Minerals (mg)	
Sodium (Na)	1.6-17
Calcium (Ca)	3-31
Potassium (K)	40-3500
Magnesium (Mg)	0.7-13
Phosphorus (P)	2-15
Zinc (Zn)	0.05-2
Copper (Cu)	0.02-6
Iron (Fe)	0.03-4
Manganese (Mn)	0.02-2
Chromium (Cr)	0.01-0.3
Selenium (Se)	0.002-0.1
Vitamins (mg)	
Phylloquinone (K)	ca. 0.025
Thiamin (B_1)	0.00-0.01
Riboflavin (B_2)	0.01-0.02
Pyridoxine (B_6)	0.01-0.32
Niacin[2]	0.10-0.20
Pantothenic acid	0.02-0.11
Ascorbic Acid (C)	2.2-2.5

- **Data are collected from Bogdanov et al. (2008)**

In terms of proteins, honey normally includes roughly 0.5% of proteins, such as enzymes and free amino acids. The main enzymes that honey included are diastase (amylase), invertase (sucrose, α-glucosidase), as well as catalase and glucose oxidase. Diastase is essential for decomposition of glycogen or starches into smaller sugar units. Invertase plays a role in resolving sucrose into fructose and glucose. Glucose oxidase produces hydrogen peroxide (H_2O_2) and gluconic acid from glucose (Alvarez-Suarez et al., 2010). Honey just contains a small amount of vitamins, mainly phylloquinone (K), thiamin (B_1), riboflavin (B_2), pyridoxine (B_6), niacin, pantothenic acid, and ascorbic acid (C) (Conti, 2000; Nanda, Sarkar, Sharma, & Bawa, 2003). The common minerals contained in honey are sodium (Na), calcium (Ca), potassium (K), magnesium (Mg), phosphorus (P), zinc (Zn), copper (Cu), iron (Fe), manganese (Mn), chromium (Cr), and selenium (Se) (Conti, 2000; Iskander, 1995; A. Stocker, Schramel, Kettrup, & Bengsch, 2005; Terrab, Hernanz, & Heredia, 2004). The mineral content of honey could be influenced by honey varieties (monofloral, poly floral, non-floral, and floral or non-floral species), and geographical origin. Thus, the concentrations of over 20 minerals can be used to identify and trace the geographical and floral/ non-floral origin, and to characterize the quality of honey varieties (Tudoreanu, Codreanu, Crivineanu, & Goran, 2013). Furthermore, honey also contains a small amount of choline (0.3-25 mg/kg), benefiting function of cardiovascular and brain, as well as composing and repairing cellular membrane, and acetylcholine (0.06-5 mg/kg), acting as a neurotransmitter (Heitkamp & Busch-Stockfisch, 1986). The reason why different honey types have a variety of tastes, aromas and colors are because they have various botanical origins (Bogdanov, Ruoff, & Persano Oddo, 2004). The major parameter influencing honey

taste is sugar content. Aromas are contingent on the complex mixtures of volatile compounds (Arvanitoyannis, Chalhoub, Gotsiou, Lydakis-Simantiris, & Kefalas, 2005). Colors depend on phytochemicals (e.g. phenolic substance), and chemical substances naturally existing in plants (Abu-Tarboush, Al-Kahtani, & El-Sarrage, 1993; R. H. Liu, 2003; J. Sun, Chu, Wu, & Liu, 2002; Vattem, Ghaedian, & Shetty, 2005). However, nectar from a few plants (e.g. *Rhododendron ponticum*) used by bees has been reported to contain toxic ingredients, mainly diterpenoids and pyrazolidine alkaloids. These poisonous honey may induce symptoms of headache, stomach ache, nausea, vomiting, unconsciousness, delirium, and sight weakness (Edgar, Roeder, & Molyneux, 2002). In addition, some cases have revealed that dormant endospores of the *Clostridium botulinum*, a type of bacterium being harmful to infants, may be contained in honey, which can convert into toxin-producing bacteria causing illness and sometimes even death in the immature stomach and intestinal tract of infants. Hence, infants under 12 months of age are advised to avoid feeding with honey (Caya, Agni, & Miller, 2004; Shapiro, Hatheway, & Swerdlow, 1998).

1.1.2 Manuka Honey

Manuka honey is a unifloral honey derived from the pollen of *Leptospermum scoparium*, the botanical name given to New Zealand's Manuka tree (Old, 2013; Weston, Mitchell, & Allen, 1999). In fact, New Zealand's indigenous people, the Maori, had been using the Manuka tree as a medicinal product for hundreds of years before Captain Cook arrived in 1769 (Old, 2013). Manuka honey was originally applied in the medical field

for its antibacterial effects during the 1940s and 1950s before the discovery of antibiotics and synthetic drugs. At present, it still plays a role in wound healing area (R. Cooper, 2007). Some honeys contain ingredients associated with non-peroxide antibacterial activity, which is derived from the large amount of methylglyoxal (MGO) present in those honeys. However, the exact MGO content in honey is contingent on the botanical origin of honey (Adams et al., 2008; Mavric, Wittmann, Barth, & Henle, 2008). Manuka honey has been recognized to exhibit a high level of "non-peroxide" antibacterial activity against various bacteria such as *Helicobacter Pylori* causing stomach ulcers and cancers (Al Somal, Coley, Molan, & Hancock, 1994; León-Ruiz et al., 2013; Russell, Molan, Wilkins, & Holland, 1990). Previous studies have identified that some Manuka honey and Viper Bugloss honey types can remain antibacterial activity in the test in presence of catalase, supporting the presence of non-peroxide activity (Allen, Molan, & Reid, 1991). MGO present in Manuka honey derived from nectar collected from the blossom of Manuka trees, principally responsible for this non-peroxide activity, has been reported to have a synergistic effect on a number of antibiotics such as piperacillin. However, this bioactivity just present in some kinds of Manuka honey from specific areas (Adams et al., 2008; Mavric et al., 2008; Mukherjee et al., 2011). Previous studies have shown that Manuka honey possesses a bactericidal mode of activity against 30 multi-drug resistant (MDR) Gram-negative rods with the potential to infect wounds, suggesting the clinical potential to inhibit pathogens that commonly colonized wounds. Manuka honey has been applied in licensed wound dressings in the UK (P. Molan, 1997). Apart from the well-known antibacterial activity, Manuka honey has been reported to have effective antioxidant activity. Previous studies

have revealed that Manuka honey contains a characteristic high content of methyl syringate. This antioxidant is not only able to neutralize superoxide radicals, but also to bind iron, suggesting to have a preventive effect against the formation of extremely damaging hydroxide radicals created by hydrogen peroxide (Brangoulo & Molan, 2011; Inoue et al., 2005). Furthermore, medicated Manuka honey has been regarded to be effective as a treatment for exomphalos major (Nicoara, Singh, Jester, Reda, & Parikh, 2014). It has been shown that Manuka honey poses a positive effect on prevention against dental plaque development and gingivitis, and is able to substitute refined sugar in the manufacture of candy (English, Pack, & Molan, 2004).

1.1.3 Thyme Honey

In terms of thyme honey, it is produced from the nectar of different species of thyme plants. Its principal locality is Greece, Italy, Spain and Morocco (Ricciardelli, 1998). Thyme honey has unique characteristics such as light amber color, distinctive floral, pungent aroma, as well as strong herbal, resinous and savory flavor (Dimou, Marnasidis, Antoniadou, Pliatsika, & Besseris, 2009). The climatic conditions at the period during blossoms of botanical origin of thyme honey determine that this honey production is changeable, challenging and limited, as a result of which, this sort of honey has to be packaged and traded as a blend of thyme and other kinds of honey (Dimou et al., 2009). Previous studies have indicated that Thyme honey is very rich in compounds related to anticancer properties (e.g. polyphenols and phenolic acids). Thyme honey extracts exhibit significant inhibition of cell viability in prostate cancer and endometrial cancer

cells (Tsiapara et al., 2009). In another study, Thyme honey has been suggested to contain a unique monoterpene, the trihydroxy ketone E-4-(1, 2, 4-trihydroxy-2, 6, 6-trimethylcyclohexyl)-but-3-en-2-one, which exerts a significant apoptotic activity in PC-3 prostate cancer cells. This inhibition of trihydroxy ketone on PC-3 cells is partly due to the reduction of NF-κB (nuclear factor-kappaB) activity and IL-6 (interleukin-6) secretion. The trihydroxy ketone could also impose an important anti-microbial effect on many human pathogenic bacteria and fungi (Kassi et al., 2014).

1.2 Oxidants

Oxidant is a by-product of normal body process, which is derived from oxygen (Rahman & Adcock, 2006). Oxygen is the most abundant chemical element by mass in the Earth's biosphere, air, sea and land (Emsley, 2011). It is crucial for the respiration of most organisms. Despite the fact that oxidative respiration is of great significance to the massive majority of organisms except few species of anaerobic bacteria, it still leads to generation of radicals detrimental to health that may directly or indirectly contribute to diseases such as cancer, cardiovascular disease, arteriosclerosis, immune-system decline, brain dysfunction, and cataracts (Ames, Shigenaga, & Hagen, 1993; Parthasarathy, Steinberg, & Witztum, 1992; Serafini, 2006). Molecular oxygen possesses two unstable electrons that are not spin-paired in the outer shell. These electrons are highly reactive to bind to a spin-matched pair of electrons, which result in the reactions between these electrons and other electron pairs especially those found in double bonds. The intermediates, as the precursor of most reactive oxygen species (ROS), are formed due to

the removal of electrons to gain a relative stableness such as superoxide anion (O^{2-}) (Turrens, 2003). Since the electrons are transferred to oxygen molecules, the breaking-up of electron pairs leads to the formation of free radicals (McCord, 2000). Dismutation of superoxide anion catalyzed by superoxide dismutase or converted spontaneously contributes to the formation of hydrogen peroxide (H_2O_2), which is probably reduced to water by either spontaneous reactions or by the action of catalase. Hydrogen peroxide, as a catalyst, may undergo the Fenton reaction to form the hydroxyl radical, while superoxide anion may react with other radicals, including nitric oxide (NO) to form peroxynitrite. These two compounds, namely hydrogen peroxide and NO, are also powerful oxidants (Buettner & Jurkiewicz, 1996; Turrens, 2003). Furthermore, molecular oxygen and H_2O participates in the reactions in mitochondria to generate Adenosine Triphosphate (ATP), a nucleoside triphosphate used in cells as a coenzyme responsible for intracellular energy transfer (Bhattacharyya, Chattopadhyay, Mitra, & Crowe, 2014). However, this reaction is likely to contribute to the formation of toxic ROS as well (Aruoma, 1994; Bhattacharyya et al., 2014).

1.2.1 Free Radicals

A free radical is any atoms, molecules or ions that have unpaired valence electrons in the outer shell, which can be formed during normal physiological processes of all living beings (Dasgupta & Klein, 2014; Erbas & Sekerci, 2011). The chemical reactivity of an atom or molecule can be changed by the unpaired electrons, and be greater than the normal ones (Halliwell, 1994). Free radicals can be summarized according to their

valence as positively charged, negatively charged or electrically neutral (Battino, Bullon, Wilson, & Newman, 1999). A number of factors are able to result in the formation of free radicals *in vivo*, including radiation (e.g. ionizing and ultraviolet), redox reactions catalysed by metal ions (e.g. $Fe^{3+} + e^{-} \rightarrow Fe^{2+}$), and enzymic catalysis (e.g. flavoproteins and hemoproteins) (Freeman & Crapo, 1982; T. F. Slater, 1988).

Normally, free radicals are generated at a low rate without detriment to health, and subsequently neutralized by the cellular defense system. However, an increasing amount of free radical may exceed the capacity of the protective system (Sjödin, Westing, & Apple, 1990). The excess free radicals can attack numerous systems, including immune system, and lead to diseases such as cancer, cardiovascular disease, arteriosclerosis, immune-system decline, brain dysfunction, cataracts and much other disorders (Ames et al., 1993; J. Gutteridge, 1995; Parthasarathy et al., 1992; Serafini, 2006).

1.2.2 Reactive Oxygen Species (ROS)

Reactive oxygen species (ROS) are defined as molecules and free radicals generated from molecular oxygen (Turrens, 2003). ROS include free radicals (e.g. hydroxyl, superoxide, NO and peroxyl), hydroperoxyl, alkoxyl, carbon dioxide radical, and non-free-radical species (e.g. hydrogen peroxide, ozone, singlet oxygen, and hypochlorous acid) (Aruoma, 1994; Halliwell, 2006). ROS can be classified according to their longevity as short-lived diffusible entities (e.g. hydroxyl, alkoxyl and peroxyl radicals), medium lifetime entities (e.g. nitroxyl and superoxide radicals), as well as non-radicals H_2O_2, organic

hydroperoxides, and hypochlorous acid (Simon, Haj-Yehia, & Levi-Schaffer, 2000). In the human body, ROS are the natural by-products of normal metabolism of oxygen benefiting homeostasis and cell signaling. Under normal circumstances, ROS can be eliminated by both enzymatic antioxidants (e.g. superoxide dismutase, catalase and glutathione peroxidase) and non-enzymatic antioxidants (e.g. Vitamin E, C, and glutathione) existed in cells (Tierney, Croft, & Hayes, 2010). However, during times of environmental stress (e.g. exposure heat or UV), the balance between ROS and production of antioxidants can be destroyed by the increasing level of ROS, which is a situation known as 'hyperoxia.' The growing tendency of ROS can also be caused by certain conditions, including hypercholesterolemia, aging, hypertension, smoking, nitrate intolerance, hyperthermia, chemotherapeutic agents, Hepatitis C, diabetes and obesity (Boots, Haenen, & Bast, 2003; Chapple, 1997; Finkel & Holbrook, 2000; Furukawa et al., 2004; Harrison, Griendling, Landmesser, Hornig, & Drexler, 2003; Korenaga et al., 2005; Kushiro et al., 2005; Landmesser et al., 2003; Rytilä et al., 2006). Meanwhile, with accumulation of ROS in human body, cellular components can be damaged by oxidation, leading to cell death and tissue injury, as well as oxidative damage to cellular membranes, tissues, and enzymes (Maltepe & Saugstad, 2009; Matanjun, Mohamed, Mustapha, Muhammad, & Ming, 2008; Niki, 2012; Tierney et al., 2010). This ongoing increased ROS bind to other free radicals to obtain a stable configuration, and will not cease until all radicals form a stable and inert covalent bond (Buettner & Jurkiewicz, 1996; Wardman & Candeias, 1996). Sustained cell death and tissue injury may result in the onset of a wide range of chronic diseases stated in human, including cancers, inflammatory diseases, atherosclerosis, neurological diseases, and even Alzheimer's disease (Chauhan &

Chauhan, 2006; Matanjun et al., 2008; Temple, 2000; Tierney et al., 2010). A number of diseases have been reported to be associated with ROS such as retinal damage, schizophrenia, skin-aging, nephritis, reperfusion injury, asthma and diabetes mellitus (Harman, 2002; Nath, Gupta, Prasad, Pandav, & Thakur, 1999; Nazeer, Saranya, & Naqash, 2014; Reddy & Yao, 1999). Apart from the diseases, ROS have been reported to promote apoptotic processes as a catalyzer for many enzymes, and to delay the process of muscle recovery (Calió et al., 2014; Ha & Zemel, 2003). Hence, consumption of antioxidants is advocated to be beneficial to health due to their preventive effects against excess free radicals produced in the body (Matanjun et al., 2008).

1.2.3 The Two Sides of ROS

Low concentration of ROS is beneficial to health, which plays a role in cellular responses against infectious agents (Alexieva, Markova, Nikolova, Aragane, & Higashino, 2010). ROS exhibit an essential effect on cell signaling and homeostasis (C. E. Cooper, Patel, Brookes, & Darley-Usmar, 2002; Finkel & Holbrook, 2000; Harrison et al., 2003; Nemoto, Takeda, Yu, Ferrans, & Finkel, 2000; R. Stocker & Keaney, 2004). For example, hydrogen peroxide is supposed to be the most important signaling messenger concerning the specificity of its production, reaction and removal (Forman, Maiorino, & Ursini, 2010).

Moreover, ROS generated by phagocytic cells are considered to have a preventive effect against infection, while cytosolic ROS are beneficial to regulate the proliferative response

(Finkel, 1998). Previous studies have shown that ROS play a crucial role in plenty of biological processes such as protein phosphorylation, transcription factor activation, normal cell growth, induction and maintenance of the transformed state, programmed cell death, immune function, regulation of vascular tone, monitoring of oxygen tension in the control of ventilation and erythropoietin production, cellular senescence, adaptation to stress, and the regulation of lifespan (Alexieva et al., 2010; D'Autréaux & Toledano, 2007; Finkel, 2003; Rajendran et al., 2014; Serafini, 2006; Van Raamsdonk & Hekimi, 2010). However, during times of environmental stress (e.g. exposure heat or UV), the balance between ROS and production of antioxidants can be destroyed by the increasing level of ROS (Matanjun et al., 2008; Tierney et al., 2010). This condition may pose a detrimental influence on cell structures, which is known as oxidative stress contributing to oxidative damage (Devasagayam et al., 2004; Joyner-Matos, Downs, & Julian, 2006; Sies, 1991). The excess ROS are able to result in direct injury to tissues, as well as to alter the biologically essential molecules (e.g. lipids, nucleic acids, proteins, carbohydrates and DNA) by oxidation (Devasagayam et al., 2004; Kirkham & Rahman, 2006; Livingstone, 2003; Niki, 2012; Parthasarathy et al., 1992; Stadtman, 1992). Lipid peroxidation leads to aging in organisms and some chronic diseases related to aging such as cancer (Sakata, 1997). In contrast, low levels of certain free radical and ROS are able to activate the growth of fibroblasts and epithelial cells (Battino et al., 1999). Therefore, synthesizing the above factors, the ROS can be considered to act as a double edged sword exhibiting both advantageous and disadvantageous effects (Niki, 2012).

1.2.4 Oxidative Stress and Disease

The term “oxidative stress” means an imbalance between the formation of free radicals and the protective antioxidant activity in a certain organism. Oxidative stress occurs due to either the lack of antioxidants or the excess generation of ROS (Halliwell, 2006; MacNee, 2000; Palmer & Kitchin, 2010; Redón et al., 2003). This imbalance may induce extensive damage to DNA, protein, and lipid, as a major sort of endogenous damage contributing to aging, accumulated with age. Protection against oxidation is considered to benefit prevention of aging and some chronic diseases associated with age such as cancer, cardiovascular disease, arteriosclerosis, immune-system decline, brain dysfunction, Huntington’s disease, diabetes, septic shock, rheumatoid arthritis, AIDS, atherosclerosis and cataracts (Ames et al., 1993; Baynes & Thorpe, 1999; Chandra, Samali, & Orrenius, 2000; Furukawa et al., 2004; Ohara, Peterson, & Harrison, 1993; Parthasarathy et al., 1992; Salvemini & Cuzzocrea, 2002).

1.3 Antioxidants in General

Antioxidants mean the substances possessing a distinctively preventive effect against oxidation in a low concentration, including every type of molecule found *in vivo* (Halliwell & Gutteridge, 1990). The ROS can react with antioxidants instead of attacking cells. One antioxidant molecule can only neutralize a single free radical, as a result of which, a constant replenishment of antioxidant resources endogenously or through supplementation is needed to counter-balance the excess free radicals. A number of natural and synthetic compounds are advocated to be effective to protect

against oxidative stress (Heo & Jeon, 2009). Natural antioxidants are supposed to be better than synthetic antioxidants amongst consumers due to the concerns regarding the toxic and carcinogenic effects of synthetic antioxidants. Natural antioxidants can be classified as phenolic compounds (e.g. flavonoids, tocopherol and phenolic acids), nitrogen compounds (e.g. alkaloids, amino acids/ peptides, chlorophyll substances, and amines), carotenoid derivatives, and ascorbic acid (Hall & Cuppett, 1997; Hudson, 2012; Larson, 1988). Phenolic compounds normally contained in honey are phenolic acids, mainly hydroxybenzoic acid, chlorogenic acid, protocatechuic acid, caffeic acid, vanillic acid, benzoic acid, p-coumaric acid, ellagic acid, and cinnamic acid; flavonoids, mainly apigenin, naringenin, kaempferol, luteolin, pinocembrin, chrysin, and galangin; and polyphenols (Beretta, Granata, Ferrero, Orioli, & Facino, 2005; Estevinho, Pereira, Moreira, Dias, & Pereira, 2008). The most plentiful categories of polyphenols are flavonoids and phenolic acids (Bravo, 1998). Flavonoids compounds always exhibit low molecular weight, and bind to sugar molecules, which can be classified as flavonols, flavones, flavanones, anthocyanidins and isoflavones (King & Young, 1999). The flavonoid categories are shown in Table 3, while the basic structure of flavonoids is shown in Figure 1 below.

Table 3. Flavonoid categories

Class	Dietary sources
Flavanol	
Epicatechin	
Catechin	Green and black tea
Epigallocatechin	Red wine
Epicatechin gallate	
Epigallocatechin gallate	
Flavone	
Chrysin	Fruit skin
Apigenin	Celery, parsley
Flavanone	
Naringin	Peel of citrus fruits
Taxifolin	Citrus fruits
Flavonol	
Kaempferol	Endive, leek, broccoli, radish, grapefruit, black tea
Quercetin	Onion, lettuce, broccoli, cranberry, apple skin, berries, olives
Myricetin	Cranberry, red wine, grapes
Anthocyanidins	
Malvidin	Red grapes, red wine
Cyanidin	Cherry, raspberry, strawberry, grapes
Apigenin	Colored fruits and peels

- **Data are collected from Rice-Evans, Miller, and Paganga (1996)**

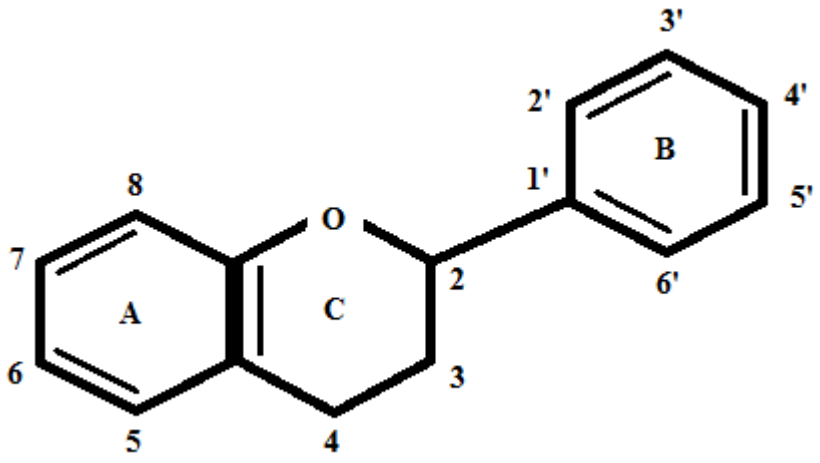
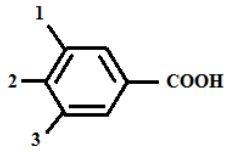
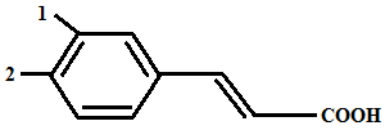
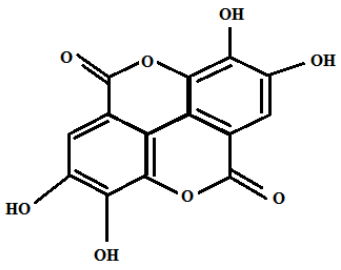
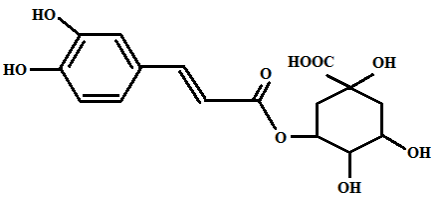


Figure 1. The basic structure of flavonoids. Redrawn from Bravo (1998)

The antioxidant activity of flavonoids can be reflected in different ways. Flavonoids are

able to trap reactive oxygen substance directly, as well as to inhibit the formation of enzymes responsible for superoxide anions, and to prevent the peroxidation process (Rice-Evans et al., 1996). Phenolic acids can be classified as either benzoic acid or cinnamic acid derivatives according to the distinct structures as shown in Table 4.

Table 4. Structure of phenolic acids

<table>
<tr><td colspan="4">Benzoic acid derivatives</td><td colspan="3">Cinnamic acid derivatives</td></tr>
<tr><td colspan="4">  </td><td colspan="3">  </td></tr>
<tr><td>Phenolic acid</td><td colspan="3">Position</td><td>Phenolic acid</td><td colspan="2">Position</td></tr>
<tr><td></td><td>C1</td><td>C2</td><td>C3</td><td></td><td>C2</td><td>C3</td></tr>
<tr><td>Gallic acid</td><td>OH</td><td>OH</td><td>OH</td><td>Caffeic acid</td><td>OH</td><td>OH</td></tr>
<tr><td>Protocatechuic acid</td><td>H</td><td>OH</td><td>OH</td><td>p-Coumaric acid</td><td>H</td><td>OH</td></tr>
<tr><td>Syringic acid</td><td>OCH_3</td><td>OH</td><td>OCH_3</td><td>Cinnamic acid</td><td>H</td><td>H</td></tr>
<tr><td>3-Hydroxybenzoic acid</td><td>H</td><td>H</td><td>OH</td><td>Ferulic acid</td><td>OCH_3</td><td>OH</td></tr>
<tr><td>Vanillic acid</td><td>H</td><td>OH</td><td>OCH_3</td><td></td><td></td><td></td></tr>
<tr><td colspan="7">Others</td></tr>
<tr><td colspan="4">Ellagic acid</td><td colspan="3">Chlorogenic acid</td></tr>
<tr><td colspan="4">  </td><td colspan="3">  </td></tr>
</table>

- **Data are collected from Fukumoto and Mazza (2000)**

Phenolic acids include hydroxybenzoic acid, and hydroxycinnamic acids (King & Young, 1999). Berries and nuts are regarded to contain the hydroxybenzoic acids, while the hydroxycinnamic acids are rarely found in free form, which includes coumaric, ferulic and caffeic acid. The majority of fruits are suggested to contain plenty of caffeic

acid, occupying 75% to 100% of the total hydroxycinnamic acids contents, while cereal grains have abundant ferulic acid (D Archivio et al., 2007). Tannins are high molecular weight polyphenols, which can either bind and precipitate or shrink proteins and other organic molecules such as amino acids and alkaloids, and which can be categorized into condensed tannin, the polymers of catechins or epicatechin, hydrolysable tannins, and the polymers of gallic or ellagic acids (King & Young, 1999). In terms of the antioxidant activity of phenolic compounds, the antioxidant property of monomeric phenolics is influenced by their numbers and molecular weight, as well as the arrangement of phenolic substituents, and extended conjugation. The anti-oxidability of flavonoids is positively proportional to the content of hydroxyl groups (Hodnick, MllosavljeviĆ, Nelson, & Pardini, 1988). The scavenging ability of phenols has been suggested to be related to the activity of reducing and chelating ferrous ion that catalyzes lipid peroxidation, which is derived from their exceptional structures of hydroxyl groups containing an aromatic ring (Al-Mamary, Al-Meeri, & Al-Habori, 2002; Aruoma, 1994; Halliwell & Gutteridge, 1990). In addition, it has been reported that vitamins also exhibit antioxidant activity directly by intrinsic free radical scavenging mechanism and/ or indirectly as cofactors for enzymes (Vertuani, Angusti, & Manfredini, 2004). In terms of vitamin C applied in this research, it is found consist in fruits and vegetables in high concentrations as a potent reducing agent (Buettner & Jurkiewicz, 1996).

1.4 Antioxidants in Honey

The antioxidant activity of honey is contingent on its antioxidants, including glucose

oxidase, ascorbic acid, phenolic acids, catalase, flavonoids, carotenoid derivatives, Maillard reaction products, organic acids, amino acids, and proteins (Al-Mamary et al., 2002; Aljadi & Kamaruddin, 2004; Frankel, Robinson, & Berenbaum, 1998; Gheldof & Engeseth, 2002; Inoue et al., 2005; Nasuti, Gabbianelli, Falcioni, & Cantalamessa, 2006; Schramm et al., 2003; Vela, de Lorenzo, & Perez, 2007). Its antioxidant effects appear mainly due to its phenolic compounds, which play significant roles in scavenging free radicals and/ or chain-breaking of the oxidation reactions to counterbalancing an excess of oxidants (Kumar, Jindal, Sharma, & Nanda, 2013; Moure et al., 2001; Jihua Wang et al., 2012). However, the antioxidant activity varies dramatically with different kinds of the floral source used by bees, seasonal and environmental factors, as well as processing ways (Al-Mamary et al., 2002; Gheldof & Engeseth, 2002). Even though the entire antioxidant activity of honey should be derived from the combination of a variety of bioactive substances, the proportion of phenolic compounds, to some degree, can reflect the total antioxidant activity of honey (Beretta et al., 2005). However, the content of phenolic compounds present in honey is not always positively proportional to antioxidant effects, suggesting that different types of polyphenols exhibit variable scavenging capacity (Al-Mamary et al., 2002; Küçük et al., 2007). Evidence shows that darker honey is probably to have a greater antioxidant activity than light colored honey. Honey with a higher concentration of water always has higher antioxidant activity (Estevinho et al., 2008; Frankel et al., 1998; Gheldof et al., 2002). The measurement of antioxidant activity with regard to honey polyphenols can be achieved *in vitro* by comparison between the oxygen radical absorbance capacity (ORAC) and the total phenolic concentration (Gheldof & Engeseth, 2002). Consumption of honey enhances plasma antioxidants has

been observed in previous studies, which demonstrates that the bioavailability and bioactivity of honey can be transferred efficiently from honey to plasma (Schramm et al., 2003).

1.5 Mechanism of Antioxidant Activity

Antioxidants are traditionally classified into two categories: primary or chain breaking antioxidants, and secondary or preventative antioxidants.

1.5.1 Primary Antioxidants

With gaining or loss of electrons of a free radical, a second radical can be formed which is able to undergo the same reaction and then continue to generate more unstable products until termination occurs. Primary antioxidants can cease the further radical chain reaction of the free radicals. They delay or inhibit the initiation of reaction by scavenging or by inactivating free radicals as shown in the equation below.

L• + AH -> LH + A•

AH scavenge the free radicals of lipid radical (L•), halting radical initiation.

LO• + AH -> LOH + A•

AH scavenge the free radicals of alkoxyl (LO•), stopping the propagation step and forming a low reactivity antioxidant radical A• that prevents the further reaction from occurring.

LOO• + AH -> LOOH + A•

AH scavenge the free radicals of peroxyl (LOO•), interrupting the propagation step and forming a low reactivity antioxidant radical A• that prevents the further reaction from occurring.

For instance, polyphenol (PPH), a type of flavonoid with a strong chain-breaking antioxidant, can inhibit lipid peroxidation by rapidly providing the peroxyl radical (ROO•) with a hydrogen atom to generate alkyl hydroperoxide (ROOH) and polyphenol phenoxyl radical (PP•) as shown in the equation below.

ROO• + PPH -> ROOH + PP•

The polyphenol phenoxyl radical (PP•) can bind to another radical, including another phenoxyl radical, or react with a hydrogen atom donated by polyphenol to intervene in the initiation of a new chain reaction (Fuhrman & Aviram, 2001).

1.5.2 Secondary Antioxidants

Secondary antioxidants can scavenge initiating radicals before the occurrence of new radical chain reactions to obtain the effects of retarding the rate of chain initiation. For instance, metal chelators, a type of antioxidant, can chelate metal ions, including Fe^{2+} and Cu^{2+}, to prevent metal-catalyzed initiation reaction, and to decompose lipid hydroperoxide. Iron is concerned as the major metal ion responsible for the formation of hydroxyl radicals *in vivo* because it could react with hydrogen peroxide (H_2O_2) and form hydroxyl radicals, which is known as the Fenton reaction.

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^-$

Researchers often design antioxidant assays by using Fenton reactions to generate

hydroxyl radicals without interference from other ROS as the reactions in the hydroxyl radical scavenging assay (Fuhrman & Aviram, 2001).

1.6 Cancer and Its Common Treatments

Cancer is a group of diseases involving abnormal cell growth with the potential to invade or spread to other parts of the body. Tumors are not equal to cancers, while benign tumors are not cancerous. Cancer may be diagnosed by the possible symptoms such as a lump, abnormal bleeding, prolonged cough, unexplained weight loss, and a change in bowel movements. Over 100 cancers affect humans. Cancer is a multistep process, which is initiated by an onset from a single transformed cell with characteristics of swift proliferation, invasion and metastasis. A number of factors, including various carcinogens, tumor promoters, and inflammatory agents can activate and catalyze this dynamic process. In addition to the oxidative stress in humans, which is also reported to be a carcinogenic factor leading to cancer. An imbalance of ROS in the human body can destroy the cellular components and then contribute to cell death and tissue injury. The persistent cell death and tissue injury may lead to a wide range of chronic diseases, including cancers. The whole modulation is controlled by the transcription factors, including antiapoptotic proteins, proapoptotic proteins, cell cycle proteins, protein kinases, cell-adhesion molecules, cyclooxygenase-2 (COX2), and other molecular targets (Aggarwal & Shishodia, 2006; Ames et al., 1993; Chauhan & Chauhan, 2006; Matanjun et al., 2008; Parthasarathy et al., 1992; Serafini, 2006; Shishodia, Majumdar, Banerjee, & Aggarwal, 2003; Temple, 2000; Tierney et al., 2010; Jin Wang & Lenardo,

2000).

Normally, the standard treatments for cancer are surgery, radiotherapy, and chemotherapy, which are beset by a number of serious side effects. In general, in order to obtain maximum benefits, patients are suggested to select the treatments carefully. In consideration of the complexity of distinct carcinomas and a variety of potentially useful treatments, patients are supposed to consult multidisciplinary teams that comprise hepatologists, radiologists, surgeons, pathologists and oncologists (Forner, Llovet, & Bruix, 2012). Surgical resection, transplantation, and ablation are universally considered as the most effective treatments for cancer, which provide patients with potential for a cure (Bruix & Sherman, 2011). Transarterial chemoembolization and sorafenib are the only non-curative treatments which can enhance survival rate (Llovet & Bruix, 2003). Arterial embolization without chemotherapy, external radiotherapy, and radioembolization also have been identified to have antitumor activity (Forner et al., 2012). In terms of chemotherapy of cancer, the antimetabolites (e.g. 5-fluorouracil (5-FU), 5-fluoro-2'-deoxyuridine (FUDR), methotrexate, and doxorubicin) have been verified to have anticancer effects (Oberfield, Steele, Gollan, & Sherman, 1989). The combination of different kinds of antimetabolites is likely to enhance efficacy. A number of studies have been done with regard to this area, such as a combination of 5-FU and methyl-CCNU (MeCCNU) with doxorubicin (Falkson, MacIntyre, Moertel, Johnson, & Scherman, 1984), and the combination of epirubicin, cisplatin, and 5-FU (J. E. Lee et al., 2014). However, these drugs are unable to ensure a cure for cancer, and a number of side effects such as hair loss, nausea, headaches and skin pigmentation are always

occurred with the use of these drugs. As for some severe cases, serious reactions include hypersensitivity reactions (including anaphylaxis), radiation recall, heart damage, and liver dysfunction may be occurred (Rossi, 2013). When chemo- and radio-therapy are applied systemically to inhibit and kill cancerous cells, they also commonly destroy healthy cells in the process. Therefore, chemo- and radio-therapy are limited by duration and dose affecting the effectiveness of these therapies (Premratanachai & Chanchao, 2014). Accordingly, researches for alternative anticancer drugs are on an urgent demand, especially for natural products. The majority of anticancer medicines in Europe and America are processed from natural elements which are extracted from microbes or plants (Ji, Li, & Zhang, 2009). A wide range of marine resources can be used to develop new anticancer medicines such as seaweed including fucoxanthin as an effective apoptosis inducer (Peng, Yuan, Wu, & Wang, 2011).

1.7 Colorectal Cancer (CRC)

The gastrointestinal (GI) system is of great significance to the human body, which is included in the digestive system. The gastrointestinal system consists of all the parts of the body from mouth to anus. Its specialized functions are related to convert food into energy needed for the body, and package the residue for waste disposal (Raman, Ambalam, & Doble, 2015).

Colorectal cancer is a type of cancer existed in the colon or rectum, which is caused by abnormal growth of cells (Arends, 2013). It is one of the most diagnosed cancers

worldwide, representing 9.7% of all cancers apart from non-melanoma skin cancers, compared to lung, occupying 13%, and breast, taking up 11%. The majority of colorectal cancer (around 95%) is adenocarcinomas (Ferlay et al., 2013). It has been reported that 1,360,602 (9.7% of all newly diagnosed cancers apart from skin cancers) new cases and 693,933 (8.5% of the total number of cancer deaths) deaths due to CRC worldwide in 2012, while the incidence of colorectal cancer amongst men is higher than women (Dušek, Mužík, Malúšková, & Šnajdrová, 2015). Colorectal cancer can be categorized according to different patterns based on its origin as inherited, familial and sporadic (Roper & Hung, 2013). The occurrence of colorectal cancer is attributed to the absence of genomic stability causing multiple mutations, including chromosomal instability (CIN), microsatellite instability (MSI), aberrant DNA methylation, and DNA repair defects (Ewing, Hurley, Josephides, & Millar, 2014; Munteanu & Mastalier, 2013). Colorectal cancer also can be caused by a number of environmental risks such as lifestyle factors (e.g., tobacco, nutrition and physical activity), naturally occurring exposures (e.g., radon gas, ultraviolet light, and infectious agents), medical treatments (e.g., radiation and medicines), workplace and household exposures, as well as pollution (Hagland & Søreide, 2015; Roper & Hung, 2013).

1.8 Anticancer Activity of Honey and Its Mechanisms

Natural products such as honey are suggested to have potential anticancer effects. The anticancer activity of honey has been proved against a variety of cancer cell lines and tissues, including breast, colorectal, renal, prostate, endometrial, cervical and oral

cancer (Fauzi, Norazmi, & Yaacob, 2011; Fukuda et al., 2010; Ghashm, Othman, Khattak, Ismail, & Saini, 2010; Jaganathan, Mazumdar, Mondhe, & Mandal, 2011; Samarghandian, Afshari, & Davoodi, 2011; Tomasin & Cintra Gomes‐Marcondes, 2011; Tsiapara et al., 2009). Daily oral ingestion of honey may play a role in the prevention of tumor spreading. The previous study concentrated on the effects of honey on tumor growth has shown that an obvious anti-metastatic effect occurred when oral application of honey before tumor cell inoculation (Orsolić et al., 2003). According to the study by Swellam et al. (2003), honey exhibits strong anti-tumor effects against bladder cancer both *in vitro* and *in vivo* in mice. These results testify that honey exerts effective prevention against the growth of different bladder cancer cell lines *in vitro*, namely T24, RT4, 253J and MBT-2. They also show a positive effect when administered intralesional application or oral intake in the MBT-2 bladder cancer implantation in mice models.

In fact, the anticancer activity of honey is attributed to a combination of various bioactive properties of honey (Fauzi et al., 2011; J. Gutteridge, 1995; Kris-Etherton et al., 2002; Majtán, Kováčová, Bíliková, & Šimúth, 2006; Subrahmanyam, 1998; Tomasin & Cintra Gomes‐Marcondes, 2011; AJ Tonks et al., 2003; A Tonks, Cooper, Price, Molan, & Jones, 2001; Tsutsui, Hayashi, Maizumi, Huff, & Barrett, 1997; Yeh, Huang, & Yen, 2005; Yeh & Yen, 2005). Cancer cells are characterized by uncontrolled cellular proliferation and inadequate apoptotic turnover (Nicholson, 2000). Apart from the common treatments mentioned in section 1.6, with the development of medical technology, new treatments against cancer aims to interfere with specific targeted

molecules needed in carcinogenesis (Chari, 2007; Goldman, 2003). The common drugs used for cancer treatment are apoptosis inducers (Earnshaw, 1995). Programmed cell death or apoptosis can be summarized into three phases, namely (i) an induction phase; (ii) an effector phase; (iii) a degradation phase (Susin, Zamzami, & Kroemer, 1998). The first phase is committed to stimulate pro-apoptotic signal transduction cascades via death-inducing signals. The second phase aims to bring cell death through a key regulator, mitochondrion. The third phase comprises nuclear and cytoplasmic events. Nuclear changes contain chromatin and nuclear condensation, cell shrinkage, DNA fragmentation, and membrane blebbing (Earnshaw, 1995; Susin et al., 1998). Eventually, in the cytoplasm, with the activation of a complex cascade of protein-cleaving enzymes called caspases, the cells are destined into fragmented apoptotic bodies which are phagocytosed by macrophages or other surrounding cells (Earnshaw, 1995; Susin et al., 1998). The two pathways of apoptosis are shown in Figure 2, namely the caspase 8 or death-receptor pathway, and caspase 9 or mitochondrial pathway (Fernandez-Cabezudo et al., 2013).

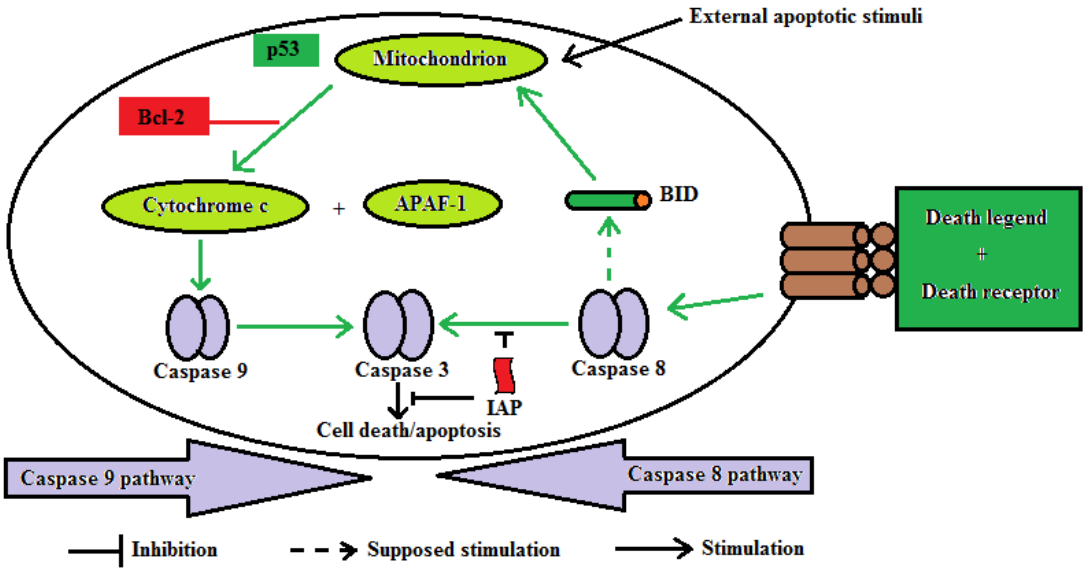


Figure 2. The two pathways of apoptosis. BID: BH3 interacting-domain death agonist; Cytochrome c: cytochrome complex; APAF-1: Apoptotic protease activating factor 1; IAP: inhibitor of apoptosis. Redrawn from S. Ahmed and Othman (2013)

The rich content of polyphenols and phenolic acids in honey has been reported to have simulative effects on apoptosis in diverse types of cancer cells through depolarization of mitochondrial membrane (Fauzi et al., 2011; Kris-Etherton et al., 2002; Yeh et al., 2005; Yeh & Yen, 2005). Honey enhances caspase 3 activation level and poly (ADP-ribose) *polymerase* (PARP) cleavage in human colon cancer cell lines due to its high content of tryptophan and phenolic (Jaganathan & Mandal, 2009). Honey also leads to apoptosis by modulation of the pro- and anti-apoptotic proteins expression in colon cancer cell lines (Jaganathan & Mandal, 2010). As can be seen in Figure 3, the expression of caspase 3, p53 and pro-apoptotic protein Bax can be increased by honey, while the expression of anti-apoptotic protein Bcl-2 can be downregulated by honey (Jaganathan & Mandal, 2010). Animal trials have proved that an adjuvant therapy that combining honey with *Aloe Vera* can promote the expression of pro-apoptotic protein Bax, and can control the anti-apoptotic protein Bcl-2 expression in Wistar rats (Tomasin & Cintra Gomes‐Marcondes,

2011).

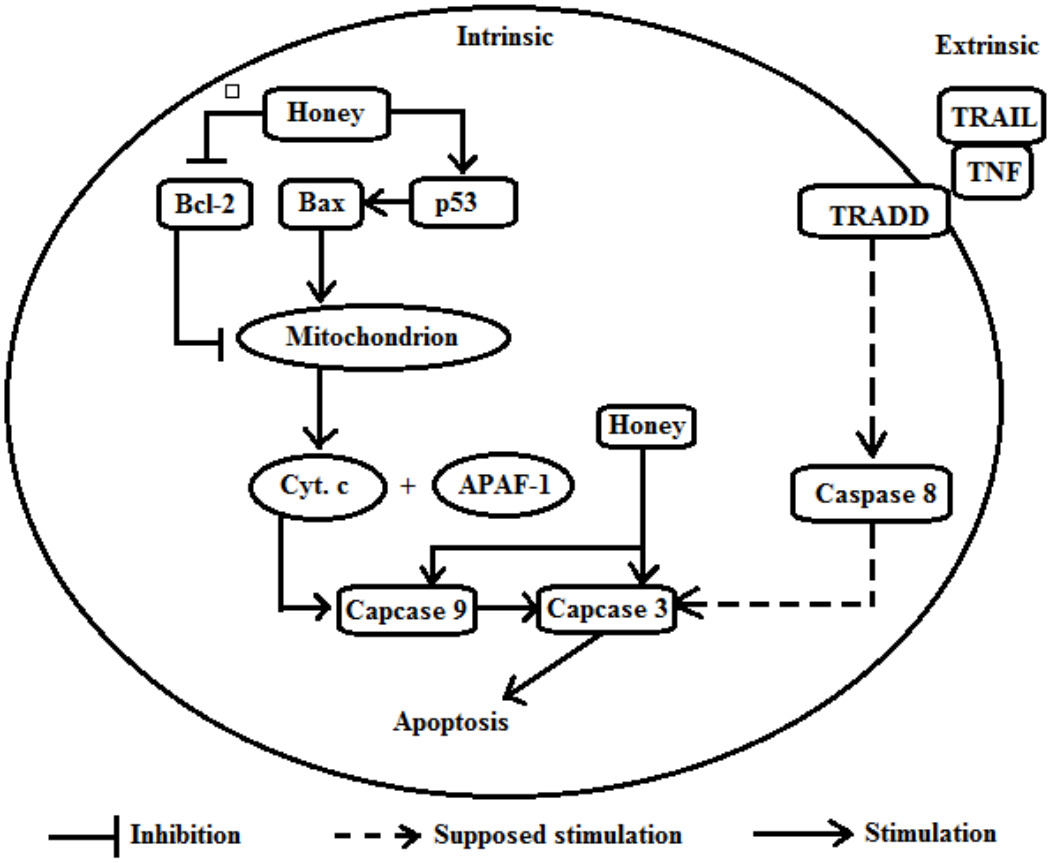


Figure 3. The mechanism of honey leading to apoptosis in colon cancer cell lines. TNF: tumor necrosis factor; TRADD: TNF receptor associated death domain protein; Cyt. c: cytochrome complex; APAF-1: Apoptotic protease activating factor 1. Redrawn from Jaganathan and Mandal (2010)

Honey is also reported to influence cell cycle arrest (Tomasin & Cintra Gomes‐Marcondes, 2011). The cell cycle includes three distinguished phases which are G_0, G_1, S and G_2M. These phases are regulated and monitored by several different proteins, including cyclins and cyclin-dependent kinases (Diehl, 2002). In animal experiments, a combination of honey and *Aloe Vera* demonstrates a marked reduction in expression of Ki67-LI in tumor cells in rats, supporting an anti-proliferative effect of honey by arresting cell cycle (Tomasin & Cintra Gomes‐Marcondes, 2011). The G_0/ G_1 phase

of glioma, colon and melanoma cancer cell lines are advocated to be blocked by the honey constituents such as flavonoids and phenolics (Jaganathan & Mandal, 2009; Y.-J. Lee et al., 2003; Pichichero, Cicconi, Mattel, Muzi, & Canini, 2010). The anti-proliferative effects of honey are contingent on the applied dose and reacted time (Pichichero et al., 2010). Bee venom also inhibits the proliferation of carcinoma cells and tumor growth *in vivo*. It is a complex mixture of substances produced in the venom gland located in the abdominal cavity. Bee venom is formed from several biologically active peptides (e.g. melittin, apamin, adolapin, mast cell degranulating peptide, and many enzymes), and non-peptide components (e.g. histamine, dopamine, phospholipase A2 (PLA2), and norepinephrine) (Habermann, 2013; Raghuraman & Chattopadhyay, 2007). The anti-proliferative activity of bee venom is derived from its simulative effects on the local cellular immune responses in lymph nodes. Bee venom can lead to apoptosis-necrosis, and lysis of tumor cells (Jang et al., 2003; X. Liu, Chen, Xie, & Zhang, 2002; Orsolic, Sver, Terzic, Tadic, & Basic, 2003). It has been reported that bee venom can contribute to apoptosis in human leukemic cells by inducing Bcl-2 and caspase 3 expression through the downregulation of mitogen-activated signal pathways, while having no influences on murine bone marrow cells (Moon et al., 2006). Also, bee venom results in stimulation of caspase 3 in synovial fibroblasts, and inhibition of cyclooxygeneses-2 expression in human lung cancer cells (Hong et al., 2005; Jang et al., 2003).

In addition, the royal jelly (RJ) proteins contained in honey can activate macrophages to release cytokines TNFα, interleukin-1 (IL1), and interlueken-6 (IL-6), while specific

honey types (e.g. pasture, jelly bush, and Manuka honey) plays a role in the stimulation of monocytes to release tumor necrosis factor-alpha and interleukin- (IL-) 1β and IL-6 (Majtán et al., 2006; Šimúth, Bíliková, Kováčová, Kuzmová, & Schroder, 2004; AJ Tonks et al., 2003; A Tonks et al., 2001). Tumor necrosis factor (TNF) has been shown to mediate tumor initiation, promotion and progression, which are growth factors for a number of tumor cells (Moore et al., 1999; Sugarman et al., 1985). The possible mechanism is associated to the binding of TNF-R to TNF-α, and adaptor protein including TNF receptor associated factor (TRAF), TNFR associated death domain protein (TRADD), and receptor-interacting protein (RIP) to regulate apoptosis and inflammation through these cytokines. The TNF-α release can regulate important cellular processes including apoptosis, cell-proliferation and inflammation (MacEwan, 2002; Šimúth et al., 2004).

Mutations of genetic structure in the human body are directly or indirectly caused by mutagenic substances which could be formed in the process of roasting and frying food, such as Trp-p-1 (3-Amino-1,4-dimethyl-5H-pyridol [4,3-b] indole), and which is linked with carcinogenicity (Tsutsui et al., 1997). Honey derived from various botanical origins are considered to exert an inhibitory effect on Trp-p-1 mutagenicity (X.-H. Wang, Andrae, & Engeseth, 2002). Honey is also reported to have a strong antimutagenic agent (Saxena, Gautam, Maru, Kawle, & Sharma, 2012). The previous study has shown that honey could cause SOS response, an error-prone repair system that is attributed to mutagenesis, in *Escherichia coli* cells exposed to radiation (e.g. UV) (Saxena et al., 2012). Another study was conducted to remove some essential genes (e.g. umuC, recA

and umuD) involved in SOS-mediated mutagenesis. These changes are significantly inhibited in the presence of honey, which confirms the strong antimutagenic effects of honey (Saxena et al., 2012).

Furthermore, honey can control the level of estrogen by its estrogenic modulatory activity. This phenomenon may play a role in inhibiting estrogen-dependent cancers such as breasts and endometrial cancers (Gruber, Tschugguel, Schneeberger, & Huber, 2002; Tsiapara et al., 2009). The complexes produced by the dimeric reaction between estrogen receptors and estrogens can be transferred into the nuclei, and be bound to specific DNA base sequences called estrogen response elements (EREs) leading to transcription and translation of the estrogenic effects in the targeted tissues (Gruber et al., 2002). Phenolic compounds contained in honey are suggested to have estrogenic modulatory activity by modulating the estrogen receptor activity (Tsiapara et al., 2009; Zaid, Sulaiman, Sirajudeen, & Othman, 2010).

On the basis of previous studies, seven different botanical origin honeys (acacia, buckwheat, fireweed, soybean, Tupelo and Christmas berry) showed a significant inhibition on Trp-p-1, compared to glucose and fructose exhibiting a similar antimutagenic activity as honey, as well as nigerose, a type of sugar contained in honey, presenting an immune protective activity (Doner, 1977; Murosaki, Muroyama, Yamamoto, Liu, & Yoshikai, 2002; Othman, 2012; X.-H. Wang et al., 2002). Evidence indicates that cancer and metastasis prevention of honey is partly derived from its activation of immune system of human body by stimulating antibodies, B and T

lymphocytes, neutrophils, monocytes, eosinophils and natural killer cells (NK-cells) production during primary and secondary immune responses in tissue culture (Abuharfeil, Al-Oran, & Abo-Shehada, 1999; N. S. Al-Waili, 2003a; Attia, Gabry, El-Shaikh, & Othman, 2008). Animal experiments on CBA mice and Y59 rats have verified this anti-metastatic activity by oral application of honey (Oršolić & Bašić, 2004). A probable mechanism of this immunomodulatory actions is that honey ingestion may be beneficial to form short-chain fatty acid (SCFA) fermentation products (Kruse, Kleessen, & Blaut, 1999). Nigerooligosaccharides (NOS), a type of sugar, present in honey has been found to possess immunopotentiation activity (Murosaki et al., 2002).

Apart from the factors mentioned above, the anti-inflammatory activity (section 1.9.2) and antioxidant activity (section 1.4) also play a role in the anticancer activity of honey. This is because both chronic inflammation and oxidative stress can result in the formation of cancer (J. M. Gutteridge & Halliwell, 1993; Subrahmanyam, 1998).

1.9 Other Therapeutic Effects of Honey

1.9.1 Antimicrobial Activity of Honey

The usage of honey as a potent medicine for a wide range of diseases can be traced from ancient times (Allsop & Miller, 1996; E. Crane, 1975, 1983; E. E. Crane, 2013; P. C. Molan, 1999a, 2001). Nowadays, the widespread use of antibiotics has contributed to a range of bacterial resistance, thus limiting the application of these agents and developing an alternative antibacterial product is in an urgent condition (Tenover, 1986).

Honey is just such an antiseptic product which has been verified to have certain antibacterial activity, especially against gram-positive bacteria (P. Molan, 1997; P. C. Molan, 1992). Previous studies have shown that undiluted honey has an inhibitory effect on 21 types of bacteria including *Escherichia coli*, *Klebsiella sp, Pseudomonas sp, Staphylococcus sp*, and two types of fungi *in vitro* (Wahdan, 1998). The discovered bacteria that sensitive to honey are shown in Table 5.

Table 5. The discovered bacteria that sensitive to honey

Actinomyces pyogenes
Bacillus anthracis
Corynebacterium diphtheriae
Epidermophyton floccosum
Escherichia coli
Haemophilus influenzae
Helicobacter pylori
Klebsiella pneumoniae
Microsporum Canis, M. gypseum
Mycobacterium tuberculosis
Nocardia asteroides
Proteus sp.
Pseudomonas aeruginosa
Salmonella sp.
Salmonella cholerae-suis
Salmonella typhi
Salmonella typhimurium
Serrata marcescens
Shigella sp.
Staphylococcus aureus
Streptococcus agalactiae, Str. Dysgalactiae, Str. uberis
Streptococcus faecalis
Streptococcus mutans
Streptococcus pneumoniae
Streptococcus pyogenes
Trichophyton rubrum, T. tonsurans, T. mentagrophytes var.
Vibrio choleriae

- **Data are collected from Bogdanov et al. (2008)**

A number of honey types have been proved to have inhibitory effects on the growth of bacterium *in vitro* at very low concentration range (from 1.8% to 10.8% (v/v)), which ensures a long period storage of honey without becoming spoiled (Carnwath, Graham, Reynolds, & Pollock, 2014; P. Molan, 2001). The antibacterial mechanisms of honey are associated with osmolarity, hydrogen peroxide, flavonoids, phenolic compounds, acidity, aromatic acids, pH and the presence of other phytochemical components, such as MGO, melanoidin, leptosin, jellins, bee defensing, and hydroxyl radicals (H. Lee, Churey, & Worobo, 2008; Mavric et al., 2008; P. C. Molan, 1992; Sherlock et al., 2010). The high osmolarity of honey is derived from the high content of carbohydrates, such as fructose, glucose, maltose and sucrose. Hydrogen peroxide is formed by glucose oxidase secreted from the hypopharyngeal glands of honeybees, while the peroxide producing capacity is contingent on honey catalase activity (J. W. White, Subers, & Schepartz, 1963; R. White & Molan, 2005). Apart from this peroxide, the low pH (3.2-4.5) and low water content of honey is also considered to exert an effect on antibacterial activity. Enzymes transform sucrose into a simple and soluble mixture of monosaccharides. The sugar molecules in honey bind to free water molecules. Therefore, microorganisms are unable to obtain enough water for the growth purpose, and are prevented from survival. The enzyme glucose oxidase transforms glucose into gluconic acid, which makes honey too acidic, and prevents microbes from growth and survival (Hadagali & Chua, 2014; J. W. White et al., 1963; Yatsunami & Echigo, 1985). However, the levels of honey antibacterial activity vary widely depending on the botanical origin, even different processing and storage conditions may affect its

antibacterial activity by changing the compositions of honey (Chen, Campbell, Blair, & Carter, 2014; Taormina, Niemira, & Beuchat, 2001). The antibacterial peroxide could be destroyed by heat, storage and light, the factors possessing a greater influence on the antibacterial capacities of blossom honey than honeydew honey, as a result of which, honey is suggested to store in a cool and dark place, and to consume when it is fresh (Bogdanov, 1997). For instance, antibacterial and antifungal activities show a reduction tendency in processed honey heated to 45 °C for 8 hours, while a decrease of antibacterial activity can be observed in processed honey stored at room temperature for 12 and 24 months, as well as in autoclaved honey (Chen et al., 2014; Elbanna et al., 2014).

1.9.2 Wound Healing and Anti-inflammatory Effects of Honey

Inflammation is a type of tissue response to trauma or pathogenic agents, which protects an organism or tissue from the injuring stimuli, such as pathogens, damaged cells or irritants (Ferrero‐Miliani, Nielsen, Andersen, & Girardin, 2007). Inflammation can be divided into two categories as either acute or chronic, with the former is the initial response of body to harmful stimuli accompanied by symptoms such as pain, heat, swelling, redness and inability to function, and with the latter is the acute inflammation prolonged for days indicated by pain, ulceration, scarring, fibrosis and non-healing of wounds. Both destruction and healing of tissue occur in the process of chronic inflammation. Chronic inflammation results in a gradual shift in the types of cell existing in the inflamed area, which can destruct the tissue, and then compromise the

survival of the organism. Thus, it can give rise to other diseases such as hay fever, atherosclerosis, rheumatoid arthritis, periodontitis and sometimes even cancer (Menke, Ward, Witten, Bonchev, & Diegelmann, 2007). In terms of anti-inflammation, the traditional drug therapy may inhibit wound healing processes because a mild, short-lived inflammation is a significant requirement to activate the healing process (R. White & Molan, 2005). Therefore, honey, as a natural product with no inhibition against wound healing processes, can be used as anti-inflammatory agents and be a substitute for drug therapy (Hadagali & Chua, 2014). A number of studies have confirmed that honey as a dressing is a much more potent medicinal treatment for burns and infected wounds than many other present alternatives (P. C. Molan, 2001; Subrahmanyam, 1998; Vermeulen, Ubbink, Goossens, De Vos, & Legemate, 2005). However, so far the exact mechanism of anti-inflammatory action of honey has not been illustrated (Hadagali & Chua, 2014). The wound treatment therapy aims to kill the infectious microorganisms existing in the wound, as well as to release any dead tissues that may provide microorganisms with a favorable environment. Inflammation not only results in the wound uncomfortable making it difficult to manage, but also prevents the repairing processes of wound healing (B Benhanifia, Boukraâ, M Hammoudi, A Sulaiman, & Manivannan, 2011; Song & Salcido, 2011). The hypotheses of decreased inflammation after honey application can be summarized by attributing to the antibacterial activity of honey or a direct anti-inflammatory effect. The direct anti-inflammatory effects have been observed in animal trials, where anti-inflammatory effects occur after application of honey in wounds excluding bacterial infection (N. Al-Waili & Saloom, 1999; Dunford, Cooper, Molan, & White, 2000; Oryan & Zaker, 1998; Subrahmanyam, 1998).

Previous researches have found that honey reduces cytokine, a type of small protein that can influence other cells, NO levels, and leukocyte (the cells of the immune system protecting the body against both infectious diseases and foreign invaders) numbers, , and increases heme oxygenase-1 levels, an enzyme that catalyzes the degradation of heme, which could support the hypothesis that honey has a direct anti-inflammatory effect and can be applied as a natural compound for the treatment of a wide range of inflammatory diseases (Kassim et al., 2012; Subrahmanyam, 1998). The presence of slough in wounds has been verified to act an inflammatory stimulus (Efem, 1988). Honey can swiftly clean the wound, relieve the pain, and decrease the exudation and edema of wounds to maintain a hygienic condition in the inflamed area by removing the dead tissue and killing the bacteria. Also, honey can promote the growth of different kinds of cell and tissue, consisting of new tissues, and decrease scarring (A. K. J. Ahmed, Hoekstra, Hage, & Karim, 2003; Alcaraz & Kelly, 2002; Bashkaran et al., 2011; Burlando, 1978; P. C. Molan, 2011; Subrahmanyam, 1993). In addition, honey is suggested to have an inhibitory effect on prostaglandin synthesis, which is responsible for the observed pain, itchiness and specific heat commonly related to inflammation (Kassim, Achoui, Mansor, & Yusoff, 2010). According to the study conducted by N. S. Al-Waili and Boni (2003), 12 individuals after 15 days daily ingestion of 250 ml of water containing 1.2 g/kg body weight of natural and unprocessed honey, the mean plasma concentration of thromboxane B_2 was reduced by 7%, 34% and 35%, and that of prostaglandin E_2 (PGE_2) by 14%, 10% and 19% at 1-, 2- and 3-hour respectively after honey ingestion. The level of Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) was decreased by 31% at 2 hours and 14% at 3 hours after honey ingestion. At day 15, plasma concentrations of

thromboxane B_2, PGE_2 and PGF_{2a} were decreased by 48%, 63% and 50% respectively. It might be concluded that honey could lower the concentrations of prostaglandins in plasma of normal individuals.

1.9.3 Nutrition and Health Effects of Honey

1.9.3.1 Oral Health

The debates regarding whether honey is detrimental to teeth are still controversial. In some cases, honey demonstrates a cariogenic effect or a far less cariogenic effect than sucrose (Bowen & Lawrence, 2005; Shambaugh, Worthington, & Herbert, 1989). However, honey intake may exert an inhibitory effect on the growth of bacteria (inducing caries) because of its antibacterial activity, and may contribute to protection against caries (Sela, 1998; Steinberg, Kaine, & Gedalia, 1996). Electron microscope studies have shown that honey ingestion induces only the feeble erosion of tooth enamel after 30 minutes, compared to fruit juice consumption leading to tooth erosion after 10 minutes (Grobler, Du Toit, & Basson, 1994).

1.9.3.2 Gastroenterology

The usage of honey as treatments for a number of gastrointestinal disorders such as diarrhea, peptic ulcers, gastritis and gastroenteritis has been reported in numerous publications. Honey is an effective inhibitor for peptic ulcers, gastritis and *Helicobacter pylori* (Al Somal et al., 1994; AT Ali, Chowdhury, & Al Humayyd, 1990). In animal

trials, honey has shown a potent effect on gastric ulcers caused by indomethacin and alcohol in rats (ATM Ali, 1995). The indomethacin-induced gastric lesions in rats could be relieved after honey intake (Nasuti et al., 2006). Postulated mechanism is that this effect could be attributed to antioxidant actions of honey, suggesting that honey plays an essential role in reducing the ulcer index, microvascular permeability, and myeloperoxidase activities of the stomach (Nasuti et al., 2006). Other functions of honey have been found to have maintenance effects on the level of non-protein sulfhydryl compounds (e.g. glutathione) in gastric tissue suffered from factors causing ulceration, and to prolong emptying process of saccharides in stomach after intake of honey compared with that after intake of a mixture of glucose and fructose (Al-Swayeh & Ali, 1997; ATM Ali, 1995; A. T. Ali et al., 1997; Pokorn & Vukmirovic, 1979). Furthermore, oligosaccharides in honey are beneficial to human digestion, which is supposed to have prebiotic effects similar to that of fructooligosaccharides (Sanz et al., 2005; Yun, 1996). Previous studies have verified that honey could promote the growth of five bifidobacteria strains *in vitro* similar to that of fructose and glucose oligosaccharides, and increase the building of *Lactobacillus acidophilus* and *Lactobacillus planetarium* compared with sucrose with no effects both *in vivo* and *in-vitro* (Shamala, Shri Jyothi, & Saibaba, 2000; Shin & Ustunol, 2005). In clinical studies with regard to infants and children, honey could decrease the duration of bacterial diarrhea, while had no effects on the length of non-bacterial diarrhea. These properties determine that honey has a mild laxative effect in individuals with the insufficient absorption of honey fructose, and can be used as a treatment for constipation (Haffejee & Moosa, 1985; Ladas, Haritos, & Raptis, 1995).

1.9.3.3 Cardiovascular Health

Honey plays a role in reduction of cholesterol, low-density lipoprotein-cholesterol (LDL-C), and triglycerides (TG), and slightly elevated high-density-lipoprotein-cholesterol (HDL-C), while artificial honey (fructose and glucose) slightly decreases cholesterol and LDL-C, and dramatically elevates insulin, C-reactive protein, and TG, the main risk factors for cardiovascular diseases (N. S. Al-Waili, 2004). In addition, honey contains NO which may act a protective function in cardiovascular diseases. In clinical studies, salivary, plasma and urinary NO metabolite concentrations showed an increasing tendency after ingestion of honey. However, concentrations of NO metabolites in honey could be influenced by honey varieties, dark or fresh honey always containing more NO metabolites than light or stored honey, while all honey types showed a reduction tendency in NO metabolites concentration after heating (N. S. Al-Waili, 2003b; N. S. Al-Waili & Boni, 2004).

1.9.3.4 Physiological Effects

Animal experiments have indicated that honey exerts an effect on the antibody production against a thymus-dependent antigen in sheep red blood cells and thymus-independent antigen in mice, and demonstrated that honey possesses an immunosuppressive activity. These features may be significant contributors to relieve pollen hypersensitivity (N. S. Al-Waili & Haq, 2004; Duddukuri, Kumar, Kumar, & Athota, 1997). In another study, individuals received a daily oral intake of 1.2 g/kg body weight of honey. The results showed that in blood serum monocytes, iron and copper

increased by 50%, 20% and 33% respectively, while lymphocyte and eosinophil percentages, magnesium, zinc, hemoglobin and packed cell volume increased slightly. In contrast, fasting sugar (5%), ferritin (11%), alanine transaminase (18%), aspartate transaminase (22%), immunoglobulin E (34%), creatine kinase (33%) and lactic acid dehydrogenase (41%) depicted a reducing tendency (N. S. Al-Waili, 2003a).

1.9.3.5 Different Health Enhancing Effects

Honey is reported to have a positive effect on hepatitis A patients through reducing the alanine aminotransferase activity by 9 to 13 times, and decreasing bilirubin production by 2.1 to 2.6 times (Baltuskevicius, Laiskonis, Vysniauskiene, Ceksteryte, & Racys, 2001). Honey has been found to reduce the incidence of radiation mucositis amongst patients undergoing cancer radiation therapy, and to decrease the need for colony stimulating factors amongst chemotherapy patients with neutropenia. Chemotherapy may contribute to a serious side effect febrile neutropenia (Biswal, Zakaria, & Ahmad, 2003; Zidan et al., 2006).

1.9.3.6 Allergy

Honey allergy is reported rarely and uncommonly, which includes reactions varying from a cough to anaphylaxis as represented symptoms. Previous studies showed that the incidence of honey allergy to the presence of components of bee origin amongst 173 food allergy patients was 2.3% (Bousquet, Campos, & Michel, 1984; Helbling, Peter,

Berchtold, Bogdanov, & Müller, 1992; Sirnik, Koch, & Golob, 1979).

1.9.3.7 Infants

Honey has a number of benefits to babies. Previous studies showed that infants were fed on honey had better blood formation, better skin color, shorter crying phases, and a higher weight gain than those were fed without honey or with sucrose. When infants on a diet with honey rather than with sucrose, an increase of hemoglobin content, and no digestion problems were observed (Frauenfelder, Krauthammer, Göldi, & Frey, 1921; Ramenghi, Amerio, & Sabatino, 2001; Takuma, 1955; Tropp, 1957). Infants were fed on a mixture of honey and milk had an improved calcium uptake, a regularly steady weight increase, an acidophilic microorganism flora rich in *B. Bifidus*, lighter and thinner faeces, suffered less frequently from diarrhoea, and more haemoglobin in blood compared with those fed by sucrose-sweetened milk (Bianchi, 1977; Rivero-Urgell & Santamaria-Orleans, 2001; Takuma, 1955). These benefits to infants of honey are derived from effects on the digestion process, which are attributed to the effects of oligosaccharides on *B. Bifidus* (Rivero-Urgell & Santamaria-Orleans, 2001). However, honey may contain dormant endospores of the *Clostridium botulinum*, a type of bacterium being harmful to infants, which can convert into toxin-producing bacteria causing illness and even sometimes death in the immature stomach and intestinal tract of infants. It is ubiquitous that *Clostridium botulinum* exists in natural food. Honey is just a non-sterilized packaged food from natural origin. The risk of a low contamination level of this bacterium seems to be inevitable. Hence, infants under 12 months of age

are advised to avoid feeding with honey (Caya et al., 2004; Shapiro et al., 1998).

1.9.3.8 Athletic Performance

Honey can be a potent carbohydrate source for athletic performance. Previous studies confirmed that honey increased the heart frequency and the blood glucose level dramatically of the athletes (Kreider, Rasmussen, Lancaster, Kerksick, & Greenwood, 2002).

1.10 Antioxidant Capacity Assays

The antioxidant effects of honey can be tested and evaluated by a range of assays such as ABTS/ TEAC (2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid/ Trolox equivalent antioxidant capacity), DPPH (2, 2-di (4-tert-octylphenyl)-1-picrylhydrazyl), ORAC (oxygen radical absorbance capacity), FRAP (ferric reducing antioxidant power), CUPRAC (Cupric ion reducing antioxidant capacity), and Folin-Ciocalteau total phenolics assays (Özyürek et al., 2011). These assays can be traditionally divided into two categories: Hydrogen atom transfer reactions (HAT) and electron transfer reactions (ET) (Prior, Wu, & Schaich, 2005).

1.10.1 Hydrogen Atom Transfer Assays

The antioxidant capacity assays on the basis of hydrogen atom transfer reactions are based on the reactions similar to the primary antioxidant mentioned in section 1.5.1.

These assays aim to assess the capacity of an antioxidant (AH) to scavenge free radicals by measuring the stable compounds formed by the reactions between antioxidant (AH) and the radicals ($X^{\bullet}$) as shown in the equation below.

$X^{\bullet} + AH \rightarrow XH + A^{\bullet}$

The reagents in hydrogen atom transfer reactions based assay are characterized by reacting rapidly (seconds to minutes), as well as solvent- and pH-independent. The relative reactivity of this assay is contingent on the energy derived from dissociating the H-donating group and ionizing antioxidant. The antioxidant reactivity hinges on the competition kinetics of the antioxidants and the added oxidisable probe for the synthetic peroxyl radicals. In the ORAC assay, for example, peroxyl radicals can be generated thermally by decomposing azo compounds, including ABAP (2, 2'- azo-bis (2-aminopropane) dihydrochloride), while fluorescein serves as the probe. Antioxidants in the sample interrupt the peroxyl radical-mediated oxidation of fluorescein. When all of the antioxidants are exhausted, the reactions between the remaining peroxyl radical and the fluorescent probe will occur and form a non-fluorescent probe. The area under the fluorescence decay curve can reflect the antioxidant capacity, which is plotted by subtracting the area under the curve of the blank from the sample containing antioxidant and fluorescence after the reactions (Fung, 2012; Prior et al., 2005).

1.10.2 Electron Transfer Assays

The antioxidant capacity assays on the basis of electron transfer reactions measure the decrease of compounds, including radicals, carbonyls and metals (M) in the sample by

transferring an electron from potential antioxidants (AH).

M (III) + AH -> $AH^{\bullet}$ + M (II)

The relative reactivity of the electron transfer reactions based assay hinges on the ionization potential of the reactive functional group and deprotonation, which lead to the reaction to be pH-dependent. The electron transfer reactions based assay is suggested to be slower than the hydrogen atom transfer reactions based assay, within minutes to hours to attain completion of reactions. The antioxidant capacity is quantified by the rate of reduction in the product rather than on the basis of kinetics in the hydrogen atom transfer reactions based assay. The majority of electron transfer reactions based assay is characterized by the color changes which can be measured by a spectrophotometer. Different types of probe applied in the assay results either in an ascension or a descension of absorbance after reactions, while the changes of color are associated with the concentration of antioxidant applied (Fung, 2012; Prior et al., 2005).

This research focused on DPPH assay and CUPRAC assay as the main measurements of antioxidant effects of Manuka honey and thyme honey.

1.10.2.1 CUPRAC Assay and Its Limitations

CUPRAC assay as the abbreviation of cupric ion reducing antioxidant capacity was firstly proposed by Özyürek et al. in 2004. It is a variant derived from the FRAP assay, which replaces Fe^{3+} with Cu^{2+}. CUPRAC assay has been widely applied in measurement for the antioxidant capacity of fruits, vegetables and plants (Reşat Apak et al., 2007; S

Esin Çelik et al., 2008; Guclu, Altun, Ozyurek, Karademir, & Apak, 2006; KÖKSAL & GÜLÇİN, 2008).

As can be seen in Figure 4, in the CUPRAC assay, the chromogenic oxidizing reagent is the bis (neocuproine (2, 9-dimethyl-1, 10- phenanthroline)) copper (II) cation (Cu (II)-Nc) serving as an out-sphere electron-transfer agent, and forms a color compound, the bis (neocuproine) copper (I) cation (Cu (I)-Nc) (Cu (I)-chelate) which is the CUPRAC chromophore formed by redox reaction with a reducing antioxidant. This reaction requires pH 7, and the absorbance should be measured at 450 nm. The CUPRAC reactions should be completed within 30 minutes (Özyürek et al., 2011).

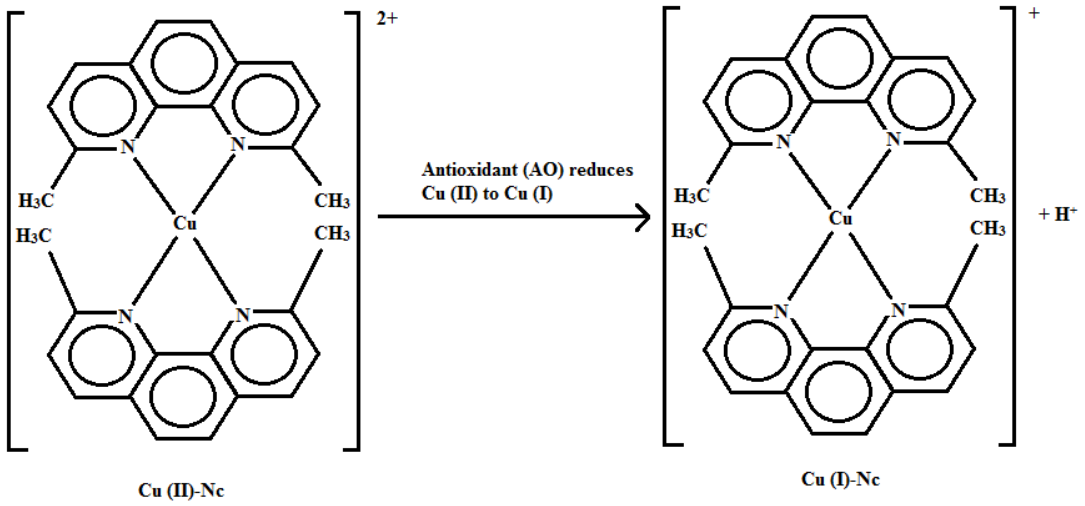


Figure 4. Reactions in CUPRAC assay. Redrawn from Özyürek et al. (2011)

The CUPRAC reagent, bis (neocuproine) copper (II) chloride (Cu (II)-Nc), reacting with *n*-electron reductant antioxidants (AO) is shown as the formula below (Özyürek et al., 2011).

$nCu\ (Nc)_2^{2+}$ + n-electron reductant -> $nCu\ (Nc)_2^{+}$ + n-electron oxidized product + nH^{+}

In this reaction, bis (neocuproine) copper (II) chelate oxidizes the reactive reducing antioxidants to the corresponding oxidized product forming a highly colored Cu (I)-Nc chelate.

CUPRAC assay has been suggested to be a favorable replacement for FRAP assay due to the following reasons (Reşat Apak et al., 2007; Resat Apak, Güçlü, Özyürek, & Karademir, 2004; Guclu et al., 2006). Firstly, the 0.6 V standard redox potential of Cu (II/ I)-neocuproine is much lower than Fe^{3+}-TPTZ with a corresponding potential of 0.7 V, suggesting that CUPRAC assay is able to assess a wider range of antioxidant compounds, including glutathione, the compound undetectable with FRAP assay (S Esin Çelik et al., 2008). Secondly, the iron reaction kinetics are acknowledged to be slower than that of copper with a duration of 30 minutes (Resat Apak et al., 2004). Thirdly, the measurement of CUPRAC contains more selections about antioxidants. It has been suggested that relative reagents used in CUPRAC assay not react with the compounds (e.g. reducing sugars and citric acid) that can be oxidizable substrates in other similar assays (Prior et al., 2005). Fourthly, the pH value of the redox reactions for CUPRAC is pH 7, which is close to physiological pH. Compared with FRAP assay requires the acidic pH (pH 3.6), which is probably to interfere the reducing capacity of protonation on antioxidant compounds. Fifthly, CUPRAC assay can measure both hydrophilic and lipophilic antioxidants simultaneously (Reşat Apak et al., 2007; Resat Apak et al., 2004).

CUPRAC assay, a colorimetric assay used to evaluate polyphenolic antioxidants, have been extensively applied to analyze polyphenol (Reşat Apak et al., 2007; Resat Apak et al., 2004; S Esin Çelik et al., 2008). Previous studies have shown that reagents used in CUPRAC assay are able to oxidize phenolic hydroxyl groups of antioxidants to their corresponding quinones, as a result of which, this assay is suitable for analysis of total antioxidant capacity in honey (S Esin Çelik et al., 2008). This is because polyphenols are abundantly contained in different types of honey as mentioned in section 1.4.

However, the limitation is that when the CUPRAC assay is used to measure a complex mixture of antioxidants, it is hard to select an appropriate reaction time to complete their oxidation with the CUPRAC reagent (Karadag, Ozcelik, & Saner, 2009).

1.10.2.2 DPPH Assay and Its Limitations

As for DPPH (1,1-Diphenyl-2-picryl-hydrazyl) assay, it was first suggested to measure antioxidant effect more than 50 years ago (Papariello & Janish, 1966). DPPH assays were initially categorized as an electron transfer reactions based assay with a marginal reaction that occurred very slowly determined by the solvent (e.g. methanol, ethanol and acetone) (Huang, Ou, & Prior, 2005). However according to the evidence showed in the subsequent studies, DPPH assays are also probably to act through the mechanism of hydrogen atom transfer reactions based assay. It is considered to have both hydrogen atoms transfer reactions and electron transfer reactions mechanisms due to the difficult

explanation of inhibitory mechanisms of the DPPH radicals without knowledge regarding the structures and compositions of antioxidant samples (Prior et al., 2005). Other electron transfer reactions based assays like Folin-Ciocalteu Reagent (FCR) and Trolox equivalent antioxidant capacity (TEAC) also demonstrate the same phenomenon (Karadag et al., 2009).

DPPH assay is on the basis of measuring the scavenging capacity of antioxidants towards it (Kedare & Singh, 2011). DPPH (2, 2-diphenyl-1-picrylhydrazyl or 1, 1-diphenyl-2-picrylhydrazyl) (Figure 5) radical, one of the few stable organic nitrogen-centered free radical, possesses a deep purple color, and can be potently scavenged by antioxidants.

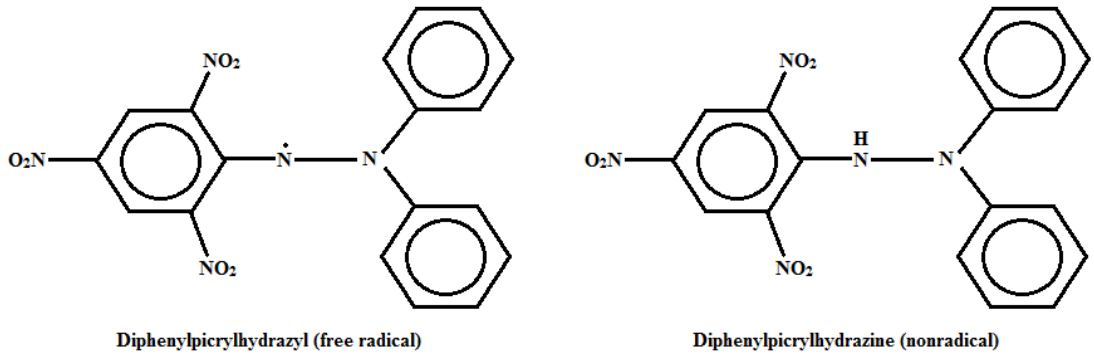


Figure 5. Structures of DPPH. Redrawn from Kedare and Singh (2011)

Antioxidants are able to decolorize the deep purple color of DPPH. The scavenging free radical capacity of the tested antioxidants can be measured by using a spectrophotometer at 517 nm (Fung, 2012). The antioxidant molecules (AH) can donate a hydrogen atom to DPPH ($Z^{\bullet}$), giving rise to the reduced form with the loss of violet color of DPPH ($Z^{\bullet}$) as shown in the equation below.

$Z^{\bullet} + AH \rightarrow ZH + A^{\bullet}$

In this reaction, the free radical (A•) will then undergo further reactions which control the overall stoichiometry, which means that a link can be provided to the reactions taking place in an oxidizing system (e.g. the autoxidation of a lipid or other unsaturated substances). Thus, the DPPH molecules (Z•) will represent the free radicals formed in the system. Its activity can be suppressed by antioxidant molecules (AH) (Kedare & Singh, 2011).

DPPH assay has been extensively applied to quantify antioxidant activity because it is simple and inexpensive, as well as can be conducted in the most rudimentary laboratories (Xie & Schaich, 2014). It can also be used to quantify antioxidants in complex biological systems, for solid or liquid samples. This method can be applied not only to measure the overall antioxidant capacity, but also to measure the free radical scavenging activity (Prakash, Rigelhof, & MIller, 2001; Sendra, Sentandreu, & Navarro, 2006).

Nonetheless, one of the most significant limitations of DPPH assay is that DPPH can only be soluble in organic solvent media, so the determination of hydrophilic antioxidants seems to be inaccessible (Arnao, 2000). DPPH assay has been reported to be much affected by light, oxygen, pH, temperature and type of solvent (Xie & Schaich, 2014). In addition, moderate-to-slow reaction with DPPH is reported to be shown by flavonoids and other complex phenols (Özyürek et al., 2011). A number of researches have been conducted with different concentrations of DPPH, incubation time, reaction solvents, and pH of the reaction mixture, which illustrate that high concentration of

DPPH seems to give rise to absorbance exceeding the accuracy of spectrophotometric measurements (Sharma & Bhat, 2009). DPPH is also reported to be sensitive to some Lewis bases, solvent types, and oxygen (Ancerewicz et al., 1998). DPPH dissolved in methanol and acetone under light results in a decrease of the absorbance (Min & Boff, 2002).

1.11 Caco-2 Cell Line as a Model of Colon Cancer

The Caco-2 cell line was originally isolated from colorectal adenocarcinoma in a human. This cell line has been widely applied in the screening of cytotoxic effects of anti-tumor drugs, and in the study of drug resistance mechanisms, as well as cellular permeability studies of polyphenols (Deprez, Mila, Huneau, Tome, & Scalbert, 2001; Konishi & Kobayashi, 2004; Manna et al., 2000; Manzano & Williamson, 2010; Zucco et al., 2005). Despite the fact that this cell line retains some undesirable colon characteristics (e.g. low paracellular permeability, and the absence of mucus), they still possess a range of pivotal morphological and functional attributes of the small intestinal epithelium, including cell polarization, expression of some brush-border enzymes, and formulation of microvilli (Schulz, 2011).

The absence of a mucus layer differentiates the Caco-2 cells from the intestinal enterocytes. Multiple transporters, receptors and metabolic enzymes, including cytochrome P450 1A (CYP1A), sulfotransferases (SULTs), UDP-glucuronosyltransferases (UGTs), and glutathione S-transferases (GSTs) can be

attached to the Caco-2 monolayer (H. Sun, Chow, Liu, Du, & Pang, 2008).

1.12 MTT Cell Proliferation Assay and Its Limitations

The MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) cell proliferation assay is the first assay developed for the 96-well plate, which is suitable for high throughput screening (HTS). MTT cell proliferation assay was first introduced by Mosmann in 1983. It is presented as a rapid, precis and simple method to evaluated living cells in mammalian cell cultures, and has been widely applied in the researches concerning chemosensitivity, radiosensitivity and toxicity of drugs in human tumor cell lines (Mosmann, 1983). MTT is a kind of yellow and water-soluble tetrazolium salt, which can be cleaved by mitochondrial dehydrogenase enzymes in living cells. Only viable cells are able to transform MTT into an insoluble formazan precipitate through the reductive cleavage of the tetrazolium ring of MTT by the succinate dehydrogenase system of active mitochondria (T. Slater, Sawyer, & Sträuli, 1963). The quantity of formazan created in this reaction is directly proportional to the number of viable cells, while it can be influenced by the factors such as the concentration of MTT, the duration of incubation, and the number of viable cells. The formazan crystals can be dissolved in plenty of organic solvents such as DMSO (Dimethyl sulfoxide). The absorbance should be determined at a wavelength of 540 nm, as well as a reference wavelength of 680 nm.

However, MTT assay also has some limitations. Particularly, poor linearity with the cell number and sensitivity to environmental conditions. The precipitation of serum proteins

can be formed after the addition of the organic solvents to media that contains serum, which is possible to interfere with absorbance readings. The 4 hours' incubation after addition of MTT may cause low cell densities or cells with lower metabolic activities. A few formazans may be carried off when the process of discarding the culture, which may interfere with the experiment results (Mao et al., 2013). Furthermore, MTT assay can be influenced by other conditions such as acidic pH, polyphenols, pyruvate analog, and nanomaterials (Ganapathy-Kanniappan et al., 2010; Gormley & Ghandehari, 2009; Han, Li, Tan, Sun, & Wang, 2010; Johno, Takahashi, & Kitamura, 2010).

1.13 Objectives

Honey shows health-promoting activity by a combination of various therapeutic effects such as antioxidant, antibacterial and anti-inflammatory activities, which have been documented and published. Nevertheless, few studies were conducted regarding the comparison of antioxidant and anticancer activities between New Zealand Manuka honey and Thyme honey, as a result of which, completing this project is of great importance to bridge the gap between theory and truth in this field. The aims of this study were : (i) to measure and compare the antioxidant activity of Manuka honey and Thyme honey by using DPPH assay and CUPRAC assay; (ii) to examine and compare the anticancer activity of Manuka honey and Thyme honey in human colon cancer Caco-2 cells; (iii) to examine the potential correlation between the antioxidant activity and anticancer activity of tested honeys.

Chapter 2: Materials and Methods

2.1 Materials

Methanol (FSBA452-4), ethanol (BSPEL975.2.5), syringe filter (NTSF2500-3), and cuvette (LPI111117) were purchased from Thermo Fisher Scientific (www.thermofisher.co.nz). DPPH (D9132), ascorbic acid (795437), copper (II) chloride (203149), ammonium acetate (A1542), neocuproine (N1501) were purchased from Sigma-Aldrich (www.sigmaaldrich.com). Manuka honey (UMF 15 +) and Thyme honey were obtained from VITABEEZ (www.vitabeez.co.nz).

All other reagents, solvents, chemicals, and facilities applied in the experiments were provided by the Applied Sciences Laboratory in Auckland University of Technology (AUT).

2.2 DPPH Assay

Two different variables were applied in the experiments. The first one aimed to measure the changes of scavenging capacity of honey according to concentrations of two types of honey. The second one concentrated on evaluating the changes of scavenging capacity of honey according to reaction time.

2.2.1 Scavenging Capacity versus Reaction Time

Preparation of Honey Stock Solution

The original stocks of Manuka honey (concentration of 0.04 g/ml) were prepared by dissolving 0.64 g of Manuka honey in 16 ml of distilled water and methanol respectively. The working stock solutions were then prepared by filtering the original stock solutions through syringe filters. The working stocks were diluted with the same solvent (water or methanol) to obtain the working solutions at concentration of 0.04, 0.02, and 0.01 g/ml. Similarly, Thyme honey working solutions in water and methanol were prepared to obtain the concentration of 0.02, 0.01, and 0.005 g/ml.

Preparation of Ascorbic Acid Standard Solution

The ascorbic acid standard solution was prepared by dissolving ascorbic acid in distilled water and methanol respectively to gain the concentration of 100 μM (17.612 μg/ml).

DPPH Assay

The free radical scavenging capacity of Manuka honey and Thyme honey was determined by using DPPH (Duan, Zhang, Li, & Wang, 2006). A DPPH solution at concentration of 63.1 μg/ml was prepared by dissolving 0.0033 g of DPPH in 50 ml of absolute methanol. The reaction was initiated by mixing 1 mL of freshly prepared DPPH with 1 mL of the honey solution in a cuvette by properly pipetting. The scavenging free radical capacity of honey solution was measured at 517 nm by using a spectrophotometer (ULTROSPEC 7000). The antioxidant activity of honey was measured at 0, 10, 20, 30, 40 and 60 min after reaction. The control group was prepared to contain the same volume of DPPH mixed with the corresponding solvent (water or methanol). Sample blank was prepared to contain 2 ml of absolute methanol. Absolute methanol of 2 ml was used to zero the

spectrophotometer as the blank. Percentage scavenging of the DPPH free radical was calculated using the following equation:

Scavenging (%) = $\left[1 - \left(\frac{Abs_{sample} - Abs_{sampleblank}}{Abs_{control}}\right)\right] X 100$

2.2.2 Scavenging Capacity versus Concentrations of Honey

Preparation of Honey Stock Solutions

The original stocks of Manuka honey (concentration of 0.04 g/ml) were prepared by dissolving 0.64 g of Manuka honey in 16 ml of distilled water and methanol respectively. The working stock solutions were then prepared by filtering the original stock solution through syringe filters. The working stocks were diluted with the same solvent (water or methanol) to obtain the working solutions at concentrations of 0.04, 0.03, 0.02, 0.01, 0.005, 0.0025 and 0.00125 g/ml. Thyme honey was prepared by using the same protocol.

Preparation of Ascorbic Acid Standard Solution

The ascorbic acid standard solution was prepared by dissolving ascorbic acid in distilled water and methanol respectively to gain the concentrations of 0.04, 0.03, 0.02, 0.01, 0.005, 0.0025 and 0.00125 μg/ml.

DPPH Assay

Same as the "DPPH assay" in section 2.2.1. The absorbance was determined at 30 minutes after reaction.

2.3 Methods for CUPRAC Assay

Preparation of Stock Solution

The original stocks of Manuka honey in water and methanol was prepared by dissolving 1.312 g of Manuka honey in 16 ml of distilled water and methanol respectively. The working stocks were then obtained by filtering the original stocks through syringe filters to give a concentration of 0.082 g/ml. The working stock solutions was further diluted by the same solvent (water or methanol) to obtain working solutions at the concentrations of 0.082, 0.0615, 0.041, 0.0205, 0.01025, 0.005125 and 0.0025625 g/ml. Thyme honey was prepared by using the same protocol.

Preparation of Other Solutions

Ascorbic acid was prepared by dissolving in distilled water and methanol respectively to gain the concentration gradients of 82, 61.5, 41, 20.5, 10.25, 5.125 and 2.5625 μg/ml. The copper (II) chloride ($CuCl_2$) of 0.2151 g and ammonium acetate (NH_2Ac) (pH 7) of 12.3328 g was dissolved in 160 ml distilled water as stock solutions to give the concentration of 10 mM and 1 M respectively. Neocuproine of 0.125 g was dissolved in 80 ml absolute ethanol to gain the concentration of 7.5 mM (Saliha Esin Çelik, Özyürek, Güçlü, & Apak, 2010).

CUPRAC Assay

An aliquot of 1mL each of Cu (II), Nc, and NH_2Ac buffer solutions were added to a cuvette. Honey solution (1.0 mL) and distilled water (0.1 mL) were added to the initial mixture to reach the final volume of 4.1 mL. After mixture with gently pipetting, the

absorbance was determined at 450 nm after 30 min incubation at room temperature by using the spectrophotometer (ULTROSPEC 7000), which was zeroed by a cuvette containing 4.1 ml of distilled water. The standard calibration curves of each antioxidant compound were plotted as Absorbance vs. Concentration. The CUPRAC molar absorptivity of each antioxidant was found from the slope of the calibration line concerned. The scheme for normal measurement of antioxidants is summarized as:

Cu (II) (1 mL) + Nc (1 mL) + NH_2Ac buffer (1 mL) + Sample (1 mL) + H_2O (0.1 mL)

2.4 Methods for Cell Viability MTT Assay

2.4.1 Cell Culture

The old culture medium was discarded carefully with the aid of a pipette. After washing Caco-2 cells with 5 mL of sterile pre-warmed PBS (Phosphate buffer saline), 1 ml of trypsin was added into culture flask. The flask was then kept in cell incubator for around 15 min to detach cells. An aliquot of 4 ml of completed medium was then added to stop trypsinization. All the mixture in the flask was transferred to a 15 ml centrifuge tube and centrifuged for approximately 5 min. The supernatant was then carefully removed, and 1 ml of completed medium was added to re-suspend Caco-2 cells.

2.4.2 Cell Counting

10 µL of cell suspension was mixed with 10 µL of Trypan Blue on a piece of parafilm. 10 µL of the mixture was then added to a hemocytometer. After counting the cells in 4 square

areas, the total number of cells in 1 ml culture medium was calculated by the following equation:

$$\text{Number of cells/ml} = \text{Average number of cells per square} \times 2 \times 10^4$$

2.4.3 Seeding Cells

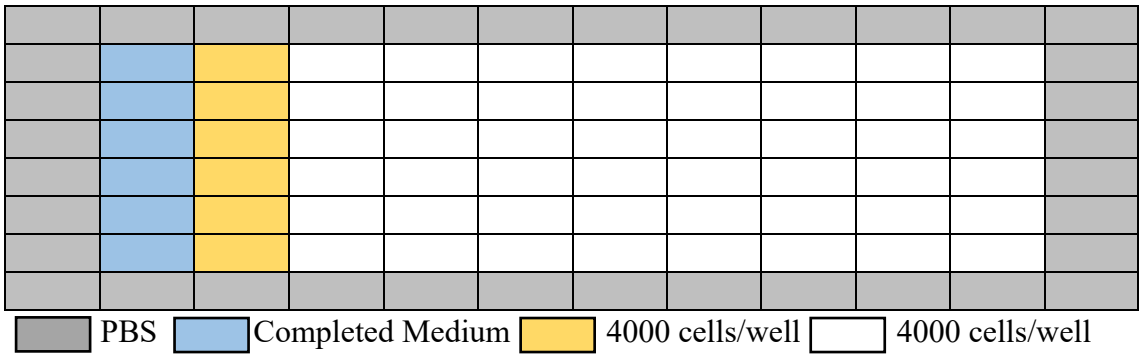


Figure 6. Seeding plan of Caco-2 cell line.

As shown as in figure 6, gray wells were loaded with 200 µL of PBS and blue wells 200 µL of completed medium as blank group. While yellow wells seeding 200 µL of cancer cells (the density of 4000 cells/well) were used as control group (no honey treatment), white wells seeding cancer cells at the same density were used to test the anti-proliferative effects of honey at various concentrations and at different time periods. Given the minor effects of tested honey determined at 24 hours in our preliminary studies, the anti-proliferative effects of honey were further investigated only at 48 and 72 hours.

2.4.4 Adding Honey as the Treatment

A fresh stock solution of Manuka honey or Thyme honey was prepared by dissolving 1.2 g of honey in 6 ml of completed medium respectively. After filtration through sterile

syringe filters, the stock solution (0.2 g/ml) was diluted by completed medium to obtain working stock solution at concentrations of 0.2, 0.15, 0.1, 0.05, 0.025, 0.0125 and 0.00625 g/ml. After cell seeding onto the 96-well plate for 24 hours, an aliquot of 100 µL of honey solution at various concentrations was added into each well in 96-well plate.

2.4.5 MTT Assay

After adding honey as the treatment for certain incubation time, all the supernatant were replaced with 100 µL of new completed medium. 10 µL of MTT was then added to each well and incubated for approximately 4 hours at 37 °C and 5% CO_2 until the purple precipitate was clearly visible. Then 80 µL of supernatant was removed, and 150µL of DMSO was added to each well to dissolve the purple precipitate. After shaking for 5 min, the absorbance (OD value) was measured by a plate reader (FLUOstar Omega, Alphatech) at the wavelength of 540 nm with a reference wavelength of 680 nm.

The OD value-concentration curves can be plotted to reflect the inhibitory effects of Manuka honey and Thyme honey on Caco-2 cell line.

2.4.6 IC_{50} Calculation Method

IC_{50} is the half maximal inhibitory concentration, which is a common parameter of drug effectiveness. The IC_{50} value in this study was calculated by using the Graphpad Prism 6.0. The data were fit into a nonlinear regression dose-response model with variable slope

(four parameters), using the following equation.

$$Y = Bottom + (Top-Bottom)/(1+10^{((LogIC_{50}-X)*H)})$$

Where Bottom and Top stands for the minimum and maximum viability values determined in MTT assay, respectively; X for logarithm concentration; and H for Hill slope.

2.5 Statistic Analysis

The statistical analysis was performed by a one-way or two-way analysis of variance (ANOVA) with a post-hoc test (Tukey's multiple comparison post-tests) by using PRISM® software (Graphpad, Version 6.0). All data analyzed using ANOVAs met the assumptions of equal variance and homogeneity. Student's unpaired t test was conducted for comparisons between two groups with a significance level of $P < 0.05$.

Chapter 3: Results

3.1 The Stability of DPPH Solution Mixed with Distilled Water and Absolute Methanol

The tested honeys were dissolved in distilled water and methanol in this study. Thus, it was essential to explore the stability of DPPH solution (methanol as the solvent) mixed with distilled water (1:1) and absolute methanol (1:1). The absorbance of DPPH solution mixed with distilled water (p-value < 0.05) and methanol (p-value > 0.05) were shown in Figure 7. These results indicated that DPPH was not stable when it was mixed with

distilled water, as a result of which, the measured scavenging capacity of antioxidants dissolved in distilled water would be smaller than the actual. This phenomenon was probably due to the insolubility of DPPH in water mentioned in section 1.10.2.2 (Arnao, 2000). In other words, the free radical scavenging capacity of Thyme honey in different solvents could not be compared by using DPPH assay. In contrast, the absorbance of DPPH in absolute methanol was nearly invariant within 60 minutes, suggesting that DPPH was stable in absolute methanol within this period.

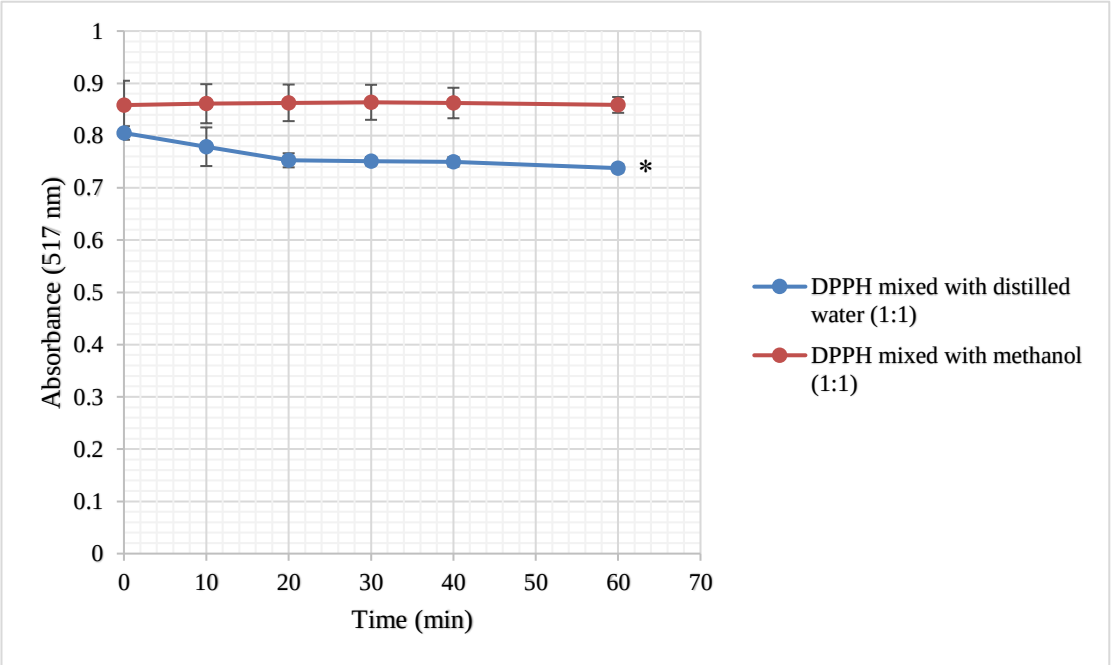


Figure 7. The absorbance of DPPH dissolved in distilled water and methanol. Data are expressed as means ± SD (n = 3) * $P < 0.05$, compared with the values determined for the same solvent at time zero.

3.2 Antioxidant Activity of Manuka Honey and Thyme Honey Tested by DPPH Assay

3.2.1 Time Course of Free Radical Scavenging Capacity

The samples of Manuka honey and Thyme honey dissolved in distilled water and absolute methanol respectively were tested for evaluating the correlation between antioxidant effects of two types of honey and incubation time. The results were shown in Figure 8 to 13.

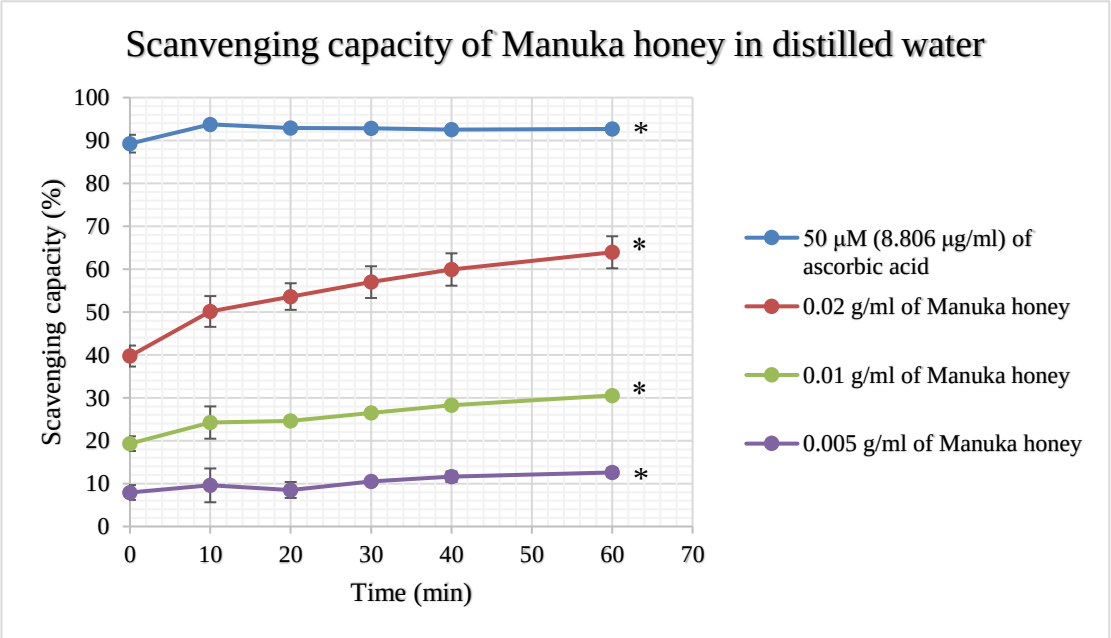


Figure 8. Time-dependent free radical scavenging capacity of Manuka honey dissolved in distilled water. Data are expressed as means ± SD (n = 3) * $P < 0.05$, compared with the values determined for the same concentration at time zero.

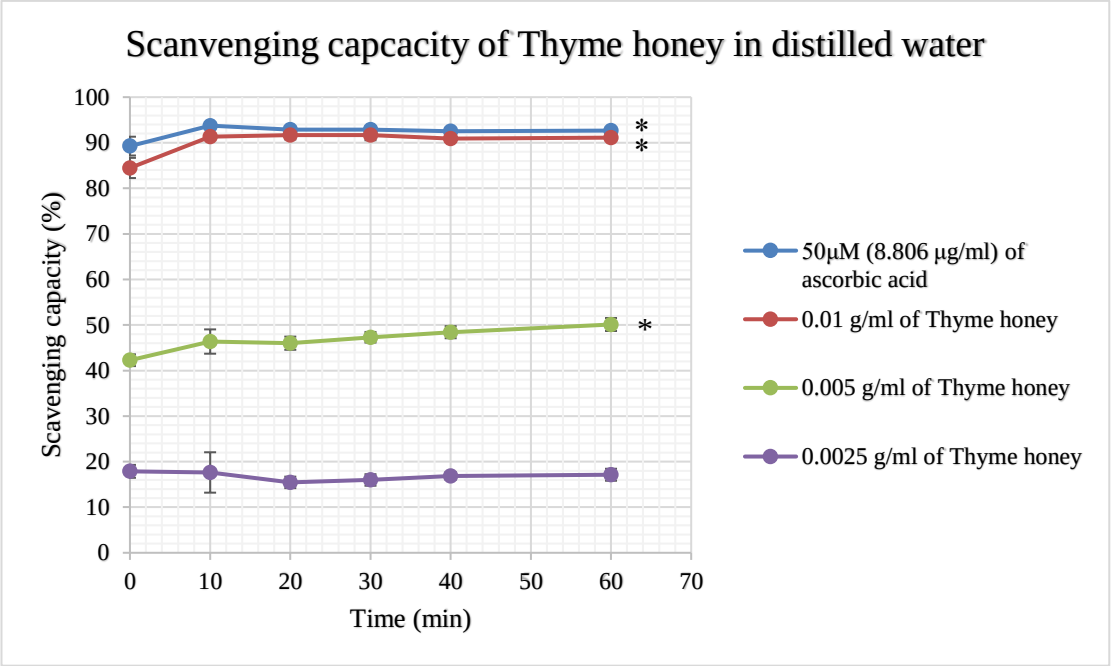


Figure 9. Free radical scavenging capacity of Thyme honey dissolved in distilled water. Data are expressed as means ± SD (n = 3) * $P < 0.05$, compared with the values determined for the same concentration at time zero.

The radical scavenging capacities of both tested honeys (apart from 0.0025 g/ml of Thyme honey (p-value > 0.05)) and ascorbic acid in distilled water appeared to be time-dependent, suggesting that honey as an electron donor could transform DPPH radicals to more stable substances and terminate the further reactions. In general, the scavenging capacity of Thyme honey was greater than Manuka honey at the same concentration and incubation time (Figure 8 and 9). However, the growth rate of scavenging capacity of Manuka honey was greater than Thyme honey at the same concentration within this period. At a time period of 60 minutes, the percentages of scavenging capacity of Manuka honey ranged in concentrations of 0.02, 0.01 and 0.005 g/ml were 63.93%, 30.55% and 12.60% respectively, while those were 91.10%, 50.10% and 17.13% for Thyme honey at concentrations of 0.01, 0.005 and 0.0025 g/ml respectively. At the two mutual concentrations (0.01 and 0.005 g/ml), Thyme honey exhibited much higher scavenging

effects than Manuka honey. Interestingly, even 0.01 g/ml of Thyme honey possessed greater effects than that of 0.02 g/ml of Manuka honey, while a similar condition appeared between 0.0025 g/ml of Thyme honey and 0.005 g/ml of Manuka honey. The increased scavenging capacities of both tested honeys in distilled water appeared to fluctuate slightly from 0 to 30 minutes.

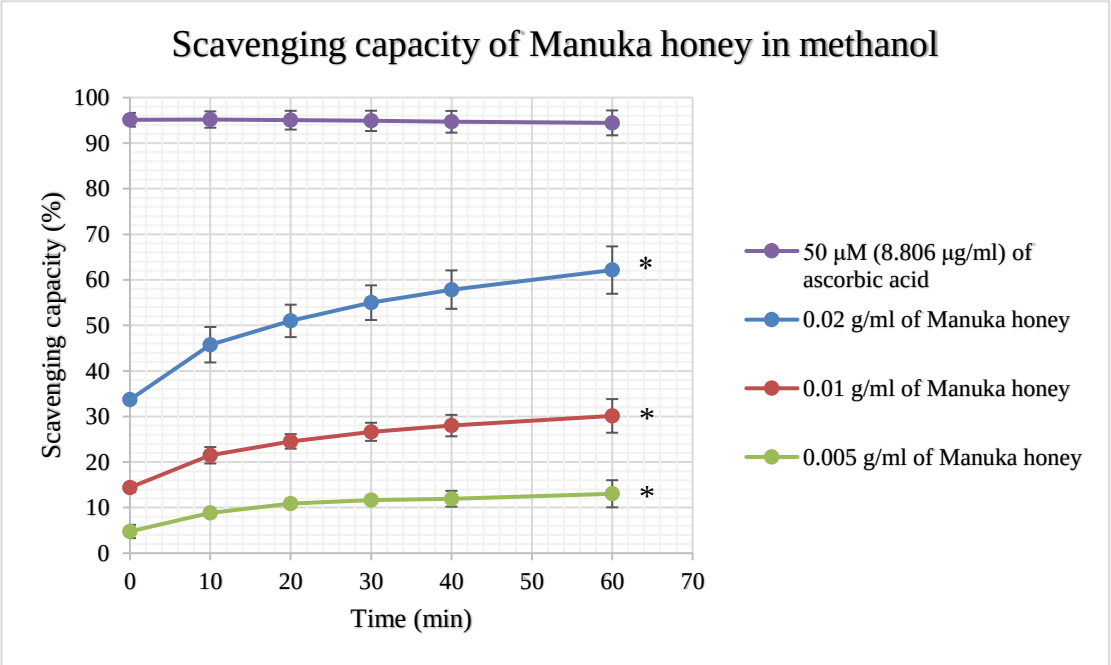


Figure 10. Free radical scavenging capacity of Manuka honey dissolved in methanol. Data are expressed as means ± SD (n = 3) * $P < 0.05$, compared with the values determined for the same concentration at time zero.

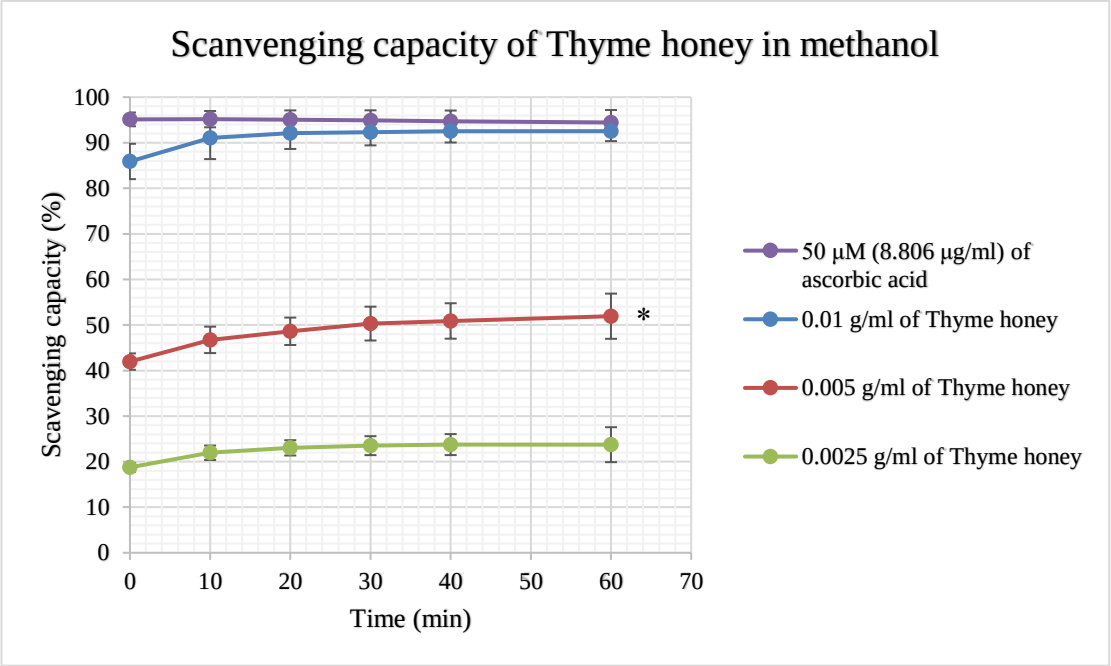


Figure 11. Free radical scavenging capacity of Thyme honey dissolved in methanol. Data are expressed as means ± SD (n = 3) * $P < 0.05$, compared with the values determined for the same concentration at time zero.

When the solvent changed to absolute methanol (Figure 10 and 11), a similar condition occurred as honey in distilled water. The radical scavenging capacity was observed in all tested honeys and ascorbic acid in methanol. Manuka honey (all concentrations) and 0.005 g/ml of Thyme honey appeared to be time-dependent (p-value < 0.05), compared with Thyme honey at concentrations of 0.01 and 0.0025 g/ml exhibited slight increase (p-value > 0.05), and ascorbic acid almost remained stable within this period. The scavenging capacity of Thyme honey was greater than Manuka honey at the same concentration and incubation time, while the growth rate of Manuka honey was higher than Thyme honey at the same concentration within this period. Thyme honey possessed higher scavenging ability than that of Manuka honey at the two mutual concentration levels (0.001 and 0.005 g/ml). The scavenging ability of 0.01 g/ml of Thyme honey

(92.57%) was even much higher than 0.02 g/ml of Manuka honey (53.79%), which was similar to the results of honeys in distilled water.

Interestingly, despite the fact that DPPH was insoluble in distilled water, which could lead to the measured scavenging capacity was smaller than the actual, the scavenging capacity of Manuka honey in distilled water was still higher than in methanol. Figure 12 to 13 depicted the difference of scavenging capacities between Manuka honey and Thyme honey dissolved in different solvents.

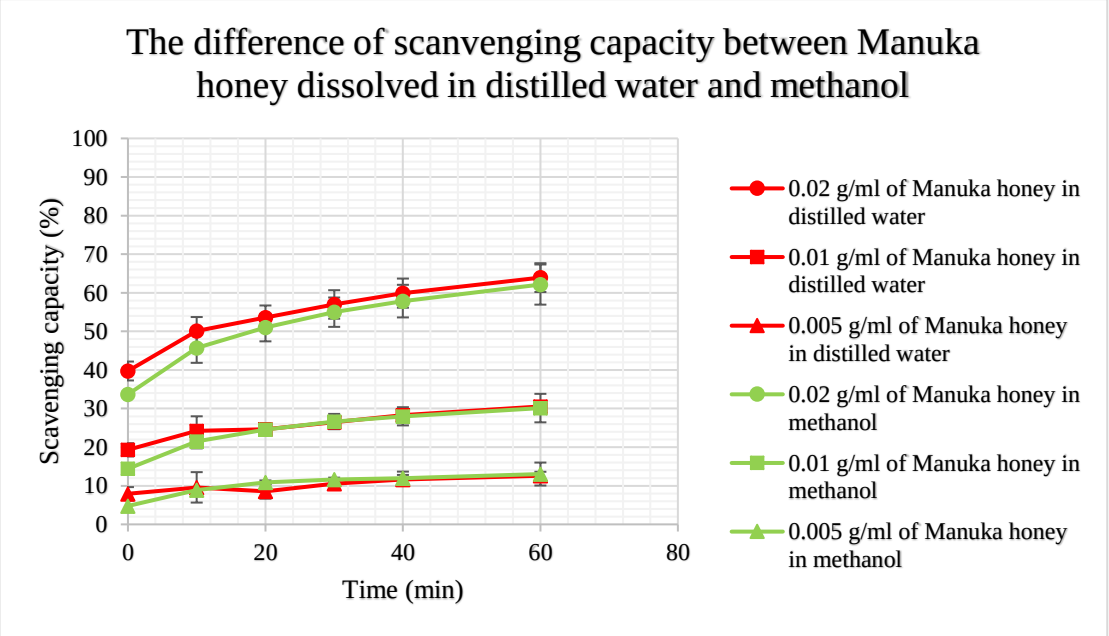


Figure 12. The difference of scavenging capacity between Manuka honey dissolved in distilled water and methanol. Data are expressed as means ± SD (n = 3).

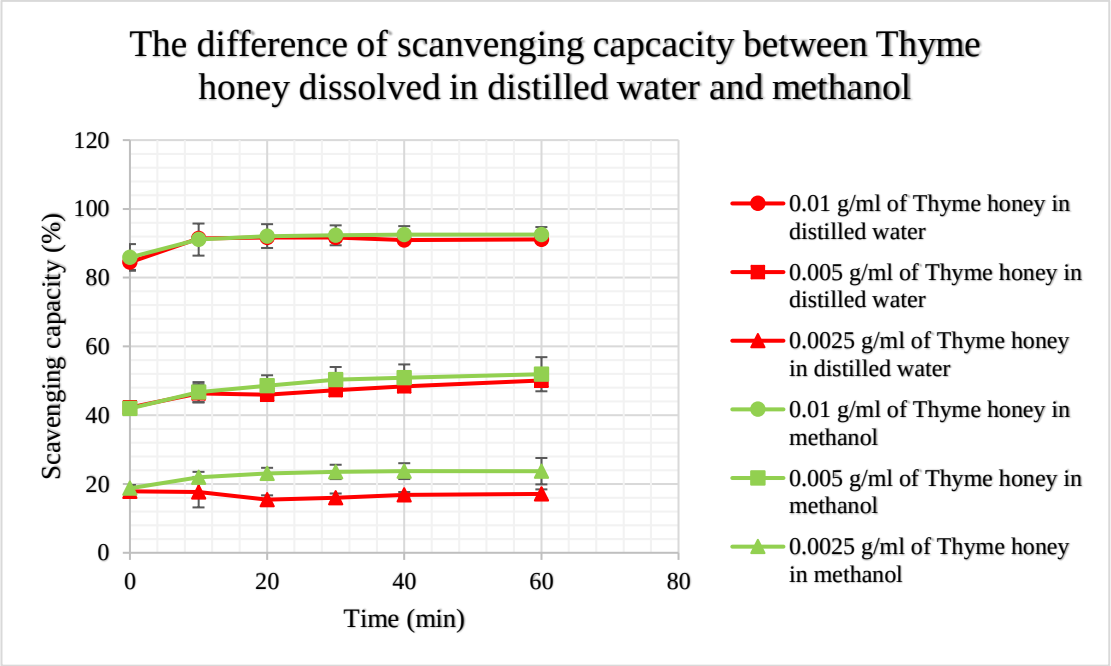


Figure 13. The difference of scavenging capacity between Thyme honey dissolved in distilled water and methanol. Data are expressed as means ± SD (n = 3).

For Thyme honey (Figure 13), the scavenging capacity of Thyme honey in methanol was greater than in distilled water. At time zero, the scavenging capacities of Thyme honey in different solvents appeared to be almost equivalent at the same concentration. While the growth rate of scavenging capacity of Thyme honey in methanol was higher than in distilled water, leading to an increasingly widening difference with the increased time and the decreased honey concentrations. Especially, a difference of 7.58% in maximum was observed in 0.0025 g/ml of Thyme honey in different solvents. This phenomenon conformed to the expectations that the measured scavenging capacity was smaller than the actual under the condition of distilled water serving as the solvent. Nonetheless, for Manuka honey (Figure 12), a totally contrary condition occurred within this period. It could be observed that Manuka honey exhibited higher scavenging capacities in distilled water than in methanol. At time zero, the scavenging capacities of Manuka honey in distilled water were greater than in methanol. The gap appeared to narrow with the

increased time and the decreased honey concentrations. When the concentration was 0.02 g/ml, the scavenging capacity of Manuka honey in distilled water was 6.05% (maximum) greater than in methanol.

3.2.2 Concentration-Dependent Antioxidant Activity of Tested Honeys

The samples of Manuka honey and Thyme honey dissolved in distilled water and absolute methanol were tested for evaluating concentration-dependent antioxidant effects of two types of honey. The results were shown in Figure 14 to 15 and Table 6 to 7.

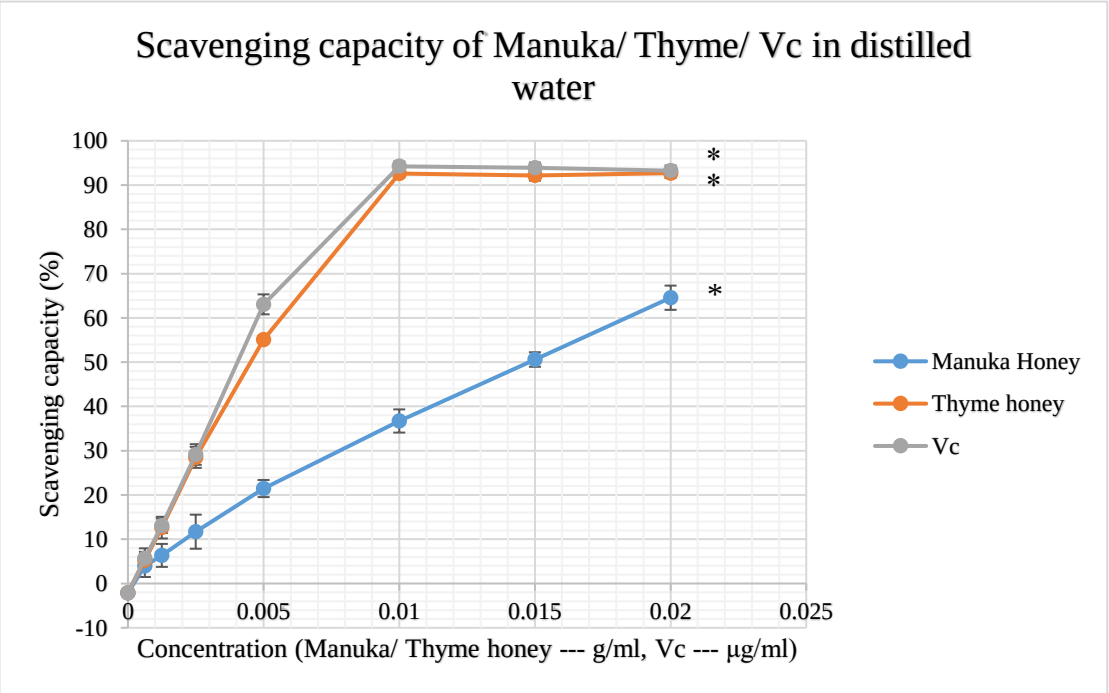


Figure 14. Comparison of scavenging capacity of Manuka honey, Thyme honey and Ascorbic acid dissolved in distilled water. Data are expressed as means ± SD (n = 3) * $P < 0.05$, compared with the values determined for the same treatment at the concentration of 0 g/ml.

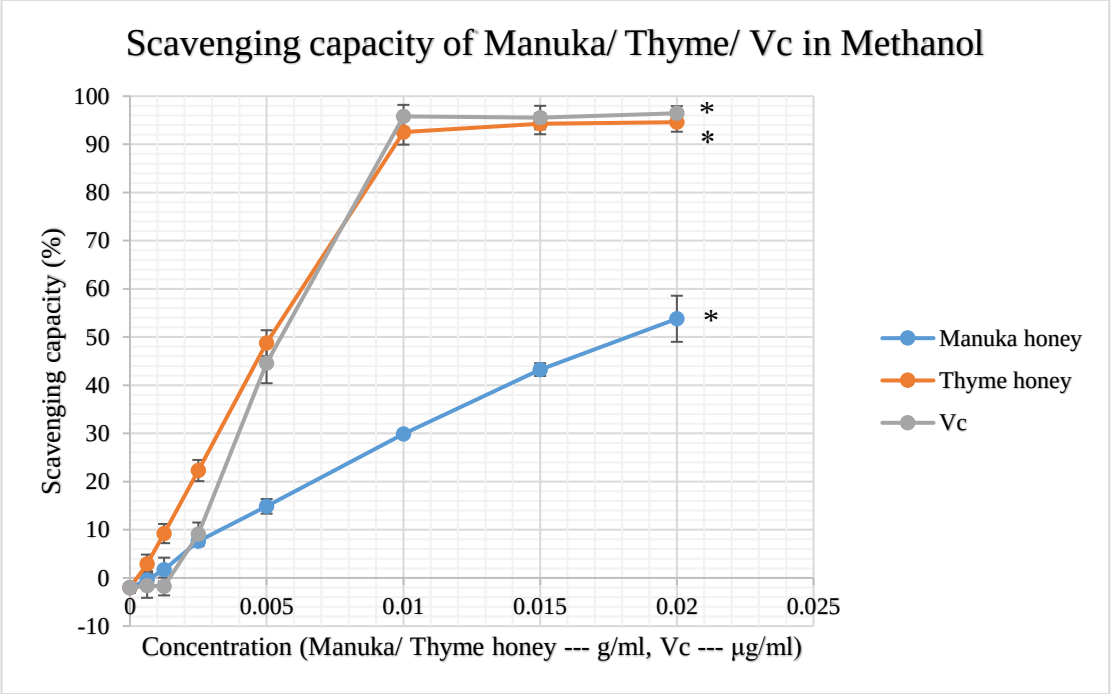


Figure 15. Comparison of scavenging capacity of Manuka honey, Thyme honey and Ascorbic acid dissolved in methanol. Data are expressed as means ± SD (n = 3) * $P < 0.05$, compared with the values determined for the same treatment at the concentration of 0 g/ml.

Table 6. DPPH assay results with solvent of distilled water (%). Data are expressed as means ± SD (n = 3).

Antioxidants \ Concentrations	0.02	0.15	0.01	0.005	0.0025	0.00125	0.000625
Manuka honey (g/ml)	64.55±2.74	50.59±1.64	36.71±2.62	21.45±1.93	11.70±3.85	6.35±2.60	3.98±2.50
Thyme honey (g/ml)	92.70±1.09	92.16±1.16	92.62±0.90	55.11±0.75	28.49±2.37	12.62±2.45	5.24±1.91
Ascorbic acid (μg/ml)	93.27±1.15	93.88±1.19	94.23±1.09	63.06±2.25	29.14±2.33	13.04±1.64	5.66±2.29

Table 7. DPPH assay results with solvent of methanol (%). Data are expressed as means ± SD (n = 3).

Antioxidants \ Concentrations	0.02	0.15	0.01	0.005	0.0025	0.00125	0.000625
Manuka honey (g/ml)	53.79±4.78	43.25±1.32	29.91±0.71	14.86±1.51	7.62±0.90	1.74±2.47	-0.41±1.82
Thyme honey (g/ml)	94.60±1.98	94.27±2.18	92.56±2.63	48.72±2.69	22.29±2.20	9.21±1.99	2.92±1.92
Ascorbic acid (μg/ml)	96.45±1.48	95.56±2.43	95.82±2.37	44.58±4.18	9.06±2.44	-1.77±1.84	-1.63±2.51

Overall, the radical scavenging capacities of both tested honeys and ascorbic acid in different solvents were observed in a concentration-dependent manner (p-value < 0.05). Thyme honey was more potent in antioxidant activity compared with Manuka honey. The scavenging capacity of Thyme honey (dissolved either in distilled water or methanol)

increased with the increased honey concentrations. Ascorbic acid (dissolved in both solvents) claimed the strongest antioxidant effects, given the concentrations of ascorbic acid were 1000 times lower than corresponding figures of tested honeys. When distilled water serving as the solvent, the inhibition-concentration curve (Figure 14) showed that Thyme honey reached maximum (92.62%) in scavenging capacity at a concentration of 0.01 g/ml and maintained a plateau from 0.01 to 0.02 g/ml, compared with Manuka honey appeared to raise linearly within the tested concentration range and peaked at 0.02 g/ml to 64.55%. When the solvent changed to methanol (Figure 15), the scavenging capacities of both types of honey were at similar patterns (concentration-dependence). The scavenging capacity of Thyme honey peaked at 0.02 g/ml to 94.60% followed by a plateau from 0.01 (92.57%) to 0.02 g/ml (94.60%), while Manuka honey showed a linear increase within the tested concentration range and peaked at 0.02 g/ml to 53.79%.

The antioxidant effects of Manuka honey in distilled water (up to 64.55%) appeared to be greater than in methanol (up to 53.79%), proving the results in section 3.1.

3.3 Antioxidant Activity of Manuka Honey and Thyme Honey Tested by CUPRAC Assay

The CUPRAC assay was conducted to investigate the concentration-dependent reducing ability of Manuka honey, Thyme honey, and ascorbic acid to convert cupric ions from Cu (II) to Cu (I). As shown in Figure 16 to 18 and Table 8, all the tested antioxidants displayed distinct levels of reducing capacity

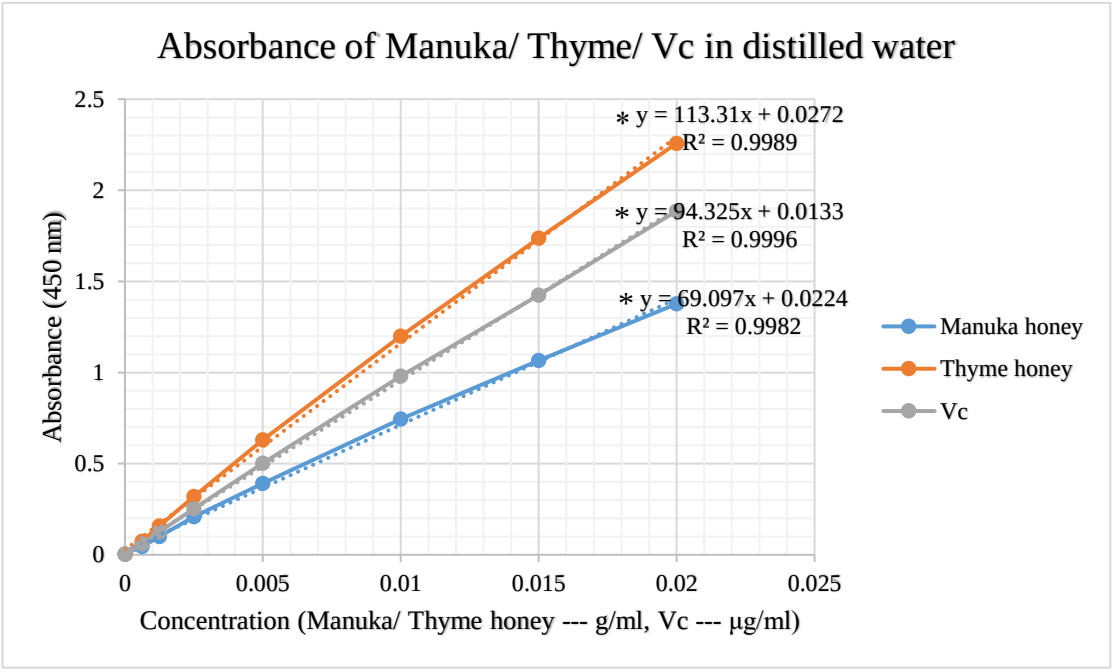


Figure 16. Comparison of reducing ability of Manuka honey, Thyme honey, and ascorbic acid dissolved in distilled water at different concentrations. Data are expressed as means ± SD (n = 3) * $P < 0.05$, compared with the values determined for the same treatment at the concentration of 0 g/ml.

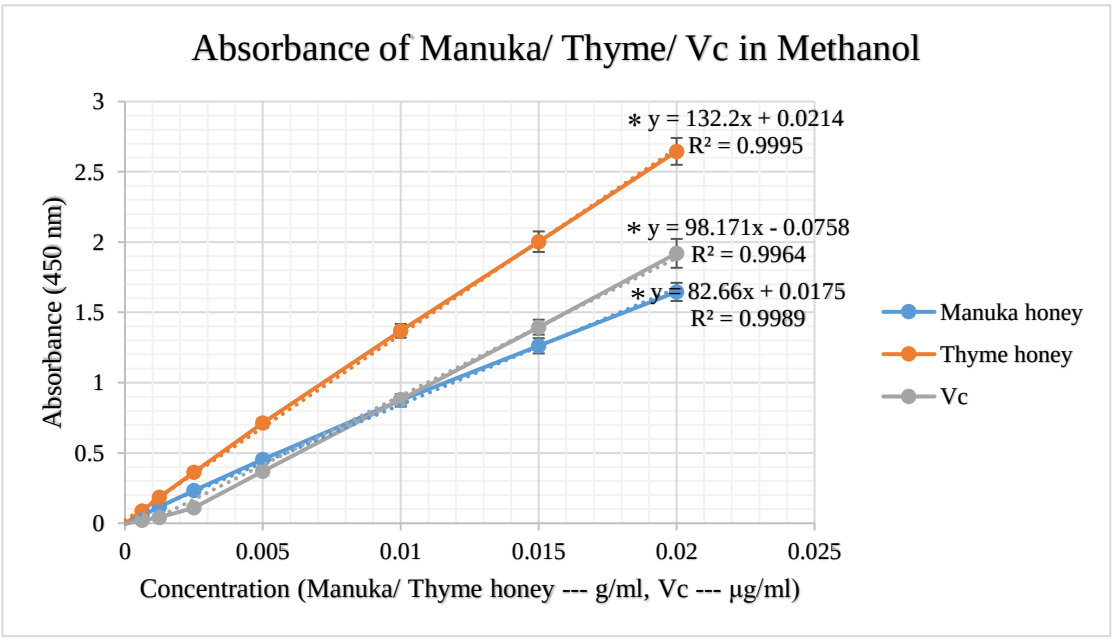


Figure 17. Comparison of reducing ability of Manuka honey, Thyme honey, and ascorbic acid dissolved in methanol at different concentrations. Data are expressed as means ± SD (n = 3) * $P < 0.05$, compared with the values determined for the same treatment at the concentration of 0 g/ml.

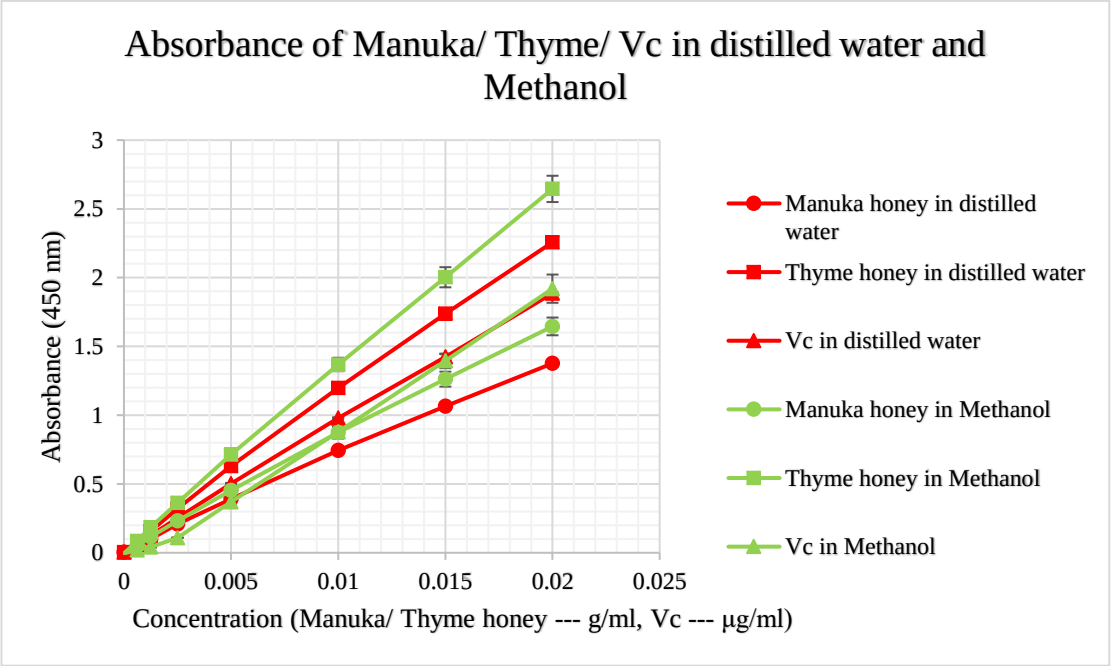


Figure 18. Comparison between Figure 16 and 17. Data are expressed as means ± SD (n = 3)

Table 8. CUPRAC assay R^2 values of Manuka honey, Thyme honey, and ascorbic acid

	Manuka honey	Thyme honey	Ascorbic acid
Distilled water	0.9982	0.9989	0.9996
Methanol	0.9989	0.9995	0.9964

Overall, all the tested honeys and ascorbic acid exhibited the capacity of reducing cupric ions (Cu (II) to Cu (I)) in a concentration-dependent manner. The reducing capacities of tested honeys were increased linearly with the increased concentrations. Both tested honeys in methanol displayed higher cupric ion reducing abilities than in distilled water. Thyme honey (in both solvents) depicted higher antioxidant capacity than Manuka honey at the same tested concentration, while ascorbic acid (10^6 times smaller than the corresponding concentrations of honeys) was between them. The R^2 values (Table 8) proved that the cupric ion reducing capacities of tested honeys exhibited a linear correlation with the increased concentrations.

3.4 Anticancer Activity of Manuka Honey and Thyme Honey

3.4.1 Concentration- and Time-Dependent Effects of Manuka Honey and Thyme Honey on Caco-2 Cell Viability

Manuka honey and Thyme honey solutions at various concentrations were added to 96-well plates with 4000 cells/well of Caco-2 cells and were treated for 48 and 72 hours. The cancer cells attached to 96-well plates for 24 hours before adding treatment. The absorbance was determined at 540 nm to compare the anti-proliferative effects between these two types of honey. The MTT assay demonstrated that both tested honeys possessed time and concentration-dependent anti-proliferative effects on Caco-2 cells as shown in Figure 19 to 22.

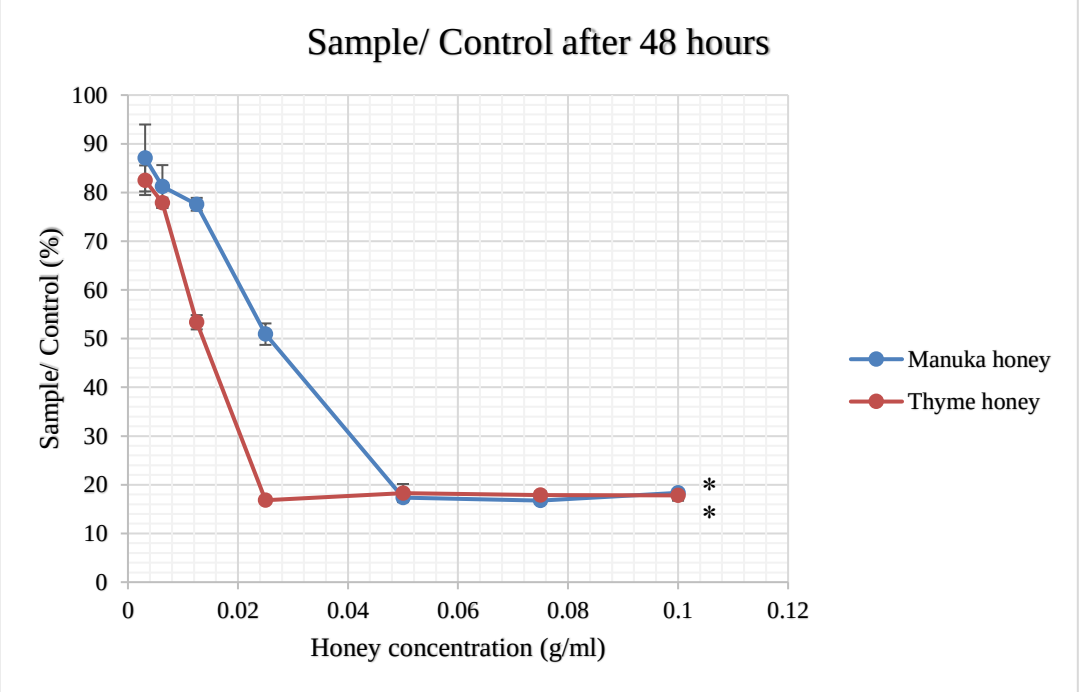


Figure 19. Growth inhibitory effects of Manuka honey and Thyme honey on Caco-2 cells treated for 48 hours. Data are expressed as means ± SD (n = 3) * $P < 0.05$, compared with the values determined for the same treatment at the lowest concentration (0.003125 g/ml).

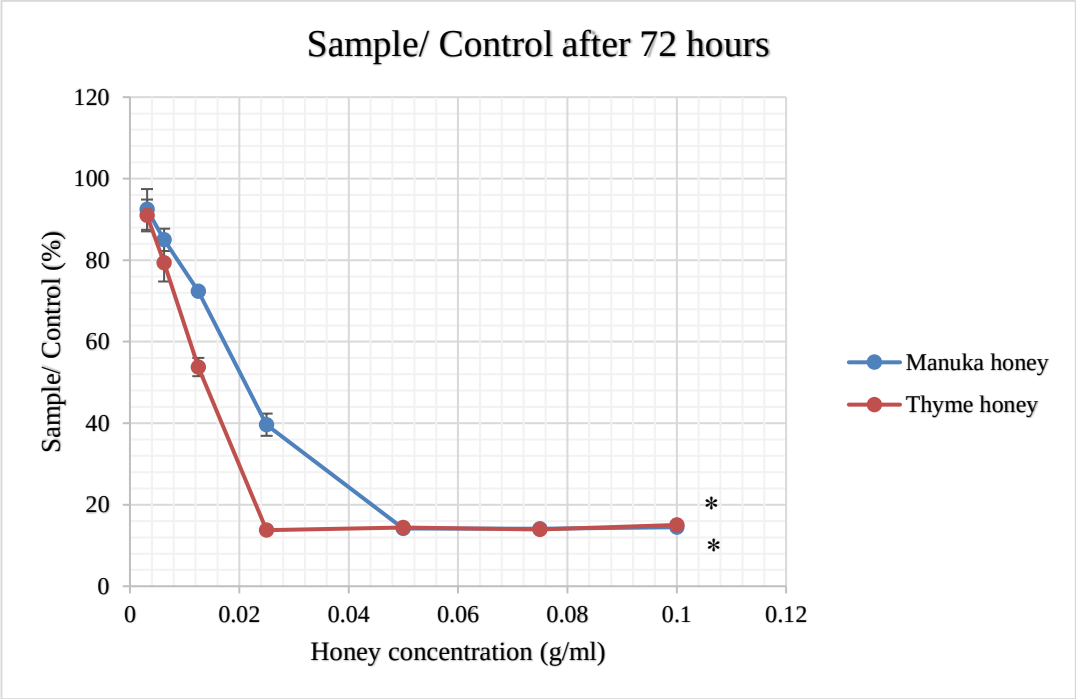


Figure 20. Growth inhibitory effects of Manuka honey and Thyme honey on Caco-2 cells treated for 72 hours. Data are expressed as means ± SD (n = 3) * $P < 0.05$, compared with the values determined for the same treatment at the lowest concentration (0.003125 g/ml).

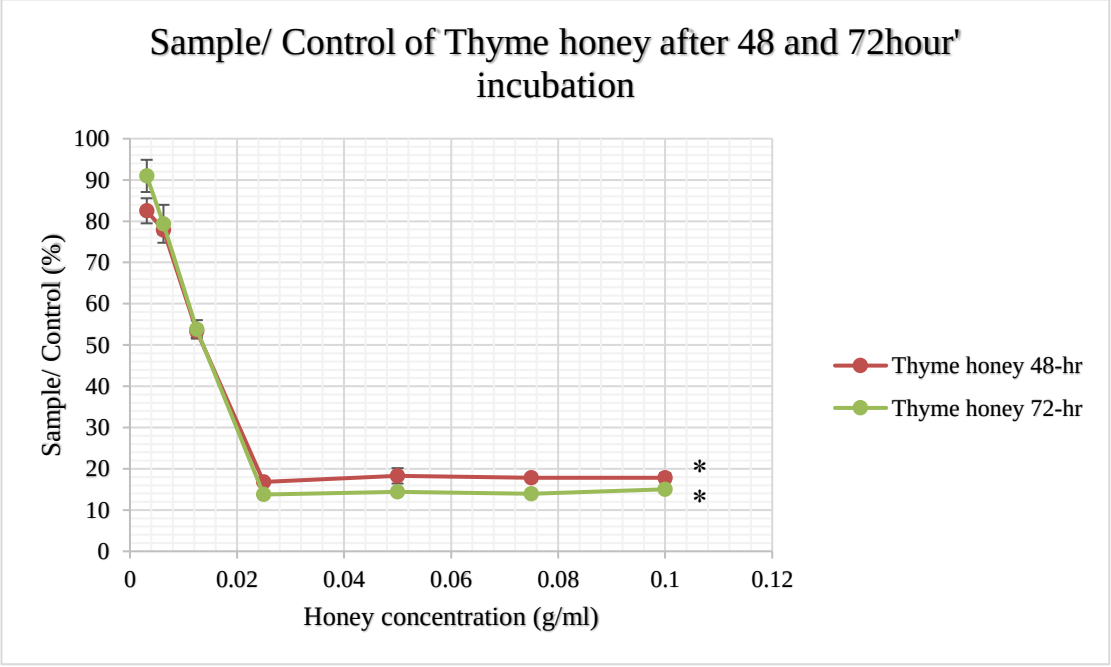


Figure 21. Growth inhibitory effects of Thyme honey on Caco-2 cells treated for 48 and 72 hours. Data are expressed as means ± SD (n = 3) * $P < 0.05$, compared with the values determined for the same treatment at the lowest concentration (0.003125 g/ml).

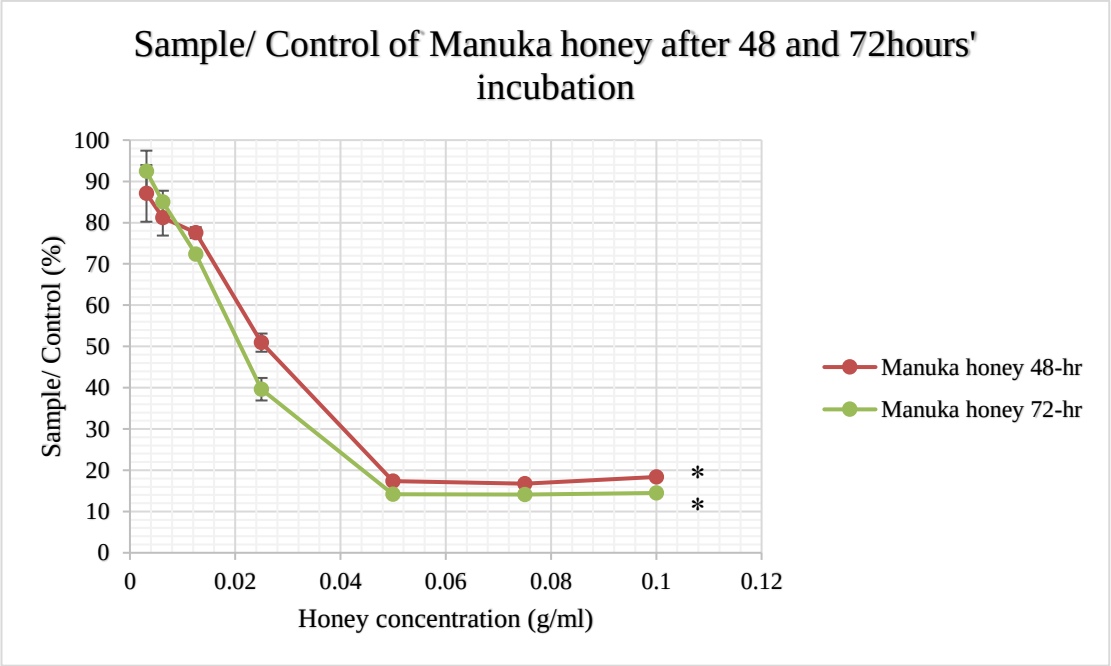


Figure 22. Growth inhibitory effects of Manuka honey on Caco-2 cells treated for 48 and 72 hours. Data are expressed as means ± SD (n = 3) * $P < 0.05$, compared with the values determined for the same treatment at the lowest concentration (0.003125 g/ml).

The anti-proliferative effects of Manuka honey and Thyme honey on Caco-2 cell line were tested by MTT assay. The values were calculated as the percentage of OD values of the sample group/ OD values of the control group. In general, the results showed that both Manuka honey and Thyme honey acted as an inhibitor against Caco-2 cell line, while the anticancer effects of the tested honeys were concentration- and time-dependent (*p*-value < 0.05). Thyme honey showed greater inhibitory effects than Manuka honey at the same concentration and incubation time. The inhibitory effects of both tested honeys treated for 72-hr were greater than 48-hr treatment at the same concentration (*p*-value < 0.05), indicating a time-dependent manner.

Figure 19 to 20 showed the cancer cells treated with different concentrations of tested honeys for different incubation time, suggesting that the cell viability after 48- and 72-hr

treatment decreased with the increased honey concentrations. Thyme honey displayed greater inhibitory effects than Manuka honey in concentration ranged from 0.003125 to 0.05 g/ml, compared with the inhibitory effects appeared to be almost equal from 0.05 to 0.1 g/ml. In Figure 21 to 22, there were two distinctive lines of Caco-2 cells after treatment of the tested honeys for two and three days, verifying that the cell viability decreased with the prolonged incubation time at the same concentration. Manuka honey could exhibit greatest inhibitory effects at a concentration of 0.05 g/ml followed by a plateau, compared with Thyme honey at 0.0125 g/ml followed by a plateau. The cell viability dropped to 13% and 14% of control after 72-hr treatment of Thyme honey and Manuka honey, respectively.

3.4.2 Anticancer potency

The IC_{50} values of Manuka honey and Thyme honey treatment for 48 and 72 hours on Caco-2 cells were shown in Figure 23 to 29 and Table 9. The Y-axis and X-axis were plotted by % of control cell viability, and the logarithm of honey concentrations respectively.

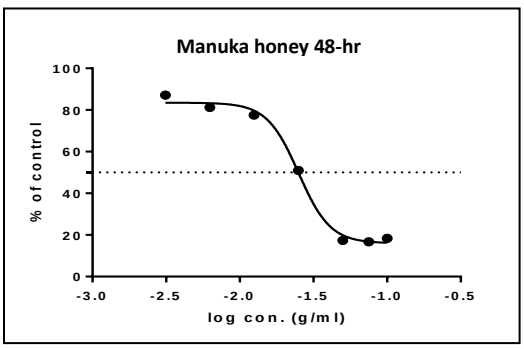


Figure 23. IC_{50} value of Manuka honey incubated for 48 hours

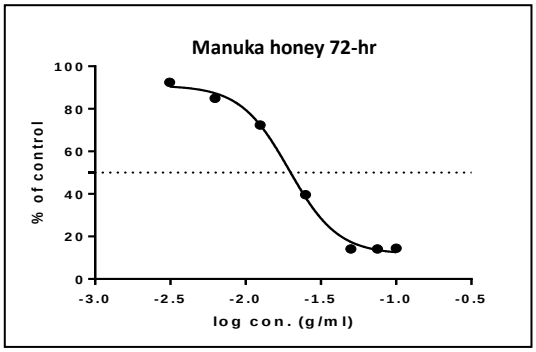


Figure 24. IC_{50} value of Manuka honey incubated for 72 hours

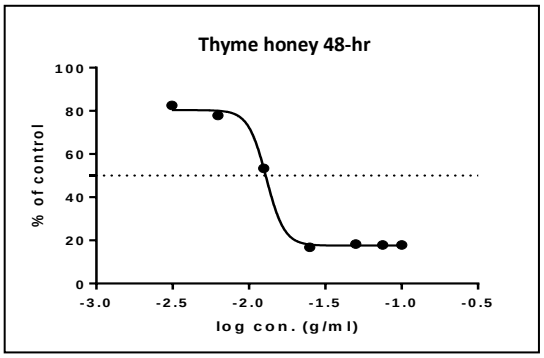


Figure 25. IC_{50} value of Thyme honey incubated for 48 hours

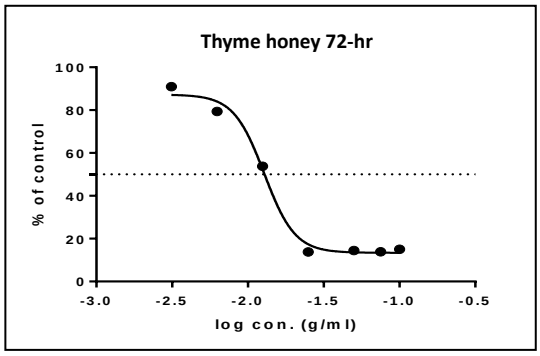


Figure 26. IC_{50} value of Thyme honey incubated for 72 hours

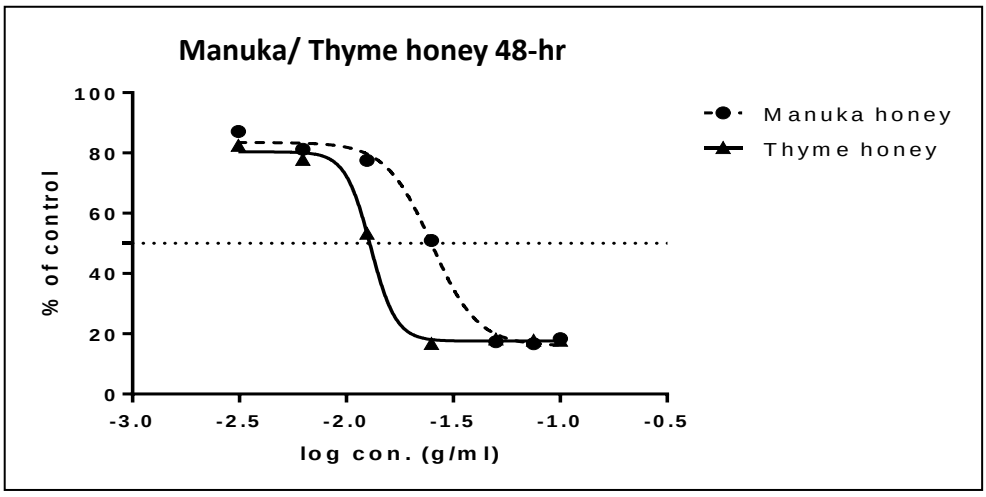


Figure 27. Comparison of IC_{50} values of Manuka honey and Thyme honey incubated for 48 hours

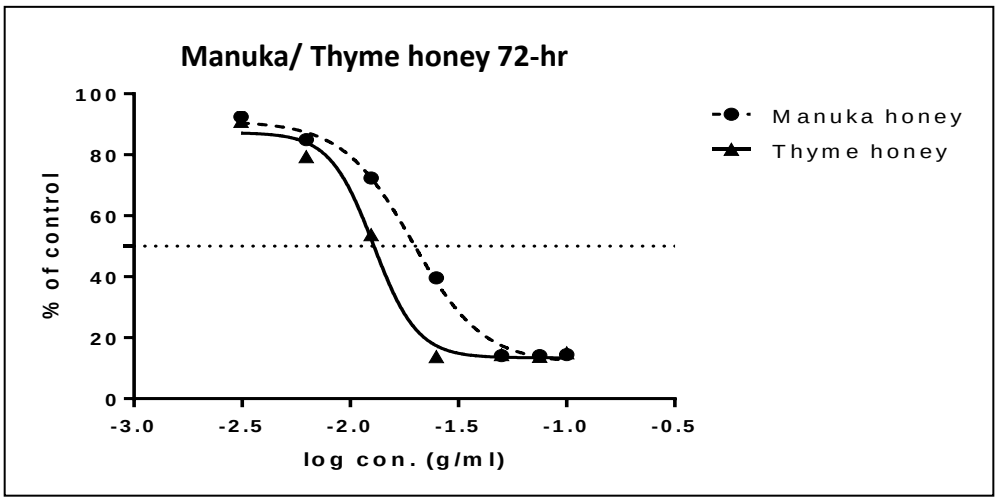


Figure 28. Comparison of IC_{50} values of Manuka honey and Thyme honey incubated for 72 hours

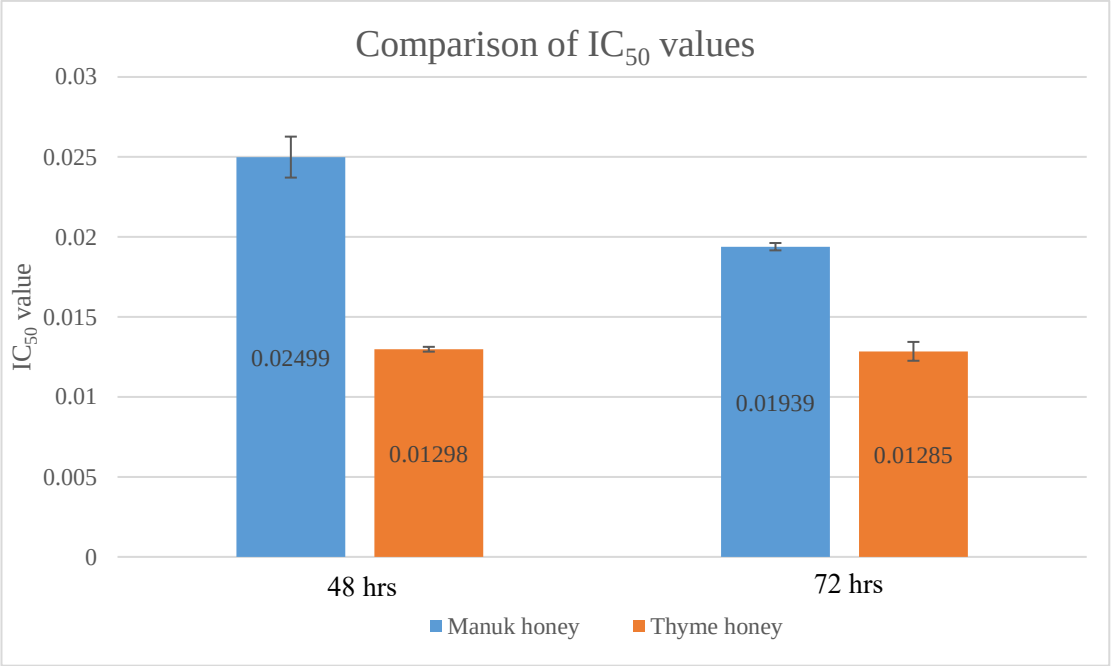


Figure 29. Comparison of IC_{50} values of Manuka honey and Thyme honey on Caco-2 cell growth for 48 and 72 hours. Data are expressed as means ± SD (n = 3)

Table 9. The differences of IC_{50} values of Manuka honey and Thyme honey treatment after 48 and 72 hours, Unit: g/ml. Data are expressed as means ± SD (n = 3); a and b, $P < 0.01$, compared with Manuka honey at 48 and 72 hours respectively.

Honey variety \ Incubation time	48 hours	72 hours
Manuka honey	0.02499 ± 0.00128	0.01939 ± 0.00023
Thyme honey	0.01298 ± 0.00015[a]	0.01285 ± 0.00059[b]
Difference	0.01201	0.00654

Representative concentration-viability curves for Caco-2 cells treated with Manuka honey and Thyme honey for 48- and 72-hr were shown in Figure 27 and Figure 28, respectively. Both tested honeys showed concentration- and time-dependent cytotoxicity effects on Caco-2 cells. All concentration-cell viability curves generally fitted well with the sigmoid model of negative exponential distribution, showing slightly decreased cell viability in the low concentration range followed by a relatively steep drop of cell viability

and then a plateau. Time-dependent IC_{50} values of both types of honey were shown in Table 9.

Thyme honey acted in a lower concentration than Manuka honey at the same incubation time. The IC_{50} values of Manuka honey varied significantly according to the incubation time from 0.02499 to 0.01939. Compared with the IC_{50} values of Thyme honey almost remained stable over the period. There was a large gap in the IC_{50} values between tested honeys treated for 48-hr, where the IC_{50} value of Manuka honey was almost twice as much as that of Thyme honey. After 72-hr treatment, the gap between the IC_{50} values appeared to be narrow but a difference of 0.00654 g/ml still existed.

Chapter 4: Discussion

4.1 Antioxidant Activity of Manuka Honey and Thyme Honey from New Zealand

In the human body, ROS, including free radicals are the natural by-products of normal metabolism of oxygen beneficial to homeostasis and cell signaling, while exhibit in all living beings (Dasgupta & Klein, 2014; Erbas & Sekerci, 2011; Tierney et al., 2010). Despite the fact that low concentration range of ROS is beneficial to health, which plays a role in cellular responses against infectious agents, an imbalance between ROS and the capacity of protective system *in vivo* leads to numerous systems under attack, including immune system, and leads to an extremely wide range of diseases and tissue damage,

sometimes even death as mentioned in section 1.2.2 (Alexieva et al., 2010; Ames et al., 1993; J. Gutteridge, 1995; Parthasarathy et al., 1992; Serafini, 2006). Therefore, antioxidant ingestion has been suggested to promote human health because of their preventive effects against excess free radicals produced in the body (Matanjun et al., 2008). Honey has been advocated to be a kind of natural antioxidant. The antioxidant activity of honey has been attributed, at least in part, to glucose oxidase, ascorbic acid, phenolic acids, catalase, flavonoids, carotenoid derivatives, Maillard reaction products, organic acids, amino acids, and proteins (Al-Mamary et al., 2002; Aljadi & Kamaruddin, 2004; Frankel et al., 1998; Gheldof & Engeseth, 2002; Inoue et al., 2005; Nasuti et al., 2006; Schramm et al., 2003; Vela et al., 2007).

The antioxidant activity of Manuka honey, Thyme honey, and ascorbic acid has been previously published. However, the profile has not been fully established to compare the antioxidant potency between NZ Manuka and Thyme honey. Since the presence of different components in the tested honeys, it was relatively difficult to measure each antioxidant component separately. Therefore, DPPH assay and CUPRAC assay were employed to evaluate the total antioxidant activity of New Zealand Manuka honey and Thyme honey dissolved in distilled water and absolute methanol, with the former indicated the free radical scavenging capacity, and with the latter measured the cupric ion reducing capacity.

The concentration gradients of honey and incubation time applied in this study were obtained after a series of repeated preliminary studies. In terms of DPPH assay, according

to the results of preliminary experiments, the absorbance measured after Thyme honey treatment was too low in the high concentration range (> 0.01 g/ml), while it was too high when Manuka honey in the low concentration range (< 0.005 g/ml). The absorbance measured after ascorbic acid treatment was too high when it kept same as the concentrations of honey. Hence, for the DPPH assay regarding incubation time vs. scavenging capacity, the concentration of ascorbic acid was used as 50 μM. The concentrations of Manuka honey were used as 0.02, 0.01 and 0.005 g/ml, while Thyme honey as 0.01, 0.005 and 0.0025 g/ml to ensure a direct comparison. For the DPPH assay concerning different concentrations vs. scavenging capacity, as well as the CUPRAC assay, the absorbance of ascorbic acid (0.02, 0.015, 0.01, 0.005, 0.0025, 0.00125 and 0.000625 μg/ml), and both tested honeys (0.02, 0.015, 0.01, 0.005, 0.0025, 0.00125 and 0.000625 g/ml) was measured at 30 minutes due to the undulatory absorbance of the mixture of DPPH and honey solutions before 30 minutes, suggesting that the reactions tended to achieve equilibrium after 30 minutes.

4.1.1 Effects of Different Solvents on Antioxidant Ability of Manuka Honey and Thyme Honey

Both Manuka honey and Thyme honey dissolved either in distilled water or absolute methanol (tested by DPPH and CUPRAC assays) demonstrated very high antioxidant activity, suggesting that these honeys contained hydrophilic and lipophilic antioxidants. In this study, the tested honeys were easier to dissolve in distilled water than in methanol. The DPPH radical scavenging and cupric ion reducing capacities of Thyme honey in both

distilled water and absolute methanol were much greater than those of Manuka honey at the same concentration and time period. Manuka honey showed greater scavenging capacity in distilled water than in methanol, while Thyme honey in different solvents was not comparable as mentioned in section 3.1. Both tested honeys exhibited higher cupric ion reducing capacity in methanol than in distilled water.

For DPPH assay, the time-scavenging capacity curve (Figure 12) indicated that Manuka honey in distilled water was 6.05% in maximum greater than in methanol in scavenging capacity at time zero and concentration of 0.02 g/ml. In contrast, Figure 13 showed the almost equal scavenging capacities of Thyme honey (all concentrations) in different solvents at time zero, and then the difference appeared to increase with the increased time caused by the unstable DPPH in water. This phenomenon could be probably summarized into two reasons: (i) Manuka honey might contain a greater percentage of hydrophilic antioxidants with scavenging capacity than the lipophilic ones; (ii) The hydrophilic antioxidant compounds in Manuka honey exhibited higher scavenging capacity than the lipophilic ones.

For the results conducted by CUPRAC assay, a different condition occurred as shown in Figure 18. Tested honeys gained higher cupric ion reducing capacity in methanol than in distilled water at the same concentration. The change of solvent from methanol to distilled water exerted greater impacts on Thyme honey (0.39 of difference in max.) than Manuka honey (0.27 of difference in max.) in the absorbance representing the cupric ion reducing capacity. This phenomenon could be probably summarized into two reasons: (i) Tested

honeys contained the higher content of lipophilic antioxidant compounds with cupric ion reducing capacity than the hydrophilic ones; (ii) The lipophilic antioxidants in tested honeys exhibited higher cupric ion reducing capacity than the hydrophilic ones.

The antioxidant effects of honey are mainly derived from its phenolic compounds (Kumar et al., 2013; Moure et al., 2001; Jihua Wang et al., 2012). Phenolic compounds normally contained in honey are phenolic acids, mainly hydroxybenzoic acid, chlorogenic acid, protocatechuic acid, caffeic acid, vanillic acid, benzoic acid, p-coumaric acid, ellagic acid, and cinnamic acid; flavonoids, mainly apigenin, naringenin, kaempferol, luteolin, pinocembrin, chrysin, and galangin; and polyphenols (Beretta et al., 2005; Estevinho et al., 2008). Mota, Queimada, Pinho, and Macedo (2008) studied the temperature-dependent aqueous solubility of two hydroxybenzoic acids (gallic and salicylic acid) and three phenylpropenoic acids (trans-cinnamic, ferulic and caffeic acids) in temperature range from 288.15 and 323.15 K. Noubigh, Abderrabba, and Provost (2007) revealed that the solubility of six phenolic compounds (ferrulic, gallic, protocatechuic and vanillic acid, as well as vanillin, sodium chloride, potassium chloride and lithium chloride) in pure water increased with increasing temperature (293.15 to 318.15 K). Lu and Lu (2007) measured the water solubility of gallic acid and its esters (methyl gallate, propyl gallate, and octyl gallate). Shahidi, Janitha, and Wanasundara (1992) reviewed the solubility of synthetic antioxidants. Unfortunately, no studies were found to focus on the systematic comparison between water- and fat-solubility of antioxidant compounds in honey. Further researches regarding the influences from different solvents to antioxidant activity of honey are needed.

4.1.2 Time-dependent Antioxidant Activity of Manuka Honey and Thyme Honey

The antioxidant effects (DPPH radical scavenging capacity) of both Manuka honey and Thyme honey dissolved in distilled water and absolute methanol (tested by DPPH and CUPRAC assays) were observed in a time-dependent manner, indicating that honey as an electron donor could convert DPPH radicals to more stable products and terminate the further reactions. Thyme honey in different solvents displayed a higher scavenging capacity than Manuka honey at the same concentration and time period, while the growth rate of scavenging capacity of Manuka honey was greater than Thyme honey at the same concentration within this period.

With the increase of incubation time, the scavenging capacity of tested honeys rised to various degrees. The time-scavenging capacity curves (Figure 12 to 13) showed that Thyme honey in distilled water was 60.55% higher than Manuka honey in the percentage of scavenging capacity at a concentration of 0.01 g/ml and 60 minutes, compared to 62.41% in methanol. The percentage of scavenging capacity of Manuka honey (0.02 g/ml) increased by 24.19% (max.) in distilled water and 28.44% (max.) in methanol within 60 min, compared with Thyme honey (0.01 g/ml) by 6.64% in distilled water and 6.65% in methanol. These data proved that the free radical scavenging capacities of tested honeys were positively proportional to incubation time, while the effectiveness of Manuka honey was more dependent on the increase of incubation time than Thyme honey.

4.1.3 Concentration-dependent Antioxidant Activity of Manuka Honey and Thyme Honey

The antioxidant effects (DPPH radical scavenging and cupric ion reducing capacities) of both Manuka honey and Thyme honey dissolved in distilled water and absolute methanol (tested by DPPH and CUPRAC assays) appeared to be concentration-dependent. Thyme honey in both solvents possessed greater antioxidant effects than Manuka honey at the same concentration.

For DPPH assay, the concentration-scavenging capacity curves (Figure 14 and 15) directly showed the concentration-dependent effects on the free radical scavenging capacity of tested honeys. The scavenging capacity of Thyme honey in both solvents increased dramatically and linearly in the concentration ranged from 0 to 0.01 g/ml followed by a plateau. The scavenging capacity of Manuka honey increased gradually and linearly with the increased concentrations. Table 6 and 7 showed that Thyme honey in both solvents exhibited greater scavenging capacity than Manuka honey at the same concentration.

In terms of CUPRAC assay, the absorbance (representing cupric ion reducing capacity) of tested honeys in both solvents was positively proportional to concentration, while Thyme honey in both solvents displayed higher absorbance than Manuka honey at the same concentration (Figure 18). The R^2 values (around 0.999) of concentration-absorbance curves demonstrated a linear correlation between the cupric reducing capacity of tested honeys with honey concentrations (Table 8).

The statistics above illustrated the concentration-dependent effects of the antioxidant activity in both tested honeys. Thyme honey in both solvents showed higher antioxidant activity than Manuka honey at the same concentration.

4.1.4 Comparison of Antioxidant Activity between Manuka Honey and Thyme Honey

Both Manuka honey and Thyme honey from New Zealand tested in this study exhibited high antioxidant activity (DPPH radical scavenging and cupric ion reducing capacities). In general, the antioxidant effects of both tested honeys appeared to be time- and concentration-dependent, and be influenced by the different solvents. Thyme honey showed greater antioxidant activity than Manuka honey. Manuka honey used in this study was UMF 15 + (UMF (unique Manuka factor): an indirect measurement of polyphenol content ranged from 5 + to 25 +). Therefore, it was possible that Manuka honey with higher UMF grade possessed higher antioxidant capacity.

Thyme honey showed higher antioxidant activity than Manuka honey in both DPPH and CUPRAC assays. This might be caused by: (i) Thyme honey contained the higher content of antioxidant compounds than Manuka honey; (ii) The antioxidant compounds in Thyme honey exhibited higher antioxidant capacity than Manuka honey. Previous study has indicated that Thyme honey (Spain) possessed a high content of vitamin C around 144.95 mg per 100 g, and high phenolic content around 121.38 mg of GAE (gallic acid equivalent) per 100 g (León-Ruiz et al., 2013). Thyme honey (Turkey), in another study, just

displayed phenolic content around 11.22 mg GAE/ 100 g (Sagdic, Silici, & Ekici, 2013), suggesting an influence from botanical origin to honey composition (Al-Mamary et al., 2002; Gheldof & Engeseth, 2002). The antioxidant effects of honey have been reported mainly derived from phenolic compounds (Kumar et al., 2013; Moure et al., 2001; Jihua Wang et al., 2012). Thus, New Zealand Thyme honey, to some degree, was considered to possess higher content of phenolic compounds than Manuka honey (UMF 15 +). However, it was possible that Manuka honey with higher UMF grade possessed higher antioxidant capacity. While ascorbic acid (vitamin C) showed a very high antioxidant activity in both DPPH and CUPRAC assays in this study. Therefore, the high antioxidant activity of Thyme honey was probably attributed to a combination of the reason (i) and (ii) above, suggesting a higher content of antioxidant compounds and a higher antioxidant ability of the antioxidants in Thyme honey.

According to the data collected in this study, the antioxidant activity of Thyme honey from New Zealand was much greater than Manuka honey in various aspects. It was considered to be an excellent antioxidant *in vitro.* Schramm et al. (2003) observed that corn syrup or buckwheat honey (1.5 g/kg body weight) consumption improved plasma antioxidants after oral intake, suggesting the efficient transfer of bioavailability and bioactivity from honey to plasma. Thus, Thyme honey was supposed to have the potential to be an effective antioxidant *in vivo*.

4.2 Anticancer Activity of Manuka Honey and Thyme Honey from New Zealand

Honey has been suggested to have anticancer activity against a variety of cancer cell lines, including lung, bladder, melanoma, osteosarcoma, lymphoid, breast, colorectal, renal, prostate, endometrial, cervical and oral cancer derived cell lines (Fauzi et al., 2011; Fukuda et al., 2010; Ghashm et al., 2010; Jaganathan et al., 2011; Premratanachai & Chanchao, 2014; Samarghandian et al., 2011; Tomasin & Cintra Gomes‐Marcondes, 2011; Tsiapara et al., 2009).

This study aimed to investigate the anti-proliferative effects against Caco-2 cells of Manuka honey and Thyme honey from New Zealand by using MTT cell proliferation assay. The Caco-2 cells were incubated as normal cell culture protocol at 37 °C with 5% carbon dioxide in an incubator. Given the minor effects of tested honey determined at 24 hours in preliminary studies, the anti-proliferative effects were further investigated only at 48 and 72 hours. Manuka honey and Thyme honey (100 μL) were dissolved in completed medium getting the concentrations of 0.1, 0.075, 0.05, 0.025, 0.0125, 0.00625 and 0.003125 g/ml were used as treatments to detect their anticancer effects. The cell density of 4000 cells/well (100 μL) was applied on 96-well plates.

4.2.1 Anti-proliferative Effects of Manuka Honey

As shown in Figure 22, Manuka honey was found to have concentration- and time-dependent cytotoxicity effects on Caco-2 cells.

The effects after 48- and 72-hr treatment showed a linear correlation within concentrations from 0.003125 to 0.05 g/ml, followed by a plateau from 0.05 to 0.1g/ml, suggesting a concentration-dependent manner. The cell viability after 72-hr treatment of Manuka honey (0.1 g/ml) was 3.88% lower than that after 48-hr treatment, showing a time-dependent manner.

The IC_{50} values of Manuka honey in Table 9 were decreased with the prolonged incubation time, suggesting that the effective concentration of Manuka honey decreased with the increase of incubation time.

4.2.2 Anti-proliferative Effects of Thyme Honey

Figure 21 illustrated that the anti-proliferative effects on Caco-2 cells of Thyme honey were found to be concentration- and time-dependent.

The cell viability after 48- and 72-hr treatment was dropped steeply in the concentration ranged from 0.003125 to 0.0025 g/ml, followed by a plateau from 0.025 to 0.1 g/ml, indicating a concentration-dependent manner. After treatment of Thyme honey (0.01 g/ml) for 72 hours, the cell viability displayed a difference of 2.83% lower than that after 48-hr treatment, proving a time-dependent manner.

As shown in Table 9, the IC_{50} values after 48- and 72-hr treatment of Thyme honey basically maintained equivalent. Thus, the effective concentration of Thyme honey

exhibiting anti-proliferative effects on Caco-2 cell line basically remained stable with the incubation time used in this study.

4.2.3 Comparison of Anti-Proliferative Effects between Manuka Honey and Thyme Honey

The cytotoxicity effects of New Zealand Manuka honey and Thyme honey were found in Caco-2 cell line in concentration- and time-dependent manners. After treatment of Manuka honey and Thyme honey for 72 hours, the cell viability was decreased to around 14% and 13% respectively, suggesting that these two types of honey were very effective to Caco-2 cell line. Manuka honey used in this study was UMF 15 + (UMF (unique Manuka factor): an indirect measurement of polyphenol content ranged from 5 + to 25 +). Therefore, it was possible that Manuka honey with higher UMF grade possessing higher anticancer capacity.

The growth inhibitory effect curves (Figure 21 and 22) showed that the cell viability could be largely decreased with the increased honey concentrations and prolonged incubation time. The inhibitory effects of Thyme honey were observed to be greater than Manuka honey at the same concentration (0.003125 to 0.05 g/ml) and incubation time. However, the highest inhibitory effects of tested honeys on Caco-2 cell line maintained approximately equivalent at the same incubation time.

As comparison about IC_{50} values (Figure 29 and Table 9) in Caco-2 cell line, the most obvious feature was that Thyme honey exhibited lower IC_{50} values than Manuka honey at the same incubation time, suggesting that Caco-2 cells were more sensitive to treatment of Thyme honey. Despite the fact that low concentration ranges of tested honeys exhibited low cytotoxicity effects on the Caco-2 cell line, these honeys as natural products containing high anticancer activity in concentration range might be necessary for further investigation and development regrading the relative extracts.

Pichichero et al. (2010) studied the anti-proliferative effects of Acacia honey on human (A375) and murine (B16-F1) melanoma cell lines. Ghashm et al. (2010) studied the anti-proliferative effects of Tualang honey on oral squamous cell carcinomas (OSCC) and human osteosarcoma (HOS) cell lines. Swellam et al. (2003) explored the antitumor effects of honey against bladder cancer cell lines (T24, 253J and RT4) *in vitro* and *in vivo* in mice. The tested honeys in these studies were able to induce an anti-proliferative effect on the cell lines in a dose- and time-dependent manner, compared to the concentration- and time-dependent inhibitory effects of Manuka honey and Thyme honey on Caco-2 cell line in this study. Given that polyphenols and phenolic acids present in honey has been proved to stimulate the apoptotic pathways and processes in cancer cells as mentioned in section 1.8 (Kris-Etherton et al., 2002; Yeh et al., 2005; Yeh & Yen, 2005), the high cytotoxicity effects of the tested honeys on Caco-2 cells in this study could be, at least in part, derived from the high content of polyphenols and phenolic acids in these honeys. Thyme honey has been reported to be very rich in compounds related to anticancer properties (e.g. polyphenols and phenolic acids) (León-Ruiz et al., 2013; Tsiapara et al.,

2009). Kassi et al. (2014) revealed that Thyme honey possessed a unique monoterpene with apoptotic activity in PC-3 prostate cancer cells. Since the anticancer activity of honey could be attributed to a combination of various bioactive properties of honey (section 1.8), the Caco-2 cells were more sensitive to treatment of Thyme honey than Manuka honey (UMF 15 +) in this study was probably due to a better combination of bioactive properties in Thyme honey. However, it was possible that Manuka honey with higher UMF grade possessing higher anticancer capacity.

According to the data collected in this study, Manuka honey and Thyme honey from New Zealand displayed high anticancer activity against Caco-2 cells. Similar anticancer effects have been recently proved for other human colon cancer WiDr and LoVo cell lines in our group (Personal communication with Dr. Yan Li, unpublished data). While they were considered to be an excellent inhibitor against human colon cancer cells *in vitro,* further in vivo investigation is warranted to elucidate the therapeutic values of extracts of tested honeys.

4.3 The Correlation between Antioxidant and Anticancer effects of honey

On the basis of data in this study, Thyme honey displayed greater antioxidant and anticancer effects than Manuka honey, suggesting that the anticancer and antioxidant effects of tested honeys could be positively correlated.

The antioxidant activity of honey has been suggested to mainly originate from phenolic compounds, including phenolic acids, flavonoids and polyphenols (Beretta et al., 2005; Estevinho et al., 2008; Kumar et al., 2013; Moure et al., 2001; Jihua Wang et al., 2012). The anticancer activity of honey has been advocated to derive from combined bioactive properties of honey, including antioxidant property (J. M. Gutteridge & Halliwell, 1993; Subrahmanyam, 1998). While polyphenols and phenolic acids in honey have been claimed to promote apoptosis in different types of cancer cells (Fauzi et al., 2011; Kris-Etherton et al., 2002; Yeh et al., 2005; Yeh & Yen, 2005). Flavonoids and phenolics in honey have been proved to block G_0/ G_1 phase in the cell cycle (Jaganathan & Mandal, 2009; Y.-J. Lee et al., 2003; Pichichero et al., 2010; Tomasin & Cintra Gomes‐Marcondes, 2011).

Oxidative stress has been suggested to be one of the major factors responsible for progression of cancer as it can cause mutations in DNA replication (Hussain, Hofseth, & Harris, 2003). The compound with stronger antioxidant activity may be more potent in inhibiting mutated DNA replication and thus cancer cell proliferation. However, further studies on cell cycle analysis may be required to confirm this hypothesis. It was evident that phenolic compounds in honey might play a pivotal role in both antioxidant and anticancer effects in this study. Therefore, along with the anticancer activity, the strong antioxidant capacities of tested honeys make them promising sources for further extraction/development of anticancer and chemopreventive compounds.

Chapter 5: Conclusion

5.1 Manuka Honey and Thyme Honey as Effective Antioxidants with Future Direction of Research

Manuka honey and Thyme honey from New Zealand were found to have effective and efficient antioxidant effects. Especially, Thyme honey showed effectiveness with regard to both DPPH free radical scavenging capacity and cupric ion reducing capacity in low concentration range and short incubation time. Despite the fact that data in this study proved that Thyme honey possessed higher antioxidant ability in various conditions, Manuka honey still had its own advantages. Manuka honey acted better in distilled water than in absolute methanol, while the cupric ion reducing capacity of Manuka honey was influenced by changes of solvent (absolute methanol to distilled water) on a lower level than that of Thyme honey.

Side effects of honey ingestion are rarely reported. Future studies are recommended to focus on the observation of antioxidant extracts from New Zealand Manuka honey and Thyme honey in plasma after oral ingestion to investigate the probability that whether Manuka honey and Thyme honey can be an alternative to clinical treatment.

5.2 Manuka Honey and Thyme Honey as Effective Anticancer Agent with Future Direction of Research

Results of this study indicated the anti-proliferative effects of New Zealand Manuka honey and Thyme honey on Caco-2 cell line *in vitro*. These effects appeared to be concentration- and time-dependent. The minimum cell viability after Manuka honey and Thyme honey treatment was almost equivalent. High concentration of these two types of honey was considered to be a potent inhibitor against Caco-2 cell line, while Thyme honey could get the highest inhibitory rate at a lower concentration than Manuka honey.

Side effects of honey ingestion are rarely reported. Further researches may be warranted to study the *in vivo* effectiveness of New Zealand Thyme honey and Manuka honey. The concentrations of certain anti-proliferative extracts from these two types of honey should be detected in plasma to explore the probability of New Zealand Manuka honey and Thyme honey as an adjunctive therapy in treating cancer.

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