Medicinal honey for cholesterol homeostasis and the structure of delivery hydrogels

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

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Declaration

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; any editorial work, paid or unpaid, carried out by a third party is acknowledged; and, ethics procedures and guidelines have been followed.

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Publications and presentations

Publications

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Oral presentation

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Table of contents

List of figures

List of tables

List of abbreviations

ACC1	Acetyl-CoA carboxylase 1
AEAC	Ascorbic acid equivalent antioxidant content
AMPK	AMP-activated protein kinase
ANOVA	Analysis of variance
AOC	Antioxidant capacity
ARG-2	Advanced rheometer generation-2
ARJ	Arjuna honey
a_T	Shift factor
CAT	Catalase
COX-2	Cyclooxygenase
COX-2	Cyclooxygenase-2
CRP	C-reactive protein
CVD	Cardiovascular disease
DPPH	2,2-diphenyl-1-picrylhydrazyl
DSC	Differential scanning calorimeter
EC	Electrical conductivity
F/G	Ratio of fructose over glucose
FA	1.0 mM fatty acid (oleic and palmitic) treatment
FAH1	Fatty acids and 1.0% honey treatment
FAH2	Fatty acids and 2.0% honey treatment
FRAP	Ferric ion reducing antioxidant power
G′	Storage modulus

G″	Loss modulus
G/W	Glucose/water
GPx	Glutathione peroxidase
GSH	Glutathione
GUG	Guggul honey
HCD	High cholesterol diet
HDL	High density lipoprotein
HDL-C	High density lipoprotein cholesterol
HMF	Hydroxymethylfurfural
HMG-CoA	3-hydroxy-3-methylglutaryl Co-enzyme A
IL	Interleukin
JIA	Jiaogulan honey
K	Constant
LDL	Low density lipoprotein
LDL-C	Low density lipoprotein cholesterol
LDLR	Low density lipoprotein receptor
LOX	Lipoxygenase-2
LPO	Lipoprotein oxidation
LXRα	Liver X receptor alpha
M	Molecular weight
MAPK	Mitogen-activated protein kinase
MC	Moisture content
MDA	Malondialdehyde
MH1	Manuka-1 honey

MH2	Manuka-2 honey
NF-κB	Nuclear factor kappa B
NO	Nitric oxide
NOS	Nitric oxide synthase
NQO1	NaD(P)H: quinone oxidoreductase 1
Nrf2	Nuclear factor erythroid 2–related factor 2
OLI	Olive honey
ORAC	Oxygen radical absorbance capacity
PGs	Prostaglandins
pH	power of hydrogen
PPARα	Proliferator activated receptor alpha
RBC	Red blood cell
RMIT	Royal Australian Institute of Technology
ROS	Reactive oxygen species
RSA	Radical scavenging activity
SEM	Scanning electron microscope
SOD	Superoxide dismutase
SREBP	Sterol regulatory element binding protein
T	Temperature
TC	Total cholesterol
TFC	Total flavonoid content
TG	Triglyceride
T_g	Glass transition temperature
TNF-α	Tumour necrosis factor α

TPC	Total phenol content
TPC	Total phenolic content
T_{α}	Alpha transition
T_{β}	Beta transition
T_{γ}	Gamma transition
VLDL	Very low-density lipoprotein
WFL	Williams, Landel and Ferry
WAXD	Wide-angle X-ray diffraction

ABSTRACT

Honey is a sweet reward of nature. Its composition is dominated by monosaccharides (70−80%), water (<20%), disaccharides and higher sugars (5−10%), and minor quantities of phenolic compounds, minerals, vitamins, enzymes and organic acids. Sugars govern its physicochemical properties and technological functions, while non-sugar components, mainly phenolic acids and flavonoids, impart health benefits and make it unique from other sweeteners. Interestingly, honey bees promote the transfer of phytochemicals from floral nectars into honey and make them concentrated in honey, thus, honey is not only an abundant source of antioxidants, but also a carrier of medicinal phytochemicals.

Oxidative stress and hypercholesterolemia are detrimental factors in the pathogenesis of atherosclerosis, the leading cause of mortality worldwide. Although different strategies have been developed to combat them, finding an effective natural remedy targeting both is challenging. Accumulated evidence emphasizes the advantages of honey in attenuating such detrimental factors, however, little is known about its mode of action on oxidative damage and lipid metabolism at a molecular level.

Recently, the use of honey as a healthy food or a complementary medicine has attracted great attention. Considerable efforts have been made to incorporate honey into delivery systems including cryogels and hydrogels to enhance its nutraceutical values and overcome its physical limitations. To date, most investigations on the physicochemical properties of honey address its quality standards, whereas understanding structural characteristics and molecular interactions within a honey matrix or a honey-incorporated network is limited.

Therefore, this PhD research was conducted with primary aims to (i) validate the physicochemical quality of four newly developed medicinal honeys and elucidate their effect on some key biomarkers of oxidative stress and lipid metabolism, (ii) establish a concrete basis

of structural characteristics of honey for technological applications in food biomedical industries, and (iii) develop a honey-gelatin template as a delivery system for honey's bio-functionality.

In the first experimental chapter, commercial manuka-1 honey with 400 mg/kg methylglyoxal (MGO 400^{+}) and four medicinal honeys (arjuna, guggul, jiaogulan and olive) newly developed in The Pangenomics Laboratory were characterized for their physicochemical and biochemical characteristics. Data indicated that all medicinal honeys tested comply with international regulations for blossom honeys, except for olive honey having substandard content of monosaccharides (49.2%). Arjuna, guggul and olive honeys demonstrated outstanding values of phenolics, flavonoids, radical scavenging activity and antioxidant content. The results encouraged the subsequent examination of their effect on oxidative stress and cholesterol homeostasis in the fatty acid-induced HepG2 cell line. Although the stimulation of *Nrf2* gene expression could not be captured for arjuna, guggul and olive honeys, all medicinal honeys up-regulated the expression of *NQO1* gene that is associated with cellular defense pathways in a concentration dependent manner, of which higher transcriptional levels were recorded at 2.0%. Manuka-1, guggul honeys (both, 1.0 and 2.0%) and arjuna honey (2.0%) significantly reduced total cellular cholesterol (TC) in the cells. In contrast, jiaogulan and olive honeys did not decrease TC. The transcriptional levels of the tested genes associated with lipid metabolism (*AMPKα*, *SREBP-2*, *HMGCR*, *LDLR*, *LXRα*, *PPARα*) varied according to honey-type and concentration. Manuka and arjuna honeys showed a good agreement between the transcriptional levels of the tested genes and the reduction of TC. Guggul honey modulated genes and decreased TC but further research at various time points is required to elucidate the mechanism. Jiaogulan and olive honeys regulated the genes in a pattern that should lead to reduced TC, but this expectation did not occur, suggesting their ability to esterify free cholesterol into storage form. Although the medicinal honeys (2.0%) activated *PPARα*,

cellular triglyceride content was not reduced upon combined treatment of fatty acids and honey to HepG2 cells.

In the second experimental chapter, a detailed profile of physicochemical and rheological properties of four blossom honeys (tulsi, alfalfa, manuka-1 and -2) were established. The honeys meet international quality regulations and contain high content of phenolics and flavonoids. Manuka-1 and tulsi honeys produce amorphous diffractograms, supported by flat micro DSC thermograms. In contrast, alfalfa and manuka-2 honeys exhibit multiple WAXD peaks that agree to endothermic transition recorded calorimetrically. Calorimetric and mechanical glass transition temperature (T_g) coincide for honey, indicating the dominant role of sugars in the metastable state of honey matrix. Finally, a good fit of modified Arrhenius and WLF equation allows the determination of free volume characteristics within glass transition state and values of activation energy required for molecular motions in the glassy state of the honeys tested.

In the last experimental chapter, molecular interactions and vitrification characteristics of a gelatin hydrogel in the present of manuka-1 honey (10–75%, w/w) were presented. Thermomechanical analyses exhibit a dramatic glass transition region when cooling samples to subzero temperatures. The implementation of synthetic polymer characterization approach pinpoints predicted mechanical T_g for high-solid hydrogels in comparison to calorimetric T_g. Partial replacement of water molecules within the hydrogels leads to stronger interactions of gelatin and honey moieties and creates amorphous systems which are significant for the development of functional foods and biomedical products. The study provides concrete background for further investigations on topical and oral delivery of bioactive compounds from honey-gelatin gels at controlled rates".

To summarize, physicochemical and biochemical characteristics of manuka-1, arjuna, guggul and olive honeys are presented here for the first time and support the development of

medicinal honeys that combat oxidative stress and hypocholesterolemia. However, their chemical profiles and their effect on pathways associated with the risk factors need to be further studied to provide solid evidence for using the medicinal honeys in the management of such risks. Comprehensive profiles of viscoelastic and structural characteristics of the blossom honeys and honey-containing hydrogels were established as a concrete foundation to maintain optimal quality of medicinal honeys and develop delivery hydrogels of honey's bio-functionality. To make honey hydrogels closer to clinical availability, swelling and release study should be conducted for the controlled delivery of honey and/or its bioactive compounds.

INTRODUCTION

Honey is a sweet reward of nature. The global productivity of honey reached 1.86 million tons, corresponding to a gross production value of $6.38 billion (Faostat, 2016). Australia is the fourth largest honey exporter in the world with gross production value was $110 million and forecasted $122 million in the year 2015-2016 and 2018-2019, respectively according to Australian Bureau of Agricultural and Resource Economics and Sciences (March quarter 2018).

Honey is composed of over 180 compounds including sugars and non-sugars. Its monosaccharides dominate in composition (75–80%), followed by water content (<20%), disaccharides and higher sugars (5–10%), and minor quantities of phenolic compounds, minerals, vitamins, enzymes and organic acids (Bogdanov, 2016; Bogdanov, Jurendic, Sieber, & Gallmann, 2008). Sugars govern physicochemical properties and technological functions, while non-sugar components, mainly phenolic acids and flavonoids impart health benefits and make honey unique from other sweeteners (da Silva, Gauche, Gonzaga, Costa, & Fett, 2016). Interestingly, the nectar and pollen foraging activities of honey bees promotes the transfer of phytochemicals from plants into honey, thus honey is not only an abundant source of antioxidants, but also a carrier of plant medicinal properties (Alvarez-Suarez, Giampieri, & Battino, 2013; Anand, Pang, Livanos, & Mantri, 2018; Yamani, Mantri, Morrison, & Pang, 2014).

Atherosclerosis is among the leading causes of mortality in contemporary societies. Its pathogenesis closely links to oxidative damage and hypercholesterolemia (Barquera et al., 2015). Several strategies have been developed to combat them, but finding an effective natural remedy targeting both is challenging. Honey has been used through human history and widely reported to have powerful antioxidant capacity and improve lipid profiles in chemical and

biological systems (Afrin et al., 2018; Ajibola, Chamunorwa, & Erlwanger, 2012; Atrott & Henle, 2009; Isidorov, Czyżewska, Jankowska, & Bakier, 2011), emphasizing its advantages in modulating atherogenic pathogenesis. However, little is known about its action modes on oxidative stress and lipid metabolism at molecular levels.

Recently, the use of honey as a healthy food or a complementary medicine has attracted great attention. Considerable efforts have been made to incorporate honey into delivery systems such as electrospun meshes, cryogels and hydrogels to enhance its values and overcome its physical limitations (El-Kased, Amer, Attia, & Elmazar, 2017; Minden-Birkenmaier & Bowlin, 2018; Wang, Zhu, Xue, & Wu, 2012). To date, most investigations on physicochemical properties of honey address its quality standards regulated by the Codex Alimentarius (2001) and European directive (2001), whereas understanding structural characteristics and molecular interactions in honey matrix and in honey-incorporated networks is still limited, thus hindering the optimization of honey quality and the development of honey-containing products.

This research was conducted with primary aims to (i) elucidate the effect of medicinal honeys on some key biomarkers of oxidative stress and lipid metabolism, (ii) establish fundamental basis of structural characteristics of honey for technological applications in food and biomedical industries, and (iii) develop a honey-gelatin template as a delivery system of honey's bio-functionality.

Primary research questions

1. Can the selected honey types modulate oxidative stress and lipid metabolism? How do they elicit such protective effects?

2. How structural properties and rheological measurements with mathematical modelling explain the quality and stability of honey?
3. How does honey influences structural behaviors of a gelatin hydrogel?

Specific research objectives

1. Evaluate quality standards of a manuka honey and four fortified honey types with plant extracts and examine their effects on cellular lipids and the expression of key genes associated with cholesterol homeostasis in fatty acid-induced HepG2 cells
2. Establish a platform of physicochemical and viscoelastic properties for honeys originated from floral nectar of medicinal plants
3. Investigate the effect of a representative honey (a commercial manuka honey containing methylglyoxal 400^{+} mg/kg) on structural characteristics of a gelatin hydrogel

Overview of the thesis

Chapter 1: Part 1 reviews (i) composition of honey and its key quality standards to ascertain its uniqueness and superiority to table sugars; and (ii) role and possible action modes of honey in relieving the multifaceted dimensions of atherosclerosis, including oxidative stress and hypercholesterolemia. Part 2 highlights the theoretical concepts of glass transition behind the structural studies in the project.

Chapter 2: Effect of manuka honey and four newly developed medicinal honeys on key biomarkers of antioxidative stress and lipid metabolism on human liver carcinogenic HepG2 cells. The study examines the quality parameters and focuses on the biochemical and molecular

analysis to explain the modulation of intracellular cholesterol triglyceride synthesis pathway by honey treatments of the fatty acid-induced HepG2 cells and suggest a relation of antioxidant activity and cholesterol reduction.

Chapter 3: Physicochemical and viscoelastic properties of honey from medicinal plants. This fundamental study generates the comprehensive information of physicochemical basic standards and structural properties of honeys originated from nectar of four medicinal plants and facilitates development of food and biomedical products containing honey as a main component.

Chapter 4: Structural variation in gelatin networks from low to high-solid systems effected by honey addition. This study examines effect of increasing incorporation of honey on structural properties including thermal, viscoelastic, phase transition and amorphous characteristics of gelatin hydro- and high-solid gels.

Chapter 5: Summary, conclusions and future directions. This chapter connects the previous chapters to present the progression of the research throughout the PhD candidature. Future directions are highlighted at the end of the chapter.

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CHAPTER 1. LITERATURE REVIEW AND BACKGROUND

1.1. Part 1: Honey and its role in relieving multiple facets of atherosclerosis

Nguyen, H. T. L., Panyoyai, N., Kasapis, S., Pang, E., & Mantri, N. (2019). Honey and its role in relieving multiple facets of atherosclerosis. *Nutrients*, *11*(1), 167.

Statement of authorship

Huong Thi Lan Nguyen (candidate) conceptualized, reviewed the literature, executed the writing of the whole manuscript, responded to reviewers and revised the manuscript.

Naksit Panyoyai read the first manuscript, executed the writing of viscosity and rheology sections that were then removed from this paper as followed reviewer's comments.

Stefan Kasapis supervised and edited the first manuscript.

Edwin Pang supervised and commented the first section of the first manuscript.

Nitin Mantri was corresponding author. He supervised, conceptualized, edited the manuscript, submitted it and granted article processing charge.

All the co-authors give full consent to Huong Thi Lan Nguyen to present the paper for examination towards the Degree of Doctor of Philosophy.

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Review

Honey and Its Role in Relieving Multiple Facets of Atherosclerosis

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Abstract: Honey, a natural sweetener has been used universally as a complete food and in complementary medicine since early antiquity. Honey contains over 180 substances, including sugars mainly fructose and glucose, water and a plethora of minor constituents such as vitamins, minerals and phytochemicals. The chemical composition of honey varies depending on floral origin, environment and geographical conditions. The sugar components dominate honey composition and they are accountable for sensory and physicochemical properties in food industry. Although present in small quantities, non-sugar components are the major contributors to the health benefits of honey. Our review summarizes and discusses composition of honey, its protective effects and possible action modes on risk factors of atherosclerosis.

Keywords: Honey; composition; antioxidants; atherosclerosis; inflammation; oxidative stress; cholesterol

1. Introduction

Atherosclerosis is a chronic disease occurring in the inner lining of arterial walls due to the progressive plaque formation [1]. Multiple risk factors are implicated in the pathogenesis of atherosclerosis, including oxidative stress, inflammatory responses, hypercholesterolemia, hypertension, diabetes and cigarette smoking [2,3] (Figure 1). The factors are interrelated and their interactions may intensify the chronic disease [4]. Different strategies developed to relieve the risk factors covering gene therapy, synthetic antioxidants, vitamins and drugs, but atherosclerosis is still a leading cause of death worldwide [1].

Dietary antioxidants have attracted great attention as one of the most favourable options to combat the risk factors. Accumulating evidence indicates plant-originated antioxidant products are far more effective than synthetic counterparts in protecting and/or strengthening the endogenous defence and repairing mechanisms [5–7]. Among those, honey has been reported to exhibit a broad range of beneficial effects [8–11]. Honey has been reported as "a rediscovered remedy" and "a source of dietary antioxidants" [12–15].

Honey is a natural sweetener, contains mainly monosaccharaides (up to 80%), disaccharides (3–5%), water (17–20%) and a wide range of minor constituents such as vitamins, minerals, proteins, amino acids, enzymes and phytochemicals [16,17]. Its composition varies depending on botanical and geographical origin, as well as environmental conditions. The sugar components determine the energy value and its physicochemical properties which are critical for technological functions of

honey [17–19]. Phytochemicals, mainly phenolic acids and flavonoids, are present in smaller quantities but they strongly determine the unique flavour, appearance and bioactivities of honey [17]. Phenolic compounds are known to offer complementary and overlapping modes of action through antioxidant activity, antibacterial and antiviral activities, modulating detoxification enzymes, stimulating the immune system, reducing platelet aggregation, modulating cholesterol synthesis and reducing blood pressure among the others [4,20,21]. Thus, their presence in the composition attributes to the relevant health benefits of honey [22]. Numerous studies have examined the phenolic profiles in honey and reported a high correlation of phenolic content with antioxidant capacity of honey [23,24].

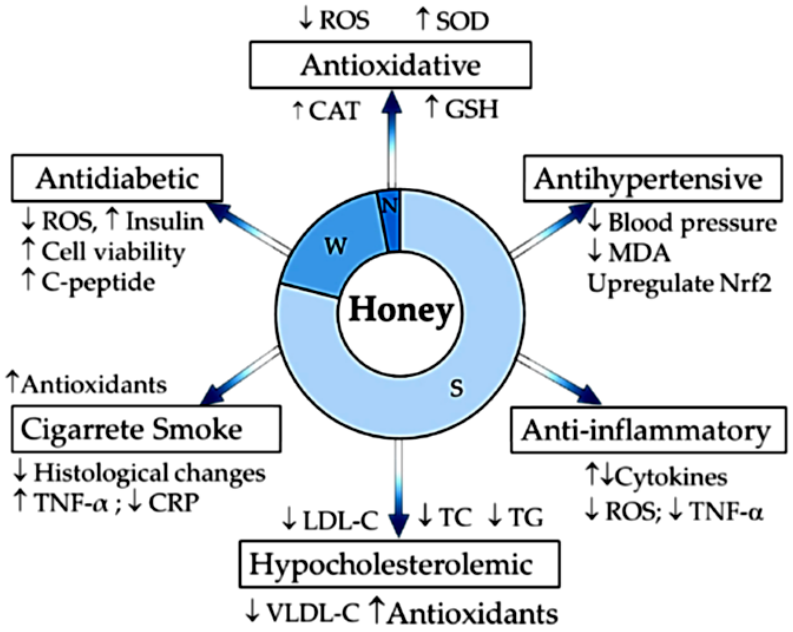

Figure 1. Summary of honey composition and its protective effects against risks in the pathogenesis of atherosclerosis. S: sugar components, W: moisture content, N: non-sugar components, ↓: decrease; ↑: increase; ROS: reactive oxygen species; SOD: superoxide dismutase; CAT: catalase; GSH: glutathione; MDA: malondialdehyde; Nrf2: nuclear factor erythroid 2-related factor 2; TNF-α: tumour necrosis factor alpha; LDL-C: low density lipoprotein cholesterol, TC: total cholesterol, TG: triglycerides, VLDL-C: very low density lipoprotein cholesterol; CRP: C-reactive protein.

Several excellent reviews have been dedicated to the characterization of honey composition and myriad of health benefits [15,17,25–29]. Our review summarizes and discusses: (i) the composition of honey and its key standards to ascertain its uniqueness, as why honey, mostly a sugar solution, elicits numerous health benefits, whereas table sugars are considered to have the reverse effect on health and contribute to growing epidemic of chronic illnesses; (ii) role of honey in relieving the multifaceted dimensions of atherosclerosis with possible action modes. Literature searches from Google Scholar, PubMed, ProQuest, Excerpta Medica dataBASE (EMBASE), ScienceDirect, Cumulative Index to Nursing and Allied Health Literature (CINAHL) and Scopus databases were performed and the keywords including "honey; antioxidant; composition; atherosclerosis, inflammation; oxidative stress; cholesterol" were entered for the reference selection.

2. Honey Composition and Antioxidant Activity

2.1. Honey Composition

Honey consists of over 180 components, including sugars, water and non-sugar components (Table 1) [30]. The sugar components in honey are mainly monosaccharides, particularly fructose

(to 40%) and glucose (35.0%) in some honey types from Asia, Europe and Turkey, followed by a small quantity of disaccharides and higher sugars (<10%) [17]. Fructose and glucose in honey are derived from the chemical conversion of disaccharides in floral nectar by bee-secreted enzymes, where fructose is the highest proportion of any sugars in almost every honey type [15]. Sugars determine the physicochemical properties of honey such as viscosity, crystallization, thermal and rheological behaviour [19]. Sugars in honey provide an energy value of 300 kcal/100 gram honey, which is equivalent to 15% of recommended daily intake of energy [30]. Significantly, fructose contributes the highest proportion in almost every honey types (up to 45.0%) and it is a sweetest sugar among the natural sugars [15]. However, fructose has a lower glycaemic index (GI), compared to sucrose and glucose (GI at 15, 65 and 100, respectively) [31–33]. Since carbohydrate-containing foods are rated according to their GI, where low GI foods are absorbed more slowly from the gastrointestinal tract, fructose-rich honey varieties may be considered as a beneficial alternative to high GI sweeteners in management of diabetes and cardiovascular diseases [30,34].

Table 1. Chemical composition of honey per 100 g [30].

Proximates (g)		**Minerals (mg)**		**Vitamins (mg)**	
Fructose	38.2	Calcium	3–31	Ascorbic acid	2.2–2.5
Glucose	31.3	Potassium	40.0–3500.0	Thiamin	0.0–0.01
Sucrose	0.7	Copper	0.02–0.60	Riboflavin	0.01–0.02
Other disaccharides	5.0	Iron	0.03–4.00	Niacin	0.1–0.2
Water	17.1	Magnesium	0.7–13.0	Pantothenic acid	0.02–0.11
Organic acids	0.5	Manganese	0.02–2.0	Pyridoxine (B6)	0.01–0.32
Proteins, amino acids	0.3	Phosphorus	2.0–15.0		
		Sodium	1.6–17.0		
		Zinc	0.05–2.00		
		Se	0.001–0.003		

The non-sugar components are at minor quantities, but they define a particular type of honey and bioactives, depending on the level of vitamins, minerals, antibiotic-rich inhibine, carotenoids, free amino acids, enzymes, proteins, Maillard reaction products and phenolic compounds present in honey composition [9,30]. Enzymes including invertase (saccharase), diastase (amylase), glucose oxidase and catalase play a critical role in honey formation. Particularly, invertase converts sucrose into monosaccharides, glucose oxidase catalyses hydrogen peroxide formation and catalase (CAT) supports the oxygen and water formation from hydrogen peroxide.

Interestingly, during nectar and pollen forage, honey bees transform phytochemicals from floral nectars of host plants into honey. The diversity of secondary metabolites in plants attributes to the variance phytochemical profiles in honey composition [35]. Phytochemicals in honey are mainly phenolic acids, flavonoids and their derivatives. Phenolic acids (e.g. caffeic, chlorogenic, coumaric, ellagic, ferulic, gallic, homogentisic, phenyllactic, protocatechuic, syringic and vanillic acids) comprise hydroxybenzoic and hydroxycinnamic acids. Hydroxybenzoic acids exert antioxidant capacity (AOC) based on the positions of OH groups in the aromatic ring, with gallic acid (3, 4, 5-trihydrozybenzoic acid) as the most effective antioxidant in this group [36]. Hydroxycinnamic acids present greater free radical scavenging ability because of the unsaturated chain bonded to the carboxyl group, imparting stability to the phenoxyl radical group. Hydroxycinnamic acids offer multiple hydroxyl groups to combat free radicals. In addition, the electron donor groups present in the benzene ring provide a greater number of resonant structures and increase the stability of the acrylic radicals in cinnamic acids [36,37].

Flavonoids (apigenin, chrysin, galangin, hesperetin, kaempferol, luteolin, myricetin and quercetin) consist of two aromatic rings A and B, joined by a 3-carbon link, usually in the form of a heterocyclic ring C [36]. Variations in the ring C result in different flavonoid classes, including flavonols, flavones, flavanones, flavanols, isoflavones, flavanonols and anthocyanidins. Substitutions in rings A and B

generate diverse compounds in each flavonoid class [38]. Depending on the molecular structures, phenolic compounds exert antioxidant capacity (AOC) in different action modes such as metal chelators, free-radical scavengers or gene modulators of enzymatic and non-enzymatic systems regulating cellular redox balance [39]. The presence of a specific phytochemical or combination thereof in honey may potentially serve as a marker for geographical and botanical origin of honey [40,41]. For examples, methylglyoxal is in manuka honey, hesperetin in citrus honey, quercetin in sunflower honey and luteolin in lavender honey [26,41–43]. The structures of common phenolic compounds in honey are presented in Figure 2.

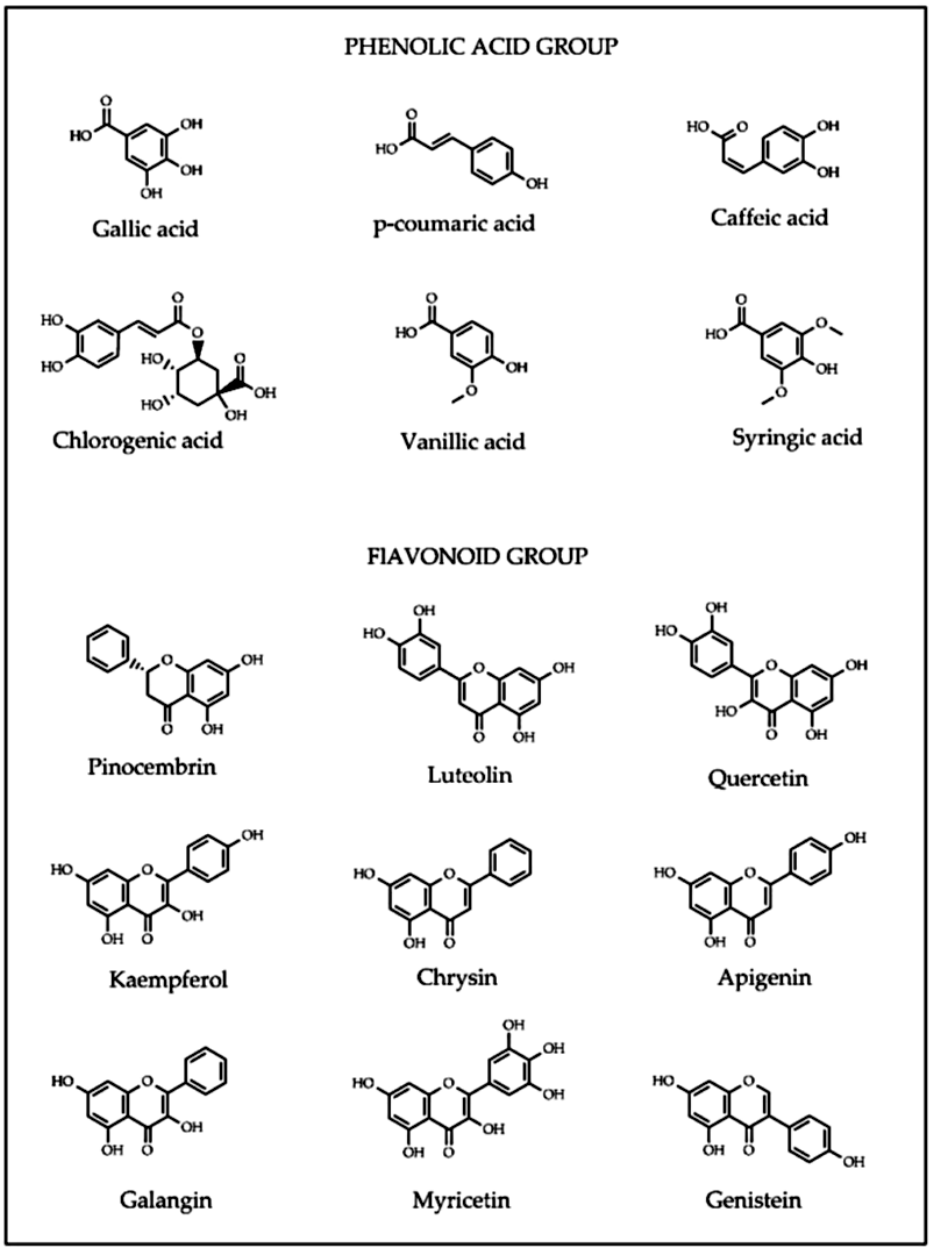

Figure 2. Common phenolic acid and flavonoid compounds identified in honey.

During pollen and nectar forage, bees are exposed to the vegetation, soil, climate and water conditions located approximately within seven km^2 in the vicinity of their hives [44]. The presence or deficiency of a particular element from the environment may be noticeable in the honey.

Thus, the composition profile of honey not only reflects the quality and origin, it is also a bio-indicator of the environment [45].

To sum up, honey composition is complex and variable depending on its botanical and geographical origin. Each constituent has its nutritional, biological and technological functions. They synergistically contribute to the overall utility of honey, making honey unique and superior to other natural sweeteners in providing energy and health benefits.

2.2. Key Compositional Standards

The variations in honey's composition, bee species, seasonal and storage conditions highlight the need for the quality standardization of different honey types. Key compositional criteria have been specified as common quality norms for commercial honey in both European Directive and in the Codex Alimentarius standard [46,47] (Table 2).

Table 2. Key compositional standards of blossom honey [46].

Criteria	Values
Moisture content (%)	≤20.0
Fructose and glucose (Sum, g/100 g)	≥60
Sucrose (g/100 g)	≤5.0
Water-insoluble content (g/100 g)	<0.1
Electrical conductivity (mS/cm)	≤0.8
Free acid (meq/kg)	≤50.0
Diastase activity (Schade scale)	≥8.0
Hydroxymethylfurfural (HMF, mg/kg)	≤40.0

Moisture content (≤20%, w/w) is an important norm for honey, because high moisture content increases the value of water activity and promotes yeast growth leading to fermentation during storage. Exclusively, the osmotolerant yeasts such *Saccharomyces* spp. can grow in a low water activity value at 0.61 using a large amount of glucose and fructose in honey to produce alcohol and carbon dioxide [48]. Honey's moisture content depends on the environmental, processing conditions during the harvesting period and storage.

Reducing sugars (glucose and fructose) and non-reducing sugars (sucrose, maltose) are physical attributes of honey, in particular crystallization process during storage. The amount of sugars in Australian honey is detailed for glucose (28.7–30.6%), fructose (32.8–36.0%), sucrose (1.1–2.2%) and maltose (1.1–2.2%) [49]. Saturated glucose is less soluble than fructose in honey, therefore glucose tends to form nuclei and expand to large crystals in aged honey, while fructose solution is stable in an amorphous state at ambient temperature. Crystallization of honey sugars depends on various factors including glucose concentration, fructose/glucose ratio (F/G) and water residue. Floral honey with a high concentration of glucose undergoes a relatively rapid crystallization compared to honeydew honey [50]. Honey possessing a F/G ratio > 1.3 does not crystallize during lengthy periods of storage, while at F/G < 1.1 its crystallization process occurs readily [51]. The crystallization process also depends on glucose and water content (G/W), whereby honey with G/W < 1.7 crystalizes slowly and with G/W > 2.0, the process is rapid and complete [52].

Electrical conductivity (EC) is a useful and reliable parameter for the determination of botanical origin of honey, since it is dependent and proportional to the content of minerals and organic acids in honey. These compounds are chemically ionizable, so they are capable of conducting electric current in solution [53]. EC is usually determined in a 20% honey solution (w/v) at 20 °C and expressed in milli or microsiemens per centimetre (mS cm^{-1}, µS cm^{-1}) [53]. Different types of honey showed varied EC values, particularly, honeydew honey (822–1213 µS cm^{-1}), heather honey (815–1092 µS cm^{-1}), citrus honey (124–262 µS cm^{-1}) and rosemary honey (89–250 µS cm^{-1}) [54,55]. Thyme honey types originated from Spain and Italy showed a similar EC range (288–559 µS cm^{-1}) [54].

Free acidity, pH and water activity represent texture, stability and shelf-life of honey [56]. Free acidity originates from organic acids. Some studies reported its range of 16.1–34.1 meq/kg for Turkish honeys and around 40.0 meq/kg for Portuguese honeys [57,58]. High acidity is an indicator of sugar fermentation. Honey is usually acidic with a pH range of 3.2–4.5. The low pH and water activity (a_w) values limit the growth of microorganisms.

Diastase activity and hydroxymethylfurfural (HMF) are markers denoting high temperatures and storage conditions. They are also dependent on the honey origin and climate region [59]. Diastase is susceptible to heating and storage factors, while HMF is almost devoid in fresh honey but present in processed and stored honey products. It is known that diastase activity is low and HMF value is high in heated honey [60,61]. Even though the enzyme activity is much more variable than the HFM value of a honey, they are both used for the selection of appropriate processing and packaging techniques among technological applications of natural honey [62].

Although honey colour is not listed amongst the standards, it attracts great attention because it is the first sign reflecting the physicochemical and biological properties of honey. Honey colour is also strongly depends on botanical origin, age, storage and processing conditions [18,26,56]. During storage, honey colour may become lighter as a result of the crystallization process, derived from the development of the white glucose crystals. Significantly, honey colour strongly correlates with the antioxidant potential of honey. Dark colour honey usually has higher ash and total phenolic content with resultant higher antioxidant capacities. For example, dark colour manuka honey (L* = 23.70, a* = 0.09; and b* = 0.15; colour intensity = 7296.7 mAU) showed a significantly stronger antioxidant power and higher phenolic content, compared to lighter colour honeys (L* = 24.90–27.31, a* = 1.42–2.10, b* = 2.66–3.59; colour intensity = 376.7–580.8 mAU) [63].

2.3. Antioxidant Capacity

The antioxidant capacity (AOC) of honey was reported to be the synergistic effect of mainly phenolic compounds along with other constituents in honey composition [28,64]. Considerable AOC values are well documented for a broad range of honey types from different botanical and geographical origins [12,18,43,58,65–73]. This notion was further supported by the fact that AOC value of honey is highly correlated to its phenolic content and colour intensity [23,24,64]. Interestingly, oxygen radical absorbance capacity (ORAC) value of honey was suggested to be equivalent to that of many fresh fruits and vegetables (3–17 μmol Trolox equivalent (TE)/g and 0.5–19 μmol TE/g fresh weight, respectively) [64].

The AOC of a sample is the basis for the quality comparisons, controls and the treatment of associated diseases [74]. The AOC of honey has been extensively examined using a number of popular chemical assays such as total phenolic content, free radical scavenging using 2,2-Diphenyl-1-picrylhydrazyl, trolox equivalent antioxidant capacity and ORAC among the others [23,71,75]. Findings from the assays, however are indicative of limits in either elucidating the total AOC due to the complexity of chemical components and the unique action mode of antioxidants [64] or potential bioactivity under physiological conditions [76]. Therefore, *in vitro, in vivo* and clinical evidence are crucial for further understanding not only AOC but also other biological activities of honey in providing health benefits, particularly attenuating the pathogenesis of atherosclerosis.

3. Honey in Relieving Multiple Facets of Atherosclerosis

3.1. Oxidative Damage

Oxidative stress occurs as a pathological condition due to an excessive generation of radical species over antioxidant defence system [77]. The radical species are represented by superoxide anion radical, hydroxyl, alkoxyl and lipid peroxyl radicals, nitric oxide and peroxynitrite [78]. They attack the cells, oxidize and damage proteins, lipids and deoxyribonucleic acids (DNA) randomly under stress conditions and excessive levels. Organisms have developed self-defence mechanisms towards

neutralizing free radicals including repairing, physical defence and antioxidant systems. Enzymatic antioxidants are represented by superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT). Non-enzymatic antioxidants include ascorbic acid, α-tocopherol, glutathione (GSH), carotenoids, flavonoids and other antioxidants. The balance between the defence systems and free radical species generation is critical for their vitality [4]. The honey's effects on oxidative stress have been the focus of several studies (Table 3). The mechanisms through which honey exerts the protection against oxidative damage resides in (i) antioxidant enzymes in its composition (such as catalase), (ii) phenolic compounds which chelate mental elements, trap or scavenge free radical species and induce cellular enzymatic and non-enzymatic antioxidant systems [24,26,39].

It was indicated that honey significantly inhibits the serum LDL oxidation compared to the sugar analogue and its ORAC values are correlated to its inhibitory effects against LDL oxidation [79]. In another study on the effects of five different honey beverages on human serum, the authors reported serum AOC in the ORAC assay increased by 7% ($p < 0.05$) after intake of buckwheat honey blended beverages (160 g/L), even though values of the serum lipoprotein oxidation and its by-product obtained from thiobarbituric acid reactive substances assay were insignificantly changed. However, this preliminary evidence potentially facilitates the ground works for long-term and epidemiological studies of the health benefits from consumption of honey-blended beverages [80].

The findings were supported by studies which reported the inhibitory effects of honey on lipoprotein oxidation of homogenates from rat liver, brain, lung, kidneys. Particularly, honey decreased the concentration of lipid peroxidation products namely H_2O_2 and malondialdehyde (MDA). This protection is related to the antioxidant activity of honey, which is comparable to those of melatonin and vitamin E [81]. In a later study, Alvarez Suarez et al. [82] found that vine honey displayed the highest capability in scavenging 2,2-Diphenyl-1-picrylhydrazyl, hydroxyl and superoxide radicals among the tested honey group and this honey elicited the remarkable inhibitory capacity against lipid oxidation in rat liver homogenate.

Table 3. Effects of honey on oxidative stress.

Honey Type	Research Model	Main Findings on Honey Effects	Reference(s)
Local honey	Rat kidney, brain, liver and lung homogenates	↓ Lipid hydroperoxides and malondialdehyde (MDA) value	[81]
Christmas vine, Morning glory, black mangrove, linen vine singing bean honey	Rat liver homogenates	Highest radical scavenging capacity in linen vine honey ↓ Lipid peroxidation	[82]
Fireweed, tupelo, Hawaiian Christmas berry clover, acacia, buckwheat, soybean honey	Human blood serum	AOC is different among honeys, ↓ Lipoprotein oxidation (LPO) Correlation of ORAC value and LPO inhibition.	[64]
Acacia, coriander, sider and palm honey	Human LDL	High antioxidant activity in xanthine-xanthine oxidase system and LDL oxidation	[79]
Buckwheat honey	Human blood serum	↑ Serum antioxidant capacity	[80]
Multifloral honey	Human red blood cells (RBC)	↓ Lipid peroxidation	[83]
Multifloral honey	RBC	↓ Extracellular ferricyanide level	[84]
Christmas vine, linen vine honey	RBC	Protection of human erythrocyte membranes from oxidative damage ↑ Defence responses and ↑ cell functions	[82,85,86]
Native multifloral honey	Endothelial cell (EA.hy926)	Protection of EA.hy926 from hydrogen peroxide and peroxyl radical Synergistic effect of phenolic antioxidants in honey	[87]
Gelam honey	Rat blood sample	↑ Antioxidant enzyme activities ↓ Hypertriglyceridemia and pro-oxidative effects	[88]
Multifloral honey	Rat plasma and heart tissue	↑ Plasma α-tocopherol and α-tocopherol/triglycerides, ↓ plasma NOx, ↓ peroxidation	[89]
Buckwheat honey	Human blood plasma	↑ Plasma antioxidant activity, ↑ defences against oxidative stress	[90]

AOC: antioxidant capacity, ORAC: oxygen radical absorbance capacity, LPO: lipoprotein oxidation, LDL: low density lipoprotein, RBC: Human red blood cells, TG: triglycerides, NOx: nitrogen oxides.

Endothelial cells play an important role in homeostasis, immune, inflammation, cell adhesion, thrombosis and fibrinolysis regulation, thus endothelial dysfunction initiates atherosclerotic progression [91,92]. Significantly, honey has been shown to enhance endothelial function through quenching lipophilic cumoxyl and cumoperoxyl radicals, suppressing cell damage, inhibiting cell membrane oxidation and decreasing reactive oxygen species (ROS) generation and GSH recovery in EA.hy926 endothelial cells [87].

Although human red blood cells (RBCs) are not directly related to atherosclerosis, their alterations may enhance the severity of atherosclerosis. RBCs are sensitive to oxidative damages due to the structural and functional characteristics, thus lipid oxidation of erythrocyte membrane causes the cell death or erythrocyte haemolysis. Honey flavonoids have been reported to prevent the peroxidation process of the lipid membrane, intracellular GSH depletion and SOD decline in RBCs, thus protect the cells from oxidative haemolysis and reduce extracellular ferricyanide [82–84]. Studies have suggested flavonoids localize in the membrane bilayer and form specific bindings to lipids and proteins in RBC membranes. As a consequence of this process, the membrane is protected from the peroxidation and strengthened against the stress factors [85,93]. Alternatively, flavonoids such as quercetin may be incorporated into RBCs to exert antioxidant effect on RBC membranes [83,86].

Gelam honey has also been evidenced to reduce MDA level, a product of peroxidation process and protect DNA oxidative damage in both the young and aged rats. The honey increased the activity of antioxidant enzymes namely erythrocyte CAT and cardiac SOD in young group, while increasing the activity of only cardiac CAT in both of the young and aged groups. The authors suggested that the reduced oxidative damage in honey-fed rats was related to the elevation of antioxidant enzyme activity under the effect of Gelam honey [88]. Another investigation revealed that honey promoted higher plasma tocopherol content, tocopherol/triglyceride level but lower plasma NOx levels and a reduced susceptibility of heart towards lipid oxidation in the honey-fed rats compared to the control [89]. The findings are consistent with results from a previous study in human plasma, where honey consumption increased plasma total phenolic content ($p < 0.05$), antioxidant and reducing capacities ($p < 0.05$) [90]. Therefore, honey substitution as a sweetener provides health benefits through the enhancement of the antioxidant defences.

The cardio-protective effect of honey has been further demonstrated in urethane-anesthetized rats administered with epinephrine whereby honey pre-treatment (5 g/kg) for one hour was found to reduce the epinephrine-induced incidence in anesthetized normal rats while honey post-treatment significantly prevented the incidence in anesthetized stressed rats. The studies suggested that the pronounced antioxidant components in honey contributed to the protection of cardiovascular system [94].

Taken together, the studies reported antioxidant capacity of honey from different origins in different models and the mechanisms through which honey exerts its antioxidant activity resides in (i) antioxidant enzymes in its composition (such as catalase), (ii) the high content of phenolic compounds which chelates mental elements, traps or scavenges free radical species and induce cellular enzymatic and non-enzymatic antioxidant systems [24,26,39]. It should be taken into considerations that the total antioxidant capacity of honey resulted from the synergistic interaction of different compounds, including phenolics, peptides, organic acids, enzymes, Maillard reaction products and other minor components. However, due to a loss of up to 40% in total phenol content and total antioxidant activity during the fractionation process [87,95], the overall effects of honey on oxidative damage obtained from the tested fractions could be underestimated. In addition, some studies evaluated the effects of honey on lipid peroxidation of tissue homogenates which may contain a wide range of compounds (proteins, intracellular lipids and others) interfering with the test specificity. Thus, it is suggested that measurement of generated lipid hydroperoxide concentration is more indicative and specific for the evaluation [82]. To date, the investigations on honey's antioxidant effects *in vitro* and *in vivo* focus on the aqueous portion of the blood (plasma) where honey antioxidants dissolve, it is suggested that the future studies should progress to evaluate the honey's effects on the lipid components of the human

body [90]. Given the limitations, the findings are supportive to the hypothesis that honey could play a role in protecting biological systems from oxidative damage.

3.2. Inflammatory Responses

Inflammation reflects a pathophysiological response of tissues characterized by signs of pain, heat, redness and swelling [96], however, prolonged inflammation is the cause of several chronic diseases such as diabetes, dyslipidaemia, hypertension, cardiovascular, obesity and pulmonary conditions. Under inflammatory conditions, mitogen-activated protein kinase (MAPK) and nuclear factor kappa B (NF-κB) pathways are activated, triggering several important proinflammatory markers including cyclooxygenase-2 (COX-2), lipoxygenase 2 (LOX-2), C-reactive protein (CRP), interleukins (IL-1, IL-6 and IL-10) and tumour necrosis factor alpha cytokine (TNF-α) [27]. Honey was found to modulate the inflammatory response in the pathogenesis of atherosclerosis through distinct inhibitory paths of (i) proinflammatory markers such as cytokines, COX-2, CRP and TNF-α [97–100] and (ii) ROS generation [101].

It was reported that the anti-inflammatory activity of honey is contributed by phenolic compounds and other minor constituents in its composition [101–104]. Kassim et al. detected a range of phenolic compounds, including chrysin, quercetin, ferulic acid, ellagic acid, hesperetin in Gelam honey. This honey reduced cytokine (TNF-α, IL 1β and IL 10) and NO levels but increased heme oxygenase-1 levels. Thus, the honey was recommended to be further investigated for treatment of different inflammatory diseases [100]. Some phenolic compounds have been individually examined for their anti-inflammatory activity. Chrysin was reported to suppress lipopolysaccharide-induced COX-2 in Raw 264.7 cells [97]. Luteolin was found to reduce intercellular adhesion molecule-1 and TNF-α and eradicate leukocyte infiltration in tissues [99]. Quercetin was demonstrated to reduce human CRP expression and also serum amyloid A and fibrinogen which are cardiovascular risk factors in mice [98].

The findings are supportive to a study on the anti-inflammatory effect of a natural honey type on bovine thrombin-induced oxidative burst in human neutrophils and rodent macrophages. It has been known that the accumulation of phagocytes, ROS production and thrombin activation occur at the sites of endothelial damage [101]. It was demonstrated that bovine thrombin-activated phagocytes produce ROS which might amplify the inflammatory responses at the site of atheromatous plaques. However, honey treatment suppressed the thrombin-induced ROS generation by the phagocytes. The findings suggested a beneficial role of honey in the pathology of atherosclerosis, particularly in ROS-induced LDL oxidation and cell signalling [101].

3.3. Hypercholesterolemia

Cholesterol is an indispensable molecule in growth and development of animal and human cells. It fulfils vital functions such a cell membrane component, a precursor for steroid hormones and bile acids and an activator in cell signalling pathways [105]. Cholesterol is combined with lipoproteins so that they are transported from one tissue to the others throughout the body. Lipoproteins are divided into high density lipoprotein (HDL), low density lipoprotein (LDL) and very low-density lipoprotein (VLDL), thus cholesterol (C) is classified accordingly into HDL-C (good cholesterol), LDL-C and VLDL (bad cholesterols) [106].

Table 4. Effects of honey on lipid profile.

Honey Type	Research Model	Main Findings of Honey Effect	Reference(s)
Honeydew honey	Rat blood serum	Similar weight gain and body fat in honey and control group; ↓ HbA1c, ↑ HDL-C	[107]
Clover honey	Rat blood serum	↓ Weight gain and adiposity, ↓ TGs but ↑ non-HDL-C levels	[108]
Native honey	Rat blood samples	↓ glucose and lipids no deteriorated effects on hyperglycaemia and dyslipidaemia	[109]
Local honey	Rat blood serum	↑ Plasma TG, HDL-C and VLDL-C but ↓ plasma LDL-C and TC	[110]
Tualang honey	Rat heart tissue	↑ Antioxidant enzyme levels in heart tissue and ↓ lipoprotein oxidation (LPO)	[111]
Tualang honey	Rat blood serum, kidneys	↓ TC and TG compared to the control at 7 days; ↓ Serum creatinine level than no honey group after 48 h; No structural effect histologically in the HCD-fed rats	[112]
Gelam, Acacia honey	Rat blood serum, internal organs	↓ Excess weight gain and adiposity index; ↓ plasma glucose, TGs, TG and obesity at similar levels to orlistat drug group	[113]
Malícia honey	Rat blood serum, liver	↓ Food consumption, ↑ glucose tolerance and SOD activity; ↓ TC, LDL and AST levels; ↑ beneficial bacteria and organic acids; Colon and liver was protected	[114]
Natural local honey	Healthy, diabetic and hyperlipidaemic human subjects, blood samples	↓ Blood lipids, homocysteine and C-reactive protein (CRP) in normal and hyperlipidaemic subjects; ↓ plasma glucose elevation in diabetics	[115]
Natural honey	Human plasma	↓ TC (3.3%), LDL-C (4.3%), TGs (19%) and CRP (3.3%) in elevated variable subjects; No increased body weight in overweight or obese participants	[116]
Natural unprocessed honey	Type 2 diabetes human subjects, weight and blood samples	↓ Body weight, TC, LDL-C, TGs ↑ HDL-C and HbA1C levels	[32]
Kanuka honey, formulated with cinnamon, chromium and magnesium	Type 2 diabetes human subject, weight and blood samples	↓ Weight Improve blood lipid profile	[117]

HbA1c: Haemoglobin A1c, HDL-C: high density lipoprotein cholesterol, LDL-C: low density lipoprotein cholesterol, VLDL-C: very low density lipoprotein cholesterol, TC: total cholesterol, TGs: triglycerides, LPO: lipoprotein oxidation, HCD: high cholesterol diet, AST: aspartate aminotransferase, CRP: C-reactive protein.

A high level of LDL-C is the main cause of plaque formation in blood vessels, which when occurred in coronary arteries, it results in blockages and heart attacks [7]. In addition, a marked elevation of lipid oxidation products and/or a reduction in plasma antioxidants promotes hypercholesterolemia [118]. Use of dietary antioxidants combined with physical exercises has been recommended as a premised lifestyle approach to control cardiovascular risks in general and cholesterol levels in particular [119].

Containing an abundant source of phenolic compounds [9,15,115], honey has been shown to improve lipid profile, particularly cholesterol levels (Table 4). The exact mechanism of honey in the improvement of this risk factor has not been clearly determined. However, phenolic compounds present in honey are reportedly associated with improvement of coronary vasodilation, prevention of blood clots and protection of LDL-cholesterol from oxidation [120]. Several natural phenolics have been reported to reduce cholesterol, including quercetin-3-β-D-glycoside, vanillin rich fraction and luteolin among the others. The phenolic compounds have been known to (i) decrease cholesterol level through the inhibition of 3-hydroxy-3-methylglutaryl co-enzyme A (HMG-CoA) reductase which is a crucial rate limiting enzyme in cholesterol biosynthesis, and/or (ii) modulate plasma LDL-C via the upregulation of LDL-receptor (LDLR) expression, of which LDLR is a cell surface glycoprotein important to the hepatic uptake and removal of plasma cholesterol [121–124]. It has been demonstrated that honey is a potential alternative for sucrose intake in individuals with poor glycaemic control and/or coronary heart disease. In a study, the long-term 52 week consumption of honey did not result in any differences in LDL-C, triglyceride (TG) or total cholesterol (TC) levels among the rat groups. However, honey diet revealed a significant increase in HDL-C levels (16% to 21%) in honey diet rats, compared to sucrose ($p = 0.044$) or sugar-free diet group ($p = 0.006$) [107].

High carbohydrate diets are connected to obesity and impaired adipose metabolism. The effects of honey on weight gain, adiposity and related biomarkers were evaluated in a study feeding rats with clover honey (honey diet group) and compared with sucrose (sucrose diet group) for 33 days. The authors found that the honey diet reduced body weight ($p \leq 0.05$) and serum TGs concentrations ($p \leq 0.05$) compared with the relevant sucrose diet. However, honey did not result in significant differences in serum HDL-C and TC [108]. In another study, honey significantly increased TG, HDL and VLDL levels and decreased plasma LDL and TC levels compared to the control group [110]. The findings are consistent with results from a recent study comparing the ameliorating effects of honey on hyperglycaemia and hyperlipidaemia in diabetic rats fed with honey for 3 weeks. The study found that use of honey (1.0 and 2.0 g/kg) increased HDL-C ($p < 0.05$) and reduced hyperglycaemia, TGs, VLDL-C, non-HDL-C, coronary and cardiovascular risk index ($p < 0.05$). However, honey at higher dose (3.0 g/kg) reduced only TGs and VLDL-C ($p < 0.05$) [109].

These results are comparable to findings from the examination of the effects of Gelam and Acacia honey on weight gain and obesity-related parameters using male Sparague-Dawley rats fed with high cholesterol diet (HCD) before treatments. The study reported a reduction in excess weight gain and adiposity index in honey group compared to control group. The honeys and the orlistat drug which elicited hepatotoxicity effects showed similar effects in significantly reduced levels of plasma glucose, triglycerides and cholesterol, obesity related parameters in rats. The authors suggested Gelam and Acacia honey are more effective than orlistat in obesity control through regulation of lipid metabolism [113]. The finding was supportive to the investigation on the renoprotective effect of Tualang honey on HCD fed rats. It was found that the TC and TG levels were markedly decreased in the honey group compared to the control at 7 days ($p = 0.025$ and 0.031, respectively). The honey group also was found to have considerably lower serum creatinine level than untreated group after 48 h ($p = 0.018$). This study indicated that the honey showed some degree of renoprotective effect biochemically [112]. Tualang honey was also examined by another research group using isoproterenol-injected rats. Isoproterenol can cause severe oxidative damage in the myocardium leading to infarct-like necrosis in the heart muscle when administered in large doses. It was reported that isoproterenol-induced rats exhibited a significant elevation of serum TC, TGs, cardiac marker enzymes (creatine kinase-MB, lactate dehydrogenase) and aspartate transaminase, cTnI and also a

decrease in antioxidant enzymes. However, the oral administration of Tualang honey (3 g/kg) for 45 days prior to isoproterenol treatment modulated TG, recovered the antioxidants and the mentioned parameters in rats [111].

The effect of honey on lipid metabolism was further confirmed by Bezerra et al. [114]. In this study, honey from *Mimosa quadrivalvis* L. produced by the *Melipona subnitida* D. (jandaira) stingless bee was evaluated for its effectiveness on lipid parameters, an antioxidant status and intestinal health of dyslipidaemic rats (1 g/kg) for 35 days. It was found that the honey group demonstrated lower food consumption, increased glucose tolerance and SOD activity, decreased total cholesterol, LDL and aspartate aminotransferase hepatic enzyme. Honey also increased beneficial bacterial population (*Bifidobacterium* spp. and *Lactobacillus* spp.) and organic acid excretion detected in faeces of dyslipidaemic rats. Taken together, honey administration showed the positive effects on the modulation of metabolic disorders and lipid profile improvement in a dose- and time-dependent manner, however, the implications of these findings need to be clarified through further animal studies.

Human clinical studies have been conducted as an addition to the *in vitro* and *in vivo* studies to further understand the effect of honey on lipid profile. Waili [115] investigated the effect of honey consumption on diabetic and hyperlipidaemic subjects for 15 days. In healthy subjects, honey consumption was found to decrease TC (7%), LDL-C (1%), TGs (2%), C-reactive protein (7%), homocysteine (6%) and plasma glucose level (PGL) (6%), while HDL-C (2%) levels were elevated. However, in patients with high blood lipid profile, the effect of honey was more pronounced in reducing TC (8%), LDL-C (11%) and CRP (75%), while sugar analogues increased LDL-C levels [115].

The findings were further supported by an independent clinical study with 55 obese individuals divided into two groups, an experimental group (n = 38) that daily consumed 70 g of Iranian natural honey and a control group (n = 17) consumed 70 g of sucrose for 30 days. The authors found honey exhibited positive effects on cardiovascular (CDV) risk factors, homocysteine and CRP without side effects and significant weight increase. In details, honey decreased TC (3%), LDL-C (5.8%), TGs (11%), fasting blood glucose (4.2%) and CRP (3.2%), while it increased HDL-C (3.3%) in individuals with the normal CDV parameters. However, honey showed a more noticeable effect in the reduction of TGs (19%) in individuals with the abnormal parameters [116].

The effect of natural honey was explored in patients with type 2 diabetes for lipid variables and body weight. The patients were divided into honey group (n = 25) and non-honey group (control, n = 23) for 8 weeks. After baseline adjustment, the fasting blood glucose in the two groups were not significantly different, however, the honey group showed a significant reduction in body weight, TC, LDL-C and TGs and dramatic increase in HLD-C (high density lipoprotein cholesterol) levels [32].

The findings are congruent with a recent study by Whitfield et al. [117]. In this study, the formulation of Kanuka honey with cinnamon, chromium and magnesium was investigated for its effect on glycaemic control, weight and lipid profile in 12 patients with type 2 diabetes. Consumption of the 53.5 g honey blend for 40 days significantly increased the body weight and improved lipid parameters in the subjects. In addition, a tendency in the increase of HDL and reduction of systolic blood pressure was also observed. However, the formulation did not affect glucose metabolism or glycaemic control.

Recently Tul-Noor et al. [125] reviewed clinical studies and undertook meta-analysis to assess the effects of honey intake on lipid risk factors, compared to sugar analogues. The authors found 10 eligible trials with a total of 444 samples, median period of 5 weeks and an average honey dose of 70 grams/day. They reported that regular administration of honey results in a reduction in LDL-C (p = 0.02), fasting triglycerides ($p < 0.001$) and an increase in HDL-C level ($p < 0.001$). They also found evidence of substantial inter-study heterogeneity for LDL-C ($p < 0.001$) and non-significant heterogeneity for fasting triglycerides and HDL-C ($p > 0.10$). However, the overall quality of the evidence in the analysis was assessed as "low quality" for LDL-C, "moderate quality" for fasting triglycerides and "moderate quality" for HDL-C according to the consistency and precision of data and publication variance. The authors recommended that honey intake showed a beneficial effect on

lipid profile including LDL-C, TGs and HDL-C in participants at different health backgrounds but trials need to be at larger, longer scale and higher quality [125].

Although human clinical studies on cholesterol-lowering effects of honey are scattered, limited in size and time course, overall these studies have indicated a consistent and promising effect of honey in improving overall lipid profile, particularly reduced LDL-C and TGs and increased HDL-C in the research objects. Natural honey contains mainly fructose and glucose components, however the studies showed its positive effects outweighed sugar analogues on lipid profile [115,116]. The results suggest a functional role attributed to the non-sugar components in honey composition. Plant phenolic antioxidants are reported to be effective in improving blood lipid profile, thus they are possibly the primary contributors to those positive effects of honey [122,124,126]. Nonetheless investigations on the underlying mechanism of honey on hypercholesterolemia have not yet provided strong evidence and need larger clinical studies to explore further the mechanism.

3.4. Hypertension

Hypertension is closely implicated in the pathogenesis of atherosclerosis. Recent studies which reported honey reduced systolic blood pressure and MDA levels in hypertensive rats [127] and alleviated the susceptibility of rat kidneys to oxidative damage through upregulating the expression of erythroid 2-related factor 2 (Nrf2), an important transcription factor regulating antioxidant defences in chronic renal failure or hypertensive rats [128]. The results have indicated that the protective effect of honey on hypertensive rats is mainly contributed by its antioxidant and anti-inflammatory activity.

3.5. Diabetes

Diabetes is implicated in inflammation, oxidation and glycation. Therefore, strong antioxidant agents potentially limit the pathogenesis of diabetes and the associated complications [129]. Gelam honey extract has been found to protect pancreatic hamster cells from hyperglycaemic conditions. Significantly, this honey decreased ROS production, glucose-induced lipid peroxidation, increased insulin content and the cell viability under hyperglycaemic conditions [130]. The findings were supported by an investigation of Jujube honey for its role in modulation of the main enzymes participating in glucose metabolism namely glucokinase and glucose 6-phosphatase in rats. Jujube honey was found to reduce MDA levels while improving the total AOC in diabetic rats ($p < 0.05$). It also decreased heat shock protein (HSP70) and glucose 6-phosphatase expressions, while increasing the glucokinase expression [131].

Moreover, a pilot study with 20 patients with type 1 diabetes and 10 healthy controls showed honey treatment reduced glycaemic index and the peak increment index in both patients ($p < 0.001$) and control ($p < 0.05$) groups compared to sucrose. In this study, honey significantly increased C-peptide level, compared to either glucose or sucrose in the control group. The results suggested honey may be used as a sugar substitute for patients with type 1 diabetes [132]. Collectively, the findings suggested potential effect of honey on diabetes management in animal models should be translated into larger clinical trials for type 1 diabetic patients.

3.6. Cigarette Smoking

The active or passive exposure to cigarette smoking is implicated in all stages of atherosclerosis and complicates cardiovascular events [133]. Tualang honey was examined for its protective effect on rats exposed to cigarette smoke. It was found that honey protected rat testis from oxidative stress caused by tobacco smoking. The honey decreased the histological changes and lipid peroxidation, but it increased the total antioxidant levels and recovered the activity of antioxidant enzymes, particularly glutathione peroxidase, SOD and catalase in the cigarette smoke-exposed rats [134].

The findings were further supported by a recent study which examined the effect of a 12-week honey administration on plasma inflammatory markers such as highly sensitive CRP, IL-6 and TNF-α among 32 non-smokers and 64 chronic smokers [135]. The study reported that TNF-α was

significantly increased, but CRP expression was significantly reduced at post-intervention among smokers with honey group. These indicated that effects of honey on TNF-α and CRP are opposite, thus it raises the needs for further investigations on the inclusive effect of honey on inflammation among chronic smokers.

4. Adverse Effects of Honey

Despite the nutritional and medicinal values, honey is prone to microbial and non-microbial contaminations. Several microorganisms including bacteria, moulds, yeast from pollen, bee intestine, human, equipment, containers and dust may infect honey. However, honey has antimicrobial properties due to the synergistic contributions of saturated sugars (~80%), acidic pH, bee defensin 1, inhibines (hydrogen peroxide, flavonoids and the phenolic acids) and low water activity [136,137]. However, spore-forming bacteria can resist for over a year in honey at low temperature [138,139], particularly the *Clostridum botulinum* causing botulism poisoning was detected in many countries [138,140–142]. Thus, raw honey that was not sterilized or qualified should not be used for infants. It was also recommended that *Clostridia* spores need to be eliminated from honey using gamma irradiation, a sterilization process which is not interfered with antibacterial activity of honey [143].

In addition, honey may contaminate with traces of pesticides, herbicides, antibiotics or heavy metals due to the bee disease control and the exposure of honey bees to environment [139]. Honey also may contain poisonous compounds, particularly grayanotoxins found in mad honey which originates from *Andromeda* flowers [144]. Thus, honey needs to be subjected to quality analysis and labelling regulations. Moreover, honey production and processing have to comply with standard protocols and legislation to assure its safety.

5. Conclusions

Honey composition is a mixture of saturated sugar and non-sugar constituents, varying accordingly to the environment, botanical and geographical origin. Dominant sugars are fructose followed by glucose, so honey is a lower GI product compared to table sugars. The non-sugar constituents such as enzymes, amino acids, vitamins, minerals, phenolic compounds are at minor quantities, but they define health benefits of honey. Each of them has its own nutritional and functional value(s) and they work together to contribute to the biological and physicochemical properties of honey, making honey a unique sweetener.

Atherosclerosis is a damaging chronic disease globally. Interestingly, several studies have emphasized the role of honey in attenuating the aforementioned risks in the pathogenesis of atherosclerosis. The beneficial effects are mainly attributed to phenolic compounds in honey composition. The mechanisms through which honey elicit the protection are associated with scavenging radical species, suppressing lipid peroxidation, strengthening enzymatic and non-enzymatic antioxidant systems and stimulating/inhibiting proinflammatory markers. However, further research in particular clinical translations will progress to better management strategies of the chronic disease, with concomitant expanded applications of honey in food and pharmaceutical industries. In addition due to possible microbial and non-microbial contaminations, honey quality should be complied with safety regulations and international standards.

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1.2. Part 2: Theories of glass transition – a structural transformation in food materials

Food products possess various structural characteristics and compositions, but they are mainly composed of carbohydrates, water, proteins and lipids (Pomeranz, 2012). Carbohydrate molecules vary in their weight, from simple sugars (fructose and glucose) to polymeric molecules (pectin, starch, gellan, carrageenan and others). Proteins represent polymeric structures with various conformations such as gelatin, bovine serum albumin. The biomaterials are extensively reported to exhibit a disordered and amorphous state, particularly in high-solid and frozen foods (Devi, Liu, Hemar, Buckow, & Kasapis, 2013). These characteristics emphasize physicochemical similarities of bio- and synthetic polymers and accommodate applications of sophisticated polymer approach in food products to explore their physical and structural transformations (Kasapis & Sablani, 2008; Roos, Karel, & Kokini, 1996; Slade & Levine, 1995).

1.2.1. Glass transition temperature and its significance

Glass transition reflects thermodynamic properties of a second-order phase transition in a time and temperature dependent manner. In details, this is a reversible transformation from the solid state to liquid (rubbery) state of amorphous materials over a temperature range. Glass transition phenomenon reflects the changes of enthalpy, heat capacity, free volume, thermal expansion coefficient (Kasapis, 2009; Roos, 2003).

Glass transition temperature (T_g) is the most critical thermal event in food science as it reflects stability and quality characteristics of biomaterials. Glass transition temperature is commonly pinpointed a single value of a midpoint of the glass transition range (Sperling, 2005). It is extensively used to predict physical and chemical behaviors of amorphous and

partially amorphous foodstuffs such as softness in snack products, sugar crystallization, the stickiness and caking (Kasapis, 2009; Roos et al., 1996). It has been known that food materials develop soft and gummy properties, decrease viscosity and increase molecular mobility beyond their T_g. On the contrary, they become rigid and most stable with limited molecular mobility below their T_g (Li, Lin, Roos, & Miao, 2019; Sablani, Kasapis, & Rahman, 2007).

1.2.2. Factors influencing glass transition temperature

Glass transition temperature determined in a food material is mainly influenced by composition, molecular weight, and plasticizers including water and other co/solutes as these factors alter thermomechanical characteristics and molecular interactions within polymeric networks (Jadhav, Gaikwad, Nair, & Kadam, 2014).

1.2.2.1. Composition of a food system

Food systems are usually composed of a biopolymer mixed with another biopolymer or co/solute. The combination develops a structural network through inter-molecular interactions forming phase-separated or crosslinked networks. Phase separation, for example, in gelatin/polydextrose mixture, depends on the total solids and gelling polymer concentrations. Some polymers have branched side chain ends, thus their presence increases viscosity and dynamics in mixtures. Molecular mobility in phase-separated systems reduced with the increase of total solids. This is observed in T_g values located at −30 ^{o}C and −22 ^{o}C for polydextrose and gelatin/polydextrose blend, respectively (Almrhag et al., 2012). In crosslinked systems, adjacent molecules are chemically linked to form a three-dimensional structure. T_g values in such systems depends on characteristics of chemical bonds (e.g. covalent, hydrogen), crosslinking density and molecular weight. A denser crosslinked system has stronger physical properties as it has several points for storing shear energy and reducing

bond rotations, thus its T_g values increases. This can be observed in the work by George, Lundin, and Kasapis (2014) showing mechanical T_g increases from −38 to 1 °C for networks of 15% protein fraction and a 65% co-solute as a result of conformational changes in the different protein fractions.

1.2.2.2. Molecular weight

The dependence of T_g on molecular weight is described in the equation (1.1) that was developed by Fox Jr and Flory (1950):

$$T_g = T_{g\infty} - \frac{K}{(\alpha_R - \alpha_G)M} \quad (1.1)$$

And equation (1.2) by Fox and Loshaek (1955):

$$\frac{1}{T_g} = \frac{1}{T_{g\infty}} + \frac{K}{T_{g(\infty)}^2 M} \quad (1.2)$$

Where K is a constant, α_R and α_G are thermal expansion coefficients in rubbery and glassy states, respectively, M is molecular weight (g/mol).

Equation (1.2) clearly indicates a linear correlation of glass transition and high molecular weight systems (Fox & Loshaek, 1955). An empirical investigation by Kasapis, Al-Marhoobi, and Mitchell (2003a) indicated T_g values of mixtures of gelatin fractions and sugars raised from −30 to −14.5 °C relevant to an increase of gelatin molecular weight from 29.2 to 68.0 kD.

1.2.2.3. Plasticizers

Plasticizers increase free volume but decrease viscosity in the micro-environment (Ferry, 1980). In addition, their structural variations significantly influence T_g of food materials (Lourdin, Coignard, Bizot, & Colonna, 1997). Water is extensively known as an effective plasticizer as it forms strong interactions (hydrogen bonds) with structural elements in food

systems (Masavang, Roudaut, & Champion, 2019; Sablani et al., 2007). Roos (2010) indicated that food materials such as starch, glutenin, maltose and fructose experience decreased T_g values when their water content increases (Figure 3).

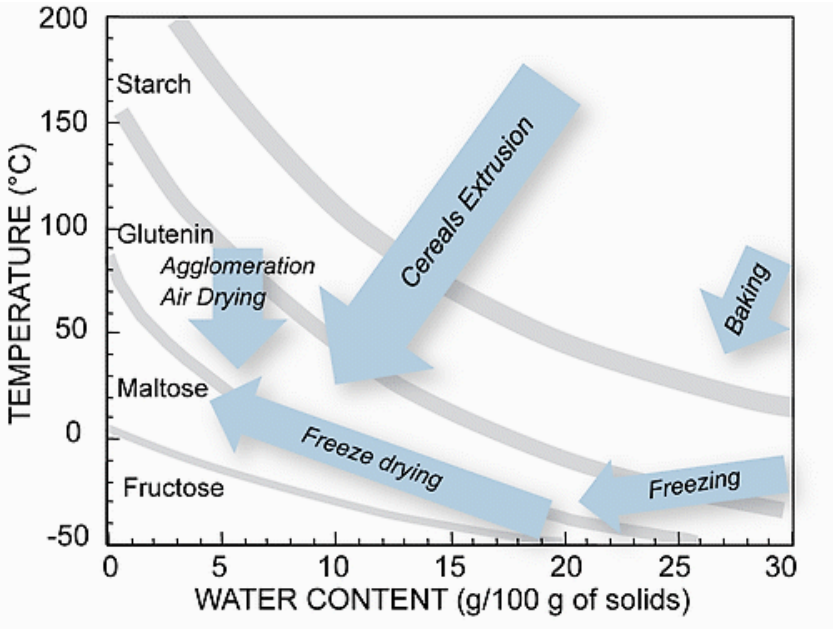

Figure 3. Starch, glutenin, maltose and fructose exhibit different ranges of glass transition temperature depending on their water content. Cereals can form glassy state after being plasticized and extruded, but fructose does not form solid state at the same conditions (Roos, 2010).

As small molecule plasticizers can increase molecular mobility leading to lower T_g values for a system, many of them have been using to control T_g of the polymers. For example, trehalose and mannitol are used to lower T_g of β-cyclodextrin polymer, making its structural transformation observable and improve its applicability in developing tablets for oral administration as cyclodextrin can complex with a wide range of active compounds (Tabary et al., 2016).

1.2.3. Structural relaxation

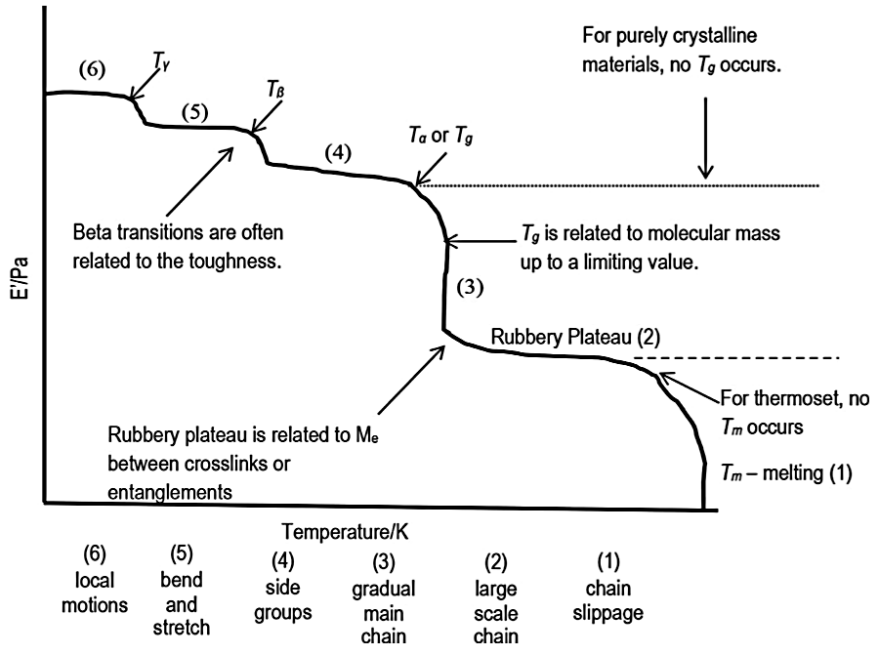

Figure 4. Molecular relaxations scanned by dynamic mechanical analyzer (Menard & Menard, 2006).

Relaxation time is defined as the time required for an equilibrium recovery of a property after interference(s), such as an applied stress to an amorphous material. Relaxation time is strongly influenced by the viscosity of the material, e.g. short relaxation times are in low viscosity systems, and *vice versa* (Champion, Le Meste, & Simatos, 2000). Figure 4 displays various molecular relaxations coupled with different degrees of molecular motions (Menard & Menard, 2006). Molecules are tightly compressed with negligible motions at very low temperatures (region 6). Early heating regime expands gradually the free volume, initiates movements of backbone and side chains, leading to a gamma transition, T_γ. Continuous heating and free volume increase generate enough space for the motions of localized groups (4–8 atoms) and side chains, thus the material experiences some toughness and a beta transition, T_β at this stage. Further heating coordinates chains to large-scale motions (region 4) in the amorphous regions and the progression of a glass transition, T_g, followed by a T_α where

segmental motions occur, and viscosity reduces (region 3). Finally, the material undergoes a melt (region 2), T_m where large-scale chains slip, and the material flows at the end of heating route.

1.2.4. Free volume theory

Free volume theory has been extensively used to explain molecular relaxations of a polymer with regard to its free volume, occupied volume and specific volume (Ferry, 1980; Li et al., 2019) as illustrated in Figure 5. The free volume indicates the available space for molecular motions in amorphous regions (v_f). The occupied volume (v_o) defines the fraction that polymer molecules locate. The specific volume (v) is the total of free volume and occupied volume. Free volume usually is around 30% of the total volume in polymer melts and collapses to 3% in the glassy state (Cangialosi, Schut, Van Veen, & Picken, 2003; Ferry, 1980).

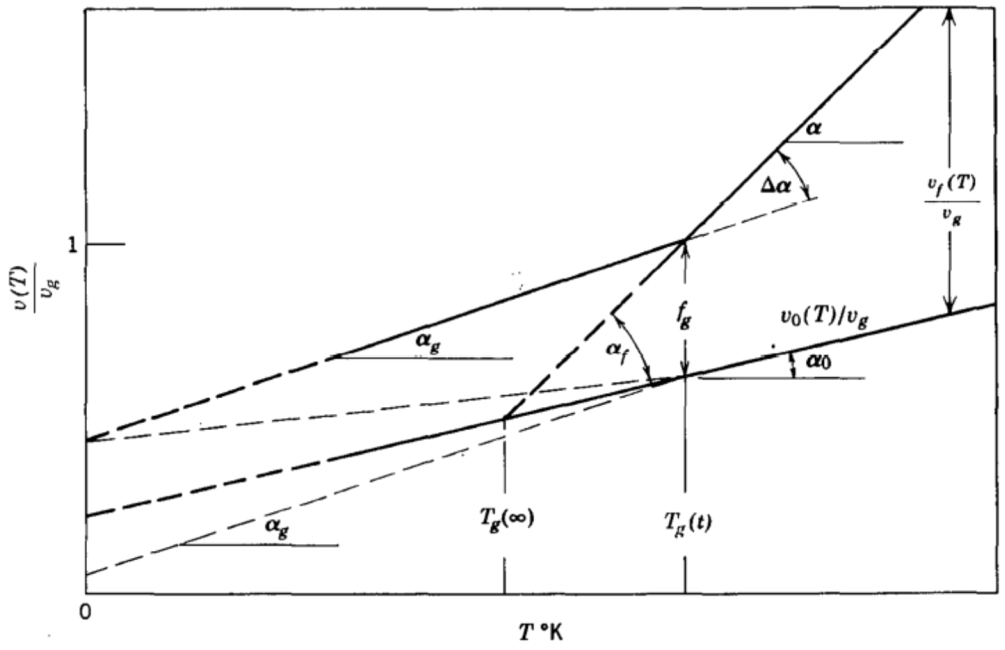

Figure 5. A representative scheme of free volume theory (Ferry, 1980)

The expansion of free volume at temperatures above T_g, is known as thermal expansion coefficient (α). The expansion coefficient at the glassy state (α_g) is lower than that in the rubbery state (Ferry, 1980).

The most used theory to explain free volume effect with regard to viscosity is the Williams-Landel-Ferry (WLF), which was first developed by Doolittle and Doolittle (1957):

$$\ln \eta = B\left(v_0/v_f\right) + \ln A \tag{1.3}$$

where, η is viscosity (Pa.s); A and B are constants.

The equation was then modified by Williams, Landel and Ferry (WLF) (1955) when they considered effect of molecular rearrangement and introduced the fractional free volume concept, f for local motions:

$$\ln \eta = (B/f) + \ln A \tag{1.4}$$

where, f is equal to v_f/v_o

As the fractional free volume linearly increases with the elevation of temperature:

$$f = f_g + \alpha_f(T - T_g) \tag{1.5}$$

where, f is the free volume at glass transition temperature and α_f is expansion coefficient

The free volume theory introduces a set of shift factors (a_T) that indicates polymer relaxation. Thus, the free volume equation (1.1) is expressed as bellow:

$$\log a_T = \frac{B}{2.303}\left(\frac{1}{f} - \frac{1}{f_o}\right) \tag{1.6}$$

A combination of the above relations was described in the framework of William, Landel, and Ferry (Ferry, 1980):

$$\log a_T = \log\left(\frac{\eta(T)}{\eta(T_o)}\right) = -\frac{(B/2.303f_0)(T-T_o)}{(f_0/\alpha_f)+(T-T_o)} \tag{1.7}$$

where, f_o is fractional free volume at a reference temperature (T_o), B is equal to 1 (Ferry, 1980)

The WFL constants (C_1^o) and (C_2^o) describe temperature dependence of viscoelasticity

in a relation with free volume (Ferry, 1980).

$$C_1^o = \frac{B}{2.303 f_o} \text{ and } C_2^o = \frac{f_g}{\alpha_f}$$

The WLF equation (1.7) is universally utilized to describe the vitrification phenomenon of polymers. However, it deviates from the progression of viscoelasticity at glassy state (Kasapis, 2009; Kasapis & Sablani, 2008).

1.2.5. Reaction rate theory

As molecules in the microstructure are almost in equilibrium state (Abiad, Carvajal, & Campanella, 2009) in glassy state, physiochemical changes in an amorphous material can be expressed by the Arrhenius model (Nelson & Labuza, 1994):

$$k = k_o e^{\left(-\frac{E_a}{RT}\right)} \quad (1.8)$$

where, *k* is rate constant at temperature *T, ko* is the pre-exponential factor, E_a is the activation energy (kJ/mol) and *R* is the ideal gas constant (8.31 J/mol K).

Equation (1.8) was then modified in the work of Arridge (1975):

$$\log \frac{k_o}{k} = \frac{E_a}{2.303\ R} \left(\frac{1}{T} - \frac{1}{T_o}\right) \quad (1.9)$$

The Arrhenius equation indicates a certain degree of activation energy (E_a) is needed for molecular mobility within their matrix in glassy state. It also explains the viscoelasticity progression in glassy region is a temperature-dependent process (Kasapis, 2009).

1.2.6. Mechanical glass transition temperature prediction

The structural transformation of elements in glass transition region and the overcome of energetic barrier for molecular mobility in glassy region is the background to predict the

mechanical glass transition temperature of a polymer material (Kasapis & Sablani, 2008; Paramita & Kasapis, 2018). This is expressed as the discontinuity of the two mathematical models defined by free volume theory (equation 1.7) and reaction rate theory (equation 1.9) (Figure 6).

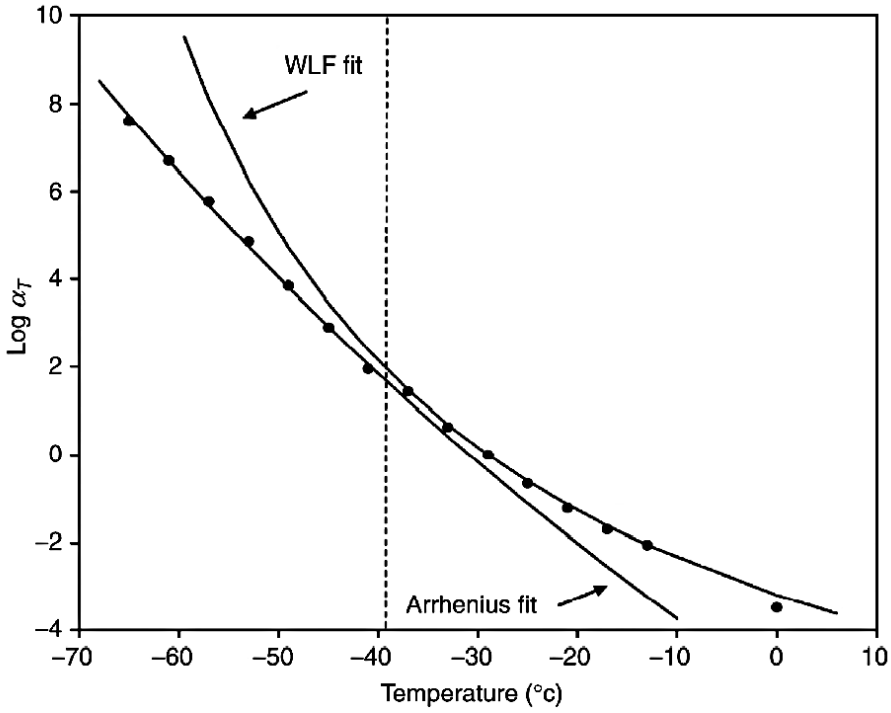

Figure 6. WLF and Arrhenius fits exhibited a discontinuity and thus pinpointed the predicted glass transition temperature of a biopolymer blend (Kasapis & Sablani, 2008).

The mechanical T_g of a system is normally deviated from its calorimetric T_g which is mainly determined by thermodynamic changes and molecular mobility with negligible effect of network formation (Kasapis, Al-Marhoobi, & Mitchell, 2003). It was noted that mechanical T_g varied up to 20 ºC from calorimetric T_g when a polymer presented in the composition of a material (Kasapis et al., 2003). This difference is due to the formation of network by the polimer and it depends on the structural elements such as molecular weight, electrostatic

interactions, thus mechanical Tg is terminologically interchangeable with the concept of network T_g (Kasapis, Al-Marhoobi, & Mitchell, 2003b; Paramita & Kasapis, 2019; Roos, 2010).

2.7. Glass transition of honey

Owing to its supersaturated sugar composition, honey is a viscous supercooled liquid and usually exhibits a Newtonian behavior. The physical characteristics enable honey to complement a current trend in the cryopreservation of biomaterials such as cells, tissues and organs (Andraca, Goldstein, & del Castillo, 2013; Recondo, Elizalde, & Buera, 2006).

Molecular mobility in honey have been explored using mostly calorimetric analysis (Bhandari, D'Arcy, & Kelly, 1999; Recondo et al., 2006; Sobrino-Gregorio, Vargas, Chiralt, & Escriche, 2017). The studies reported honey at a subzero temperature regime is a glassy solid showing a dramatic increase in viscosity and the immobilization of chemical and biological components, thus its quality is highly stable. In contrast, a rapid heating route starting at glassy state increases heat capacity and generates characteristic T_g that varies for different honeys. Lazaridou, Biliaderis, Bacandritsos, and Sabatini (2004) reported a range of T_g for honeys from −30 to −50 °C.

Further heating honey above room temperature liquefies it and increases its susceptibility to physical and chemical modifications. A high temperature regime dramatically reduces its enzyme activity, degrades proteins and releases free amino acids which herald Maillard reaction and colour changes (da Silva, Gauche, Gonzaga, Costa, & Fett, 2016). Moreover, high temperatures alter molecular structures of phenolic and flavonoid compounds affecting their antioxidant capacity. Therefore, mild temperature (40−50 °C) is strongly recommended for commercial honey in preventing crystallization process and browning

reactions during storage (Blidi, Gotsiou, Loupassaki, Grigorakis, & Calokerinos, 2017).

Although physicochemical properties of several sugar systems have been extensively explored to provide valuable database for baking and confectionary industries (Mayhew, Neal, Lee, & Schmidt, 2017; Ruiz-Cabrera & Schmidt, 2015), data for naturally occurring sugar mixtures like honey are still limited. Thus, determination of thermal and structural behavior of honey provides fundamental information for quality stability (e.g. crystallization process is minimized, bioactivities are maintained, etc.), optimization of handling and packing procedures and the development of honey-containing products to delivery its bio-functions.

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CHAPTER 2. EFFECT OF HONEY ON KEY BIOMARKERS OF OXIDATIVE STRESS AND CHOLESTEROL HOMEOSTASIS

2.1. Introduction

Atherosclerosis is a leading cause of worldwide vascular deaths and its pathogenesis closely links to oxidative stress and hypercholesterolemia (Herrington, Lacey, Sherliker, Armitage, & Lewington, 2016). It was reported that a 10% elevation of total cholesterol corresponds to a 27% higher incidence of coronary heart disease. Since most of plasma cholesterol is low density lipoprotein cholesterol (LDL-C) which forms plaque and hardens the arteries at elevated levels, LDL-C is a detrimental element associated with atherosclerotic pathogenesis (Lalor et al., 2012; Law, Wald, & Thompson, 1994).

Statins have been popularly used to reduce cholesterol levels due to their ability of inhibiting hydroxy methylglutaryl coenzyme A reductase (HMGCR), a rate-limiting enzyme in *de novo* cholesterol synthesis. However, it was reported that healthy lifestyle and dietary regimes are responsible for a significant decrease in vascular mortality in general and cholesterol levels in particular, thus, many people on the margin of abnormal cholesterol levels can return to normal cholesterol status without medications (Jenkins et al., 2005). Recently, functional foods have attracted great attention due to their nutraceutical properties that promote health and modulate hypercholesterolemia (Gul, Singh, & Jabeen, 2016).

Honey, a natural sweetener, contains monosaccharides, disaccharides, water, enzymes, proteins, amino acids, vitamins and a wide range of phytochemicals. Due to the diversity of plant metabolites in floral nectars from various plants, chemical profiles and consequently antioxidant activities of honeys vary (Anand et al., 2019; Combarros-Fuertes et al., 2019; Yamani, Mantri, Morrison, & Pang, 2014). The oxygen radical absorbance capacity of honey (3−17 μmol TE/g) is comparable to that of several fresh fruit and vegetables (0.5−19 μmol

TE/g) (Gheldof & Engeseth, 2002; Özcan & Juhaimi, 2015). The potential of honey in modulating cholesterol homeostasis has been evidenced in several investigations (Nguyen, Panyoyai, Kasapis, Pang, & Mantri, 2019). Among those, phenolic compounds such as catechin, quercetin, luteolin occurring in honey are reported to improve coronary vasodilation, prevent blood clots and LDL-C oxidation (Chen, Yang, Jiao, & Zhao, 2002; Mbikay, Sirois, Simoes, Mayne, & Chrétien, 2014; Panchal, Poudyal, & Brown, 2012; Wong, Lin, & Leung, 2015). Several flavonoids in honey are aglycon forms, making them readily bioavailable when consumed (Alvarez-Suarez, Giampieri, & Battino, 2013). However, to date, understanding the molecular mechanism through which honey elicits its protection against oxidative stress and hypercholesterolemia is very limited.

It has been reported that chronic oxidative stress closely links with dysregulated lipid homeostasis. Transcription factor NF-E2-related factors 2 (*Nrf2*) and its downstream gene, NAD(P)H:quinone oxidoreductase 1 (*NQO1*) are central in defence responses to oxidative stress (Sharath Babu, Anand, Ilaiyaraja, Khanum, & Gopalan, 2017). In addition, AMP-activated protein kinase (*AMPK*), a key energy sensor has been extensively investigated in lipid metabolism pathways because its activation induces ATP-generating pathways, including the uptake and oxidation of glucose and fatty acids (Hwang, Kwon, & Yoon, 2009).

Cholesterol metabolism is co-regulated by sterol regulatory element-binding protein (*SREBP-2*) and liver X receptors (*LXR*s) in the liver. *SREBP-2* preferentially modulates genes associated with cholesterol synthesis and uptake (*HMGCR* and *LDLR*, respectively) (Raghow, Yellaturu, Deng, Park, & Elam, 2008). *LXR*s are responsible for reducing cholesterol absorption in intestine and promoting the excretion of bile cholesterol in the liver through upregulating membrane transporters (Li et al., 2017). In addition, proliferator-activated receptor alpha (*PPARα*) is a critical molecule that controls the transcription of genes downstream of the lipid catabolism. The activation of *PPARα* enhances the β-oxidation of fatty

acids, reducing the content of cellular lipids (Zheng, Lv, Sheng, & Yang, 2010). Thus, it is interesting to explore the effect of honey on these biomarkers to determine if honey can be used in the management of atherosclerosis.

The human HepG2 cells are highly differentiated and exhibit several genotypic characteristics of normal liver cells, including cholesterol synthesis and excretion (Gerets et al., 2012), so it was used in the current study. Manuka honey and four medicinal honeys newly developed in The Pangenomics Laboratory, RMIT University were examined to investigate their physicochemical, biochemical properties, and effect on the aforementioned biomarkers in fatty acid-induced HepG2 cells.

2.2. Materials and methods

2.2.1. Honey samples

A commercial manuka honey containing methylglyoxal 400^{+} mg/kg (manuka-1) and four medicinal honeys developed from extracts of *Terminalia arjuna* bark (aruna honey), *Commiphora mukul* stem (guggul honey), *Gynostemma pentaphyllum* leaves (jiaogulan honey) and *Olea europaea* fruit retentate (olive honey) were examined for their physicochemical, biochemical properties, and effect on primary biomarkers of oxidative stress and lipid metabolism. The plant species were selected based on scientific literature reporting their phytochemical profiles and biofunctions linked to their antioxidant activities and hypocholesterolemia.

Four healthy bee colonies provided by Berley Honey were kept separately in semi-control enclosures and each was fed with a medicinal nectar, which is a solution of sugars, amino acids and a medicinal plant extract to produce medicinal honey (Table 1). Briefly, sugar proportion was prepared following the sugar pattern of *melitophilus* flowering plant species

which are friendly for honey bees (Baker, 1983; Chalcoff et al., 2017). The amino acid concentrations were prepared following those identified in flower nectar of *Prunella vulgaris* which is a bee-friendly herb wildly distributed in bee pastures during the summer (Gardener & Gillman, 2001; Kuriya, Hattori, Nagano, & Itino, 2015). The inclusion of plant extract was adjusted to the bee's preference. In addition, pollen granules A^+ premium grade (Western Australia) was daily given as a protein source to maintain bee colonies. Extracts of *T. arjuna bark*, *C. mukul* stem and *G. pentaphyllum* leaves were obtained following methods described by Sultana, Anwar, and Przybylski (2007), Jasmine et al. (2013), Liu et al. (2016) with some modifications, respectively. Olive retentate (Boundary Bend, Victoria) was directly used to replace plant extracts as it contains (mg/100 ml): total phenol content 90.5, hydroxytyrosol 15, sodium 1.9, total sugars < 1.9, fat < 0.2, protein 0.1.

Table 1. Composition of medicinal nectars

Components	Concentration	Reference
Sugars (sucrose and hexose)	50.0% (3:1, w/w)	Baker (1983); Chalcoff, Gleiser, Ezcurra, and Aizen (2017)
Amino acids	2.4 mM	Gardener and Gillman (2001)
Plant extracts	< 1.0% (w/w)	Can, Yildiz, Sahin, Turumtay, et al. (2015); Gheldof and Engeseth (2002)

2.2.2. Moisture, pH, electrical conductivity

Moisture, pH and electrical conductivity were measured following the International Honey Commission method (Bogdanov, Martin, & Lullmann, 2002). Moisture content was recorded using a Refracto 30GS refractometer (Mettler Toledo, Australia) and converted

following a Chetaway table. pH value was measured from 10% honey solutions. Electrical conductivity was measured from 20% (w/v) honey solutions using a S230 conductivity meter (Mettler Toledo, Australia).

2.2.3. Reducing sugars

The contents of D-glucose and D-fructose in honeys were analyzed following instructions from K-SUFRG 06/14 kit (Megazyme, Australia). Briefly, D-glucose was assayed by utilizing hexokinase and glucose-6-phosphate avoiding sucrose hydrolysis. The content of D-fructose was recorded after the analysis of D-glucose content, following isomerization with phosphoglucose isomerase.

2.2.4. Colour intensity

Colour intensity of honeys was measured following Saxena, Gautam, and Sharma (2010). Honey solutions (50%, w/v in Milli-Q water) were filtered using 0.45 μm sterile Millex filters (Merk, Australia) to remove particles. Results were calculated as the difference of the 450 and 720 nm absorbance readings recorded from a Lambda 35 UV-vis spectrophotometer (Perkin Elmer).

2.2.5. Protein content

Protein content was assayed following Bradford's method (1976) using a Thermo Scientific™Coomassie Protein Assay Kit (Thermo Fisher, Australia). In doing so, a volume of 20 μl of honey solution (10%, w/v) was mixed with 250 μl of Coomassie reagent in a 96 microplate. After ten-minute incubation at room temperature, the plate was read at 595 nm absorbance using a POLARstar Omega plate reader (BMG Labtech, Ortenberg, Germany). A calibration curve using bovine serum albumin standards in the range of 0 to 100 μg/ml was used to calculate the protein content.

2.2.6. Total phenolic content

Total phenolic content was assayed using Folin-Ciocalteu reagent (Singleton, Orthofer, & Lamuela-Raventós, 1999). Briefly, 0.5 ml of 10% filtered honey solution (w/v) was mixed with 2.5 ml of 0.2 N Folin-Ciocalteu reagent (Sigma-Aldrich, Australia). Subsequently, 2 ml of 75 g/l Na_2CO_3 solution was added. After 2-hour incubation at ambient temperature, the mixture was read at 760 nm absorbance using a Lambda 35 UV–vis spectrophotometer (Perkin Elmer). Data is the mean of four replicates ± SD. Gallic acid standards ranging from 0 to100 µg/ml were used to construct a calibration curve.

2.2.7. Total flavonoid content

Total flavonoid content was measured following the method described by Islam, Khalil, Islam, Moniruzzaman, Mottalib, Sulaiman, Gan, et al. (2012). Two grams of honey was diluted into 10 ml methanol and filtered with a 0.45µm sterile Millex filter (Merk, Australia). Then, 1 ml of the 20% honey solution (w/v) was added to 4 ml milli-Q water, followed by 0.3 ml of 5% $NaNO_2$ (w/v). After five minutes, 0.3 ml of 10% $AlCl_3$ (w/v) was added. Six minutes later, the mixture was neutralised with 2 ml of 1.0 mM NaOH solution and increased into 10 ml using water. The absorbance was recorded at 510 nm within 5 min using a Lambda 35 UV–vis spectrophotometer (Perkin Elmer). Catechin standards ranging from 0 to 100 µg/ml were used to construct a calibration curve against a blank consisted of honey solution and methanol without $AlCl_3$.

2.2.8. Radical scavenging activity and ascorbic acid equivalent antioxidant content

Radical scavenging activity (RSA) of the medicinal honeys was recorded by determining the reduction of radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) following Meda, Lamien, Romito, Millogo, and Nacoulma (2005) with minor modifications. Honey was diluted in methanol and filtered with a 0.45 µm sterile Millex filter (Merk, Australia). Then, 0.8 ml of

2.5% honey solution (w/v) was added to 2.7 ml of 0.024 mg/ml DPPH in methanol. The mixture was vigorously shaken for 15 seconds and incubated for 30 minutes without light at room temperature. The DPPH reduction was read at 517 nm absorbance using a Lambda 35 UV–vis spectrophotometer (Perkin Elmer). RSA was the percentage of DPPH discoloration calculated from the equation:

$$\% \text{ RSA} = [(A_{DPPH} - A_S)/A_{DPPH}] \times 100$$

where A_{DPPH} and A_S are the absorbance units of DPPH solutions in the absence and presence of honey, respectively.

The antioxidant content (AEAC) was analysed as aforementioned and referred to calibration curve of ascorbic acid standards ranging from 0–15.0 μg/ml.

2.2.9. Viscosity

Viscosity was measured using an AR-G2 rotational rheometer (TA instruments). Honeys (1.5 gram) were loaded onto a 40 mm diameter parallel plate geometry at room temperature and the analysis was set at 500 μm gap and shear rate from 0.01 to 10 cm^{-1} at 20 ^{o}C.

2.2.10. Fourier-transform infrared spectroscopy (FTIR)

FTIR spectra of the medicinal honeys were scanned using A MIRacleTM ZnSe single reflection ATR plate-linked spectrometer (Perkin Elmer, Norwalk, USA). An amount of 0.2 gram of honeys was placed onto the measuring plate and measured for 40 scans from 4000 to 500 cm^{-1}, 4 cm^{-1} resolution at room temperature.

2.2.11. Cell culture

Human liver cell line (HepG2) was kindly granted by School of Health and Biomedical Sciences, RMIT University. HepG2 cells were routinely maintained in a complete Dulbecco's modified Eagle's medium (DMEM) containing foetal bovine serum (FBS, 10%, v/v) (Thermo

Fisher, USA), glutamine (2 mmol/L), penicillin (100 U/mL), and streptomycin (100 mg/mL) in an incubator set at 37°C and 5% CO_2 atmosphere. The complete medium was changed two times a week and subcultures were performed when the cells reached a 75% confluence. Cells were counted using trypan blue reagent and Countess® Automated Cell Counter (Invitrogen, Australia). All assays used the cells at passage numbers less than twenty.

2.2.12. Cell viability

The viability of HepG2 cells under the effect of medicinal honeys was assayed using PrestoBlue® kit (Invitrogen, Australia). 100 µl of complete DMEM medium containing 3 × 10^4 cells/well was pipetted on a 96-well plate and incubated at 37 °C and 5% CO_2. Ten hours later, the media was discarded, and the cells were incubated in free-serum DMEM overnight. Honey solutions in a range of 0–20% (w/v) were prepared in 2% FBS-DMEM and applied to the cells. After 24-, 48- and 72-hour incubation with honeys, the cells were washed with PBS twice followed by adding PrestoBlue reagent 10.0% in free-serum medium followed by another two-hour incubation. The fluorescence intensity was recorded at 535 nm/590 nm using a POLARstar spectrophotometer (BMG LabTech, Germany). Cell viability was calculated as the percentage of the fluorescence unit of treated cells to control that is untreated cells.

2.2.13. Cell treatment with fatty acids

HepG2 cells (6 x10^5 cells/well) in complete DMEM medium were seeded on a 6-well plate. Ten hours later, the complete medium was discarded, and the cells were maintained in serum-free, high glucose DMEM medium overnight. A conjugation of fatty acids 1.0 mM (oleic acid/palmitic acid, 2:1) and fat-free bovine serum albumin (BSA, Roche) was prepared at a 6:1 ratio in 2.0% FBS-DMEM medium and delivered to the cells. In addition, honey was simultaneously added at final concentrations 1.0 and 2.0% (w/v). Control cells were exposed to 1.0% (w/v) BSA only. Cell pellets were collected after 24 hours.

2.14. Analysis of total cellular cholesterol and triglyceride content

Lipids were extracted from cell pellets (2.13) following Folch, Lees, and Stanley (1957). The dry lipid was dissolved in 200 µl methanol for the subsequent determination of total cellular cholesterol (TC) and triglycerides (TG) by enzymatic methods, using Amplex Red Cholesterol Assay Kit (Molecular Probes) and EnzyChrom Triglyceride Assay Kit (BioAssay Systems), respectively. Cellular protein content was assayed based on bicinchoninic acid (BCA) method using BCA Protein Assay Kit (Thermo Fisher Scientific). TC and TG contents were normalized to relevant protein content (µg/mg cellular protein) and expressed in fold change to control (1.0 mM fatty acid treatment).

2.2.15. Real-time quantitative polymerase chain reaction (RT qPCR)

Total cellular RNA was isolated from the HepG2 cells using RNeasy mini kit (Qiagen, Australia). Complementary DNA templates were synthesised from 500 ng of total RNA in 20 µl assays, using SensiFAST cDNA Synthesis kit (Bioline). Reactions were set at 25 °C for 10 minutes, followed by 42 °C for 15 minutes, 48 °C for 15 minutes, 85 °C for 5 minutes and finally cooled at 4 °C before they were stored at -20 ºC. Primers (Table 2) were obtained from Bioneer Pacific (Victoria, Australia).

RT qPCR were set up as described in SensiFAST™ SYBR No-ROX kit (Bioline) and performed at 95 °C for 2 minutes, succeeded by 40 cycles of 95 ºC for 5 seconds, 60 °C for 10 seconds, 72 °C for 10 seconds using a Rotor-Gene Q machine (Qiagen). β-actin was used as a reference gene. The primer specificity was confirmed by the analysis of melting curve. The relative quantification of gene expression was accomplished following $2^{-\Delta\Delta C_T}$ method (Livak & Schmittgen, 2001).

2.2.16. Statistical analysis

Data were analyzed using Excel (Office 365) and IBM SPSS Statistics 26 software and

expressed as mean of four replicates ± standard deviation (SD) (SPSS Inc., Chicago, IL) in all parameters. Statistical analysis was performed using by one-way analysis of variance (ANOVA) followed by Duncan's multiple comparison test. Results were considered statistically different when $p \leq 0.05$.

Table 2. Primer sequences

Gene	RefSeq Identification	Primers	Sequence (5' to 3')
AMPKα	NM_006252.4	Forward	TCGCCACTCTCCTGATGCATAT
		Reverse	GATGATGAGGCTGTGAAAGAAG
Nrf2	NM_001145413	Forward	GAGCCCAGTATCAGCAACAG
		Reverse	TTCAATGATTCTGACTCCGGC
NQO1	NM_001025434	Forward	TGCTGCAGCGGCTTTGAAGA
		Reverse	TTTCAGTATCCTGCCGAGTCT
SREBP2	NM_004599.4	Forward	GCCCTGGAAGTGACAGAGAG
		Reverse	TCACTCCCTGGGAAAGCA
HMCGR	NM_000859.3	Forward	GGTGTATCTATTCGCCGACAG
		Reverse	CTGTTGGAGTGG CAGGACC
LDLR	NM_000527.4	Forward	CATCTACTCGCTGGTGACTG
		Reverse	GGCAACCGGAAGACCATCTT
LXRα	NM_001130102.3	Forward	TCACCTTCCTCAAGGATTTCA
		Reverse	TCATCAACCCCATCTTCGAG
SREBP-1c	NM_001321096.3	Forward	CAGCTCTGCACTCCTTCAAG
		Reverse	TGCAGCTGTTCCTGTGTGAC
PPARα	NM_001001928	Forward	CAATGCACTGGAACTGGATGA
		Reverse	AGACTCCACCTGCAGAGCAA
β-actin	NM_001101.5	Forward	GGTCAGAAGGATTCCTATGTGG
		Reverse	GCACCACACCTTCTACAATGAG

2.3. Results and discussion

2.3.1. Physicochemical properties

2.3.1.1. Moisture, electrical conductivity, pH and colour intensity

Moisture content (MC) (Table 3) is a critical factor determining honey quality and affecting other physical characteristics of honey such as sugars, crystallization, viscosity and stability (Escuredo, Míguez, Fernández-González, & Seijo, 2013). MC depends on several factors such as weather and maturity of honey. Honey with high MC undergoes the fermentation caused by osmotolerant yeasts. In the present study, moisture content was considerably low in arjuna, guggul and jiaogulan honeys (14.9, 13.4 and 14.0%, respectively), compared to manuka-1 and olive honeys (18.5 and 18.4%). The results indicated MCs for the honeys tested (including the 4 we produced) are well below 20.0%, the imposed limit for natural nectar honeys (EEC, 110/2001) and consistent to previous findings which reported a moisture range of 13.0–19.0% for honeys from various origins (Escuredo et al., 2013; Kayacier & Karaman, 2008).

Table 3. Physicochemical properties of honey

Parameters	MH1	ARJ	GUG	JIA	OLI
Moisture content (%)	18.5 ± 0.8^{c}	14.9 ± 0.6^{b}	13.4 ± 0.2^{a}	14.0 ± 0.2^{ab}	18.4 ± 0.2^{c}
Electrical conductivity (mS/cm)	0.54 ± 0.01^{c}	0.24 ± 0.01^{a}	0.34 ± 0.01^{b}	0.31 ± 0.01^{b}	0.32 ± 0.01^{b}
pH	4.1 ± 0.05^{ab}	3.9 ± 0.05^{a}	4.0 ± 0.01^{ab}	4.1 ± 0.01^{ab}	4.2 ± 0.01^{b}
Colour intensity (mAU)	2201.1 ± 2.0^{e}	462.3 ± 2.6^{c}	722.7 ± 0.5^{d}	171.7 ± 0.9^{a}	243.5 ± 0.1^{b}

Data are mean ± SD of four replicate measurements with different letters in the same row indicating significant differences ($p < 0.05$), analyzed by one-way ANOVA succeeded by Duncan's multiple comparison test. MH1: manuka-1 honey containing methylglyoxal 400 mg/kg, ARJ: arjuna honey, GUG: guggul honey, JIA: jiaogulan honey and OLI: olive honey.

Electrical conductivity (EC) reflects the quantity of mineral elements, organic acids and proteins in composition of honey. EC of all medicinal honeys was less than 0.8 mS/cm, the maximal limit regulated for blossom honey (EEC, 110/2001) and comparable to the previous findings (Terrab, Recamales, Hernanz, & Heredia, 2004). We also found that EC values in the newly developed medicinal honeys (0.24–0.34 mS/cm) were lower than in manuka-1 honey (0.54 mS/cm). This could result from the variations in nectar composition and environment that honey bees were exposed to (Kavanagh, Gunnoo, Marques Passos, Stout, & White, 2019).

pH value of honey is an important feature affecting its texture, stability and shelf-life. pH measurement of the medicinal honeys showed acidic values within a limited range of 3.9–4.2. The results agree with previous findings reporting an acidic pH range of 3.7–4.5 for different natural honeys (Anand, Pang, Livanos, & Mantri, 2018; Kavanagh et al., 2019).

Colour intensity (ABS_{450}) was proportional to the content of pigments (carotenoids, flavonoids) and antioxidant activity of honey (Gheldof & Engeseth, 2002). Colour intensity of the medicinal honeys greatly varied with the highest value was for manuka-1 honey (2201.1 mAU), whereas much lower values were recorded for four novel medicinal honeys (462.3, 722.7, 171.7 and 243.5 mAU for arjuna, guggul, jiaogulan and olive honey, respectively). The findings were in line with other observations which reported a wide colour range of 125.0–3400.0 mAU for natural honeys (Beretta, Granata, Ferrero, Orioli, & Facino, 2005; Habib, Al Meqbali, Kamal, Souka, & Ibrahim, 2014; Nguyen, Panyoyai, Paramita, Mantri, & Kasapis, 2018).

2.3.1.2. Viscosity

Viscosity analysis indicated all tested honeys were Newtonian liquid, which is consistent with most of natural honeys (Oroian, 2013; Yanniotis, Skaltsi, & Karaburnioti, 2006). The novel medicinal honeys presented much higher viscosity than manuka-1 honey. Guggul honey presented the highest viscosity (316.2 Pa.s), followed by arjuna, jiaogulan and

olive honey (125.8, 100.0 and 63.1 Pa.s). Although manuka-1 honey showed the lowest viscosity (15.8 Pa.s), its viscosity is comparable with that of other natural honeys (1.8–13.8 Pa.s) (Anupama, Bhat, & Sapna, 2003). In addition, the viscosity data in the present study was inversely correlated with moisture contents of the relevant honey types (Table 3), congruent with previous findings (Saxena et al., 2010; Yanniotis et al., 2006).

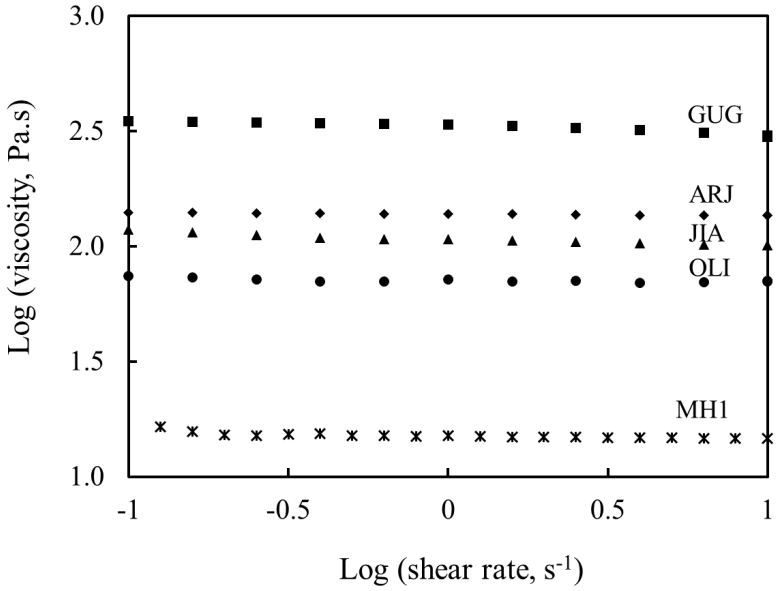

Figure 1. Viscosity and rheological behaviour of medicinal honeys. MH1: manuka-1 honey containing methylglyoxal 400^{+} mg/kg, ARJ: arjuna honey, GUG: guggul honey, JIA: jiaogulan honey and OLI: olive honey.

2.3.1.3. Fourier-transformed infrared spectroscopy

Chemometric analysis of the medicinal honeys using Fourier-transform infrared spectroscopy (FTIR) exhibited five vibrational regions assigned for specific chemical bonds in honey (Figure 2). The regions of 3700–3000 cm^{-1} and 3000–2800 cm^{-1} indicate to O–H stretching of water molecules, and C–H stretching of sugars, respectively (Tewari & Irudayaraj, 2004). A peak appears at 1655 cm^{-1} in all honeys is assigend for the bending of H–O–H molecules (Kasprzyk, Depciuch, Grabek-Lejko, & Parlinska-Wojtan, 2018). The 1540–

1175 cm^{-1} region indicated the presence of bending modes for C–O–H, C–C–H, and O–C–H groups (Pataca, Neto, Marcucci, & Poppi, 2007). The vibrations with strong intensity absorbance in 1175–940 cm^{-1} bands known as a fingerprint region for honey's sugars were specific for stretching modes of C–O and C–C from carbohydrates (Anjos, Campos, Ruiz, & Antunes, 2015). Bands in 900–750 cm^{-1} indicated the stretching modes of C–O and C–C of saccharide molecules (Nguyen et al., 2018). The results suggest a similarity in chemical links representing typical components of the four medicinal honeys and manuka-1 honey. However, other analysis techniques such as HPLC may need to use for further determination of the sugar and chemical profile.

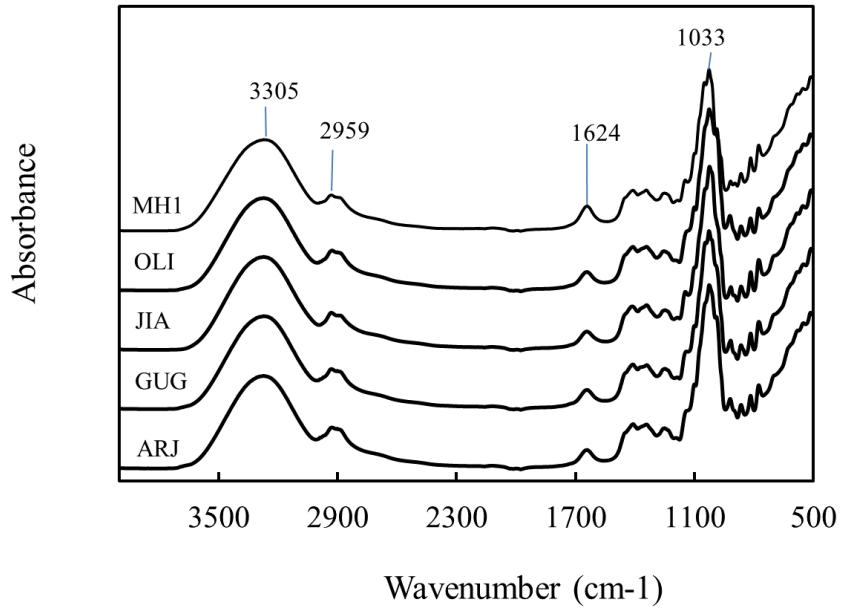

Figure 2. Chemometric analysis using Fourier-transform infrared spectroscopy (FTIR) scanned from 4000 to 400 cm^{-1}. MH1: manuka-1 honey containing methylglyoxal 400 mg/kg, ARJ: arjuna honey, GUG: guggul honey, JIA: jiaogulan honey and OLI: olive honey.

2.3.2. Biochemical characteristics

2.3.2.1. Reducing sugars

Monosaccharides (fructose and glucose) that are the major components of honey govern the physical state of honey. The data (Table 3) indicates that the total content of fructose and glucose is over 60 g/100 g for all medicinal honeys, except for olive honey (49.2 g/100 g honey). The results satisfy the regulation by Council Directive 2001/110/EC (EEC, 110/2001) and similar with other natural honey varieties having total monosaccharides within the range of 43−75 g/100 g honey (Al et al., 2009; Saxena et al., 2010). In addition, the ratio of fructose/glucose (F/G) is important for development of crystal sugar nuclei in honey, as it has been reported that honey having F/G ratio > 1 can be stored longer without crystallization, and in contrast, honey having F/G ratio < 1 develops crystals rapidly, leading to unfavored colour and texture. In the current study, all medicinal honeys presented F/G ratio > 1 that are similar to most natural honeys (Al et al., 2009; Nguyen et al., 2018).

2.3.2.2. Protein content

Protein content is influenced by the presence of amino acids, nectar sources, enzymes secreted from honey bees and storage conditions (Saxena et al., 2010). Manuka-1 honey exhibited highest protein content (128.5 mg/100 g) as this honey is categorized as “high protein” compared to other natural commercial honeys (Nguyen et al., 2018). The four medicinal honeys showed lower and varying protein contents, with the maximum value among the four honeys is for jiaogulan honey (114.4 mg/100 g), followed by olive, guggul and arjuna honey (66.6, 55.3 and 25.6 mg/100 g, respectively).

Table 4. Biochemical characteristics of medicinal honey

Parameters	MH1	ARJ	GUG	JIA	OLI
Fructose (g/100 g)	44.2 ± 2.0^{e}	33.5 ± 0.3^{b}	37.6 ± 0.4^{c}	41.4 ± 0.9^{d}	28.0 ± 2.8^{a}
Glucose (g/100 g)	28.9 ± 2.0^{b}	27.4 ± 0.1^{b}	31.9 ± 0.2^{c}	33.7 ± 0.3^{c}	21.2 ± 2.3^{a}
Protein content (mg/100 g)	128.5 ± 1.0^{e}	25.6 ± 5.0^{a}	55.3 ± 3.0^{b}	111.4 ± 3.6^{d}	66.6 ± 9.1^{c}
Total phenolics (mg GAE/100 g)	72.1 ± 2.0^{c}	73.4 ± 2.4^{c}	108.2 ± 1.8^{d}	31.6 ± 1.7^{a}	59.8 ± 0.5^{b}
Total flavonoids (mg CAE/100 g	3.9 ± 0.2^{b}	15.3 ± 0.4^{c}	4.1 ± 0.5^{b}	2.1 ± 0.1^{a}	24.0 ± 0.5^{d}
Radical scavenging activity (%)	55.5 ± 1.7^{b}	90.8 ± 0.4^{d}	91.7 ± 0.4^{d}	21.7 ± 0.3^{a}	75.2 ± 0.0^{c}
AEAC (mg/100 g)	49.6 ± 1.5^{b}	81.0 ± 0.3^{d}	81.9 ± 0.4^{d}	19.4 ± 0.3^{a}	67.1 ± 0.0^{c}

Data are mean ± SD of four replicate measurements, with different letters in the same row (a–e) indicating significant differences ($p < 0.05$) by one-way ANOVA followed by Duncan's multiple-range test. MH1: manuka-1 honey containing methylglyoxal 400^{+} mg/kg, ARJ: arjuna honey, GUG: guggul honey, JIA: jiaogulan honey and OLI: olive honey.

2.3.2.3. Total phenolic and flavonoid content

Phenolic and flavonoid compounds are markers of antioxidant activity and the content of total phenolics (TPC) and flavonoids (TFC) were commonly analyzed to predict therapeutic potential of natural products (Alvarez-Suarez et al., 2013; Can, Yildiz, Sahin, Akyuz Turumtay, et al., 2015). Table 3 showed that guggul honey contained the highest level of phenolic compounds (108.2 mg GAE/100 g), followed by manuka-1 and arjuna honeys (72.1 and 73.4 mg GAE/100 g), whereas olive honey contained a moderate level (59.8 mg/100 g) and jiaogulan honey showed the lowest level (31.6 mg/100 g) of phenolic compounds. The data are comparable with other natural honey varieties which were reported to contain a TPC range of 16.02−120.04 mg GAE/100 g (Anand et al., 2018; Can, Yildiz, Sahin, Akyuz Turumtay, et al., 2015) and supported the colour intensity data, e.g. honey with higher colour values having more phenolic content (Alves, Ramos, Gonçalves, Bernardo, & Mendes, 2013). We found a

strong correlation of TPC and colour intensity (R^2 = 0.930) for all four medicinal honeys but not for manuka-1 honey. The deviation of manuka-1 honey results from its extra high colour intensity value (2201.1 mAU), suggesting the presence of additional compounds from natural nectar, compared to the medicinal honeys.

Total flavonoid contents (TFC) greatly varied among the honeys. Although manuka-1 honey contained a higher TFC (3.9 mg/100 g) than many natural honeys (0.47–3.61 and 2.0–5.4 mg/100 g honey) (Anand et al., 2018; Valdés-Silverio et al., 2018), its TFC was similar to guggul honey (4.1 mg/100 g) and much lower than that of arjuna and olive honeys (15.3 and 24.0 mg/100 g, respectively). Jiaogulan honey had the lowest TFC (2.1 mg/100 g). This indicates that the TPC and TFC of honeys are strongly determined by nectar sources and the medicinal compounds in the honeys prepared from medicinal plant extracts may enhance the level of bioactive compounds and therapeutic properties of the four medicinal honeys.

2.3.2.4. Radical scavenging activity and ascorbic acid equivalent antioxidant content

RSA has been extensively examined in biological samples using 2,2-diphenyl-1-picrylhydrazyl (DPPH), a stable nitrogen-centered radical. The RSA varied among the tested honey types. Arjuna and guggul honeys showed the highest RSA (90.8% and 91.7%), followed by olive and manuka-1 honeys (75.2% and 55.5%) and jiaogulan honey (21.7%). The values are comparable to those in Bangladesh and Indian natural honey varieties (33.6–97.5% and 44.0–71.0%, respectively) (Islam, Khalil, Islam, Moniruzzaman, Mottalib, Sulaiman, & Gan, 2012; Saxena et al., 2010). Our data indicated that except for jiaogulan honey having low RSA, other medicinal honeys (arjuna, guggul and olive) have higher RSA than manuka-1 honey.

AEAC calculated using the calibration curve of ascorbic acid standards (R^2 = 0.998) revealed the antioxidant potential of the medicinal honeys. The AEAC was highest in arjuna and guggul honeys (81.0 and 81.9 mg/100 g, respectively), followed by olive and manuka-1 honeys (67.1 and 49.6 mg/100 g, respectively) and the lowest was found in jiaogulan honey

(19.4 mg/100 g). Except for jiaogulan honey, other medicinal honeys showed higher AEAC values compared to those for Burkina Fasan, India and Bangladesh honeys (10.20–37.87 mg AEAC/100 g) (Islam, Khalil, Islam, Moniruzzaman, Mottalib, Sulaiman, & Gan, 2012; Meda et al., 2005; Saxena et al., 2010). In addition, the AEAC data exhibited a good agreement with RSA for all tested honeys, confirming the significantly elevated antioxidant content and activity for arjuna, guggul and olive honeys.

2.3.3. Cytotoxicity of medicinal honeys

Cells exposed to biologically toxic materials may change their morphology, growth and death rate and disintegration may occur. Therefore, examination of the cytotoxicity of the novel drugs and compounds is crucial prior to further investigations (Boncler, Różalski, Krajewska, Podsędek, & Watala, 2014). The current study tested the toxicity of all honeys within concentration range of 0–10% (w/v) on the viability of HepG2 cell line. The data showed that the tested honeys affected the viability of HepG2 cells depending on time course and honey doses (Figure 3).

The medicinal honeys at 1.0% (w/v) were safe for the cells as they did not affect the viability for the whole experimental period, except for manuka-1 and jiaogulan honey which reduced cell viability to 84.4% and 74.4% after 72 hours, respectively. This difference indicated an additional effect from non-sugar components in manuka-1 and jiaogulan honey. Interestingly, 3.0% concentration of honey analogue, guggul, jiaogulan and olive honeys significantly reduced cell viability after 24, 48 and 72 hours, indicating that the honey analogue and these medicinal honeys induced cytotoxicity in a similar pattern. However, manuka-1 and arjuna honeys at 3.0% maintained cell population after 24 hours, but reduced the population after 48 and 72 hours, suggesting phytochemicals in manuka-1 and arjuna honeys may protect or promote the cells at this concentration within 24 hours but not later.

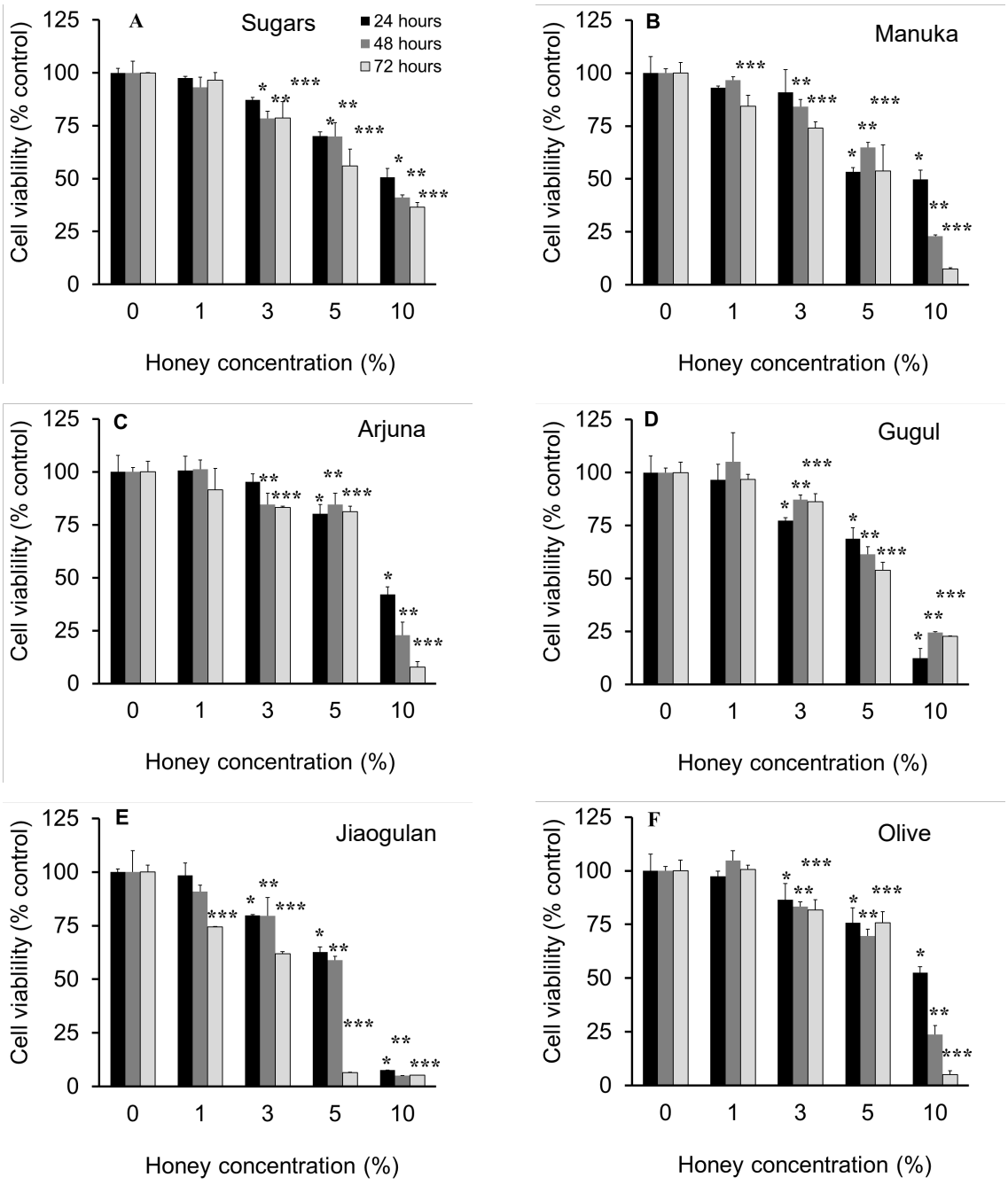

Figure 3. The viability of HepG2 under the effect of (A) honey analogue consisting of sugars, (B) manuka-1 honey containing methylglyoxal 400 mg/kg, (C) arjuna honey, (D) guggul honey, (E) jiaogulan honey and (F) olive honey for 24-hour (black), 48-hour (grey) and 72-hour (whitist) incubation. Results are the mean ± SD of four replicates. *, **, *** signifies $p \leq 0.05$ vs. control (0% honey) after 24-hour, 48-hour, and 72-hour incubation, respectively, analyzed by one-way ANOVA followed by Duncan's multiple comparison test.

All tested honeys significantly reduced cell viability at 5.0% and 10.0% concentrations. The reduced viability could be due to the high glucose levels at such honey concentrations which have been documented to induce apoptosis in human umbilical vein endothelial cells and HT22 hippocampal neuronal cells (Ceriello, dello Russo, Amstad, & Cerutti, 1996; Fan et al., 2016; Risso, Mercuri, Quagliaro, Damante, & Ceriello, 2001). Noticeably, jiaogulan honey at 5% killed 93.5% cells after 72 hours and this honey at 10% eliminated 92% of the cell population after 24 hours and maintained that level till 72 hours. The data suggest a prominent anticancer effect of 10.0% jiaogulan on HepG2 cells that are supportive to previous findings for jiaogulan extracts (Xie et al., 2012).

The results revealed that the cytotoxic effect of the tested honeys is comparable with those for sugar syrup, thyme and manuka-1 honey varieties on the viability of PC3, DU145 human prostate cancer cell lines, and THP-1 cell lines (Abel & Baird, 2018; Swellam et al., 2003; Tonks et al., 2003). Taken together, we selected honey concentrations of 1.0 and 2.0% (w/v) for a 24-hour incubation as safe limits to ensure the high stability of the cell population in honey treatments for subsequent investigation.

2.3.4. Effect of medicinal honeys on the expression of key genes associated with antioxidant defense responses

Nrf2 and *NQO1* genes have been extensively studied for their protective effect against oxidation. In this study, we found that the analogue, manuka-1 and jiaogulan honeys (Figure 4A, B and E) activated *Nrf2* genes and upregulated *NOQ1* gene expression at both concentrations (1.0 and 2.0%). The honey analogue showed lower levels of *NQO1* transcription (<1.5 fold) than those of the tested honeys (>1.5 fold), suggesting an additional effect of non-sugar components. Arjuna, guggul and olive honeys strongly increased *NQO1* mRNA abundance at high concentration (2.0%) without affecting *Nrf2* transcription (Figure 4C, D and

F). The results agree with outstanding values of phenolics/flavonoids, RSA and AEAC recorded for such honeys (Table 4). Interestingly, although jiaogulan honey exhibited modest antioxidant potential, it strongly affected the gene expression, thus, more investigation of chemical profile should be taken to determine the protective elements in jiaogulan honey and other medicinal honeys.

In addition, the findings in the current study are in line with other investigations reporting that polyphenols modulate Kelch-like ECH associated protein 1/Nrf2/antioxidant response elements (Keap1/Nrf2/ARE) gene pathway leading to the amelioration of oxidative stress *in vitro* and *in vivo* (Gu et al., 2017; Roubalová et al., 2017; Sharath Babu et al., 2017; Vigliante, Mannino, & Maffei, 2019). The unvaried *Nrf2* transcript levels by arjuna, guggul and olive honeys may result from either the modulation of non-sugar components or the experimental terminating time where *Nrf2* may have been expressed differently before 24 hours and later decreased to the level detected (Gu et al., 2017).

2.3.5. Effect of medicinal honeys on cellular lipid content

HepG2 cells were challenged with fatty acids 1.0 mM (oleic/palmitic; 2:1) in the presence of honey (1.0 and 2.0%) for 24 hours and assayed for their cellular cholesterol (TC) and triglycerides content (TG). Variations in TC and TG are shown in Figure 5. Total cellular cholesterol (TC) was elevated by fatty acids treatment, but it was reduced by the treatments of manuka-1 and guggul honeys (both, 1.0 and 2.0%) and arjuna honey (2.0%) (Figure 5B, C and D), whereas TC was not reduced by honey analogue, jiaogulan and olive honey at both concentrations (1.0 and 2.0%) (Figure 5A, E and F). Cellular triglyceride content (TG) significantly increased (3.9 fold) by fatty acids treatments, and the addition of any medicinal honey did not significantly reduce TG in fatty acid-induced HepG2 cells.

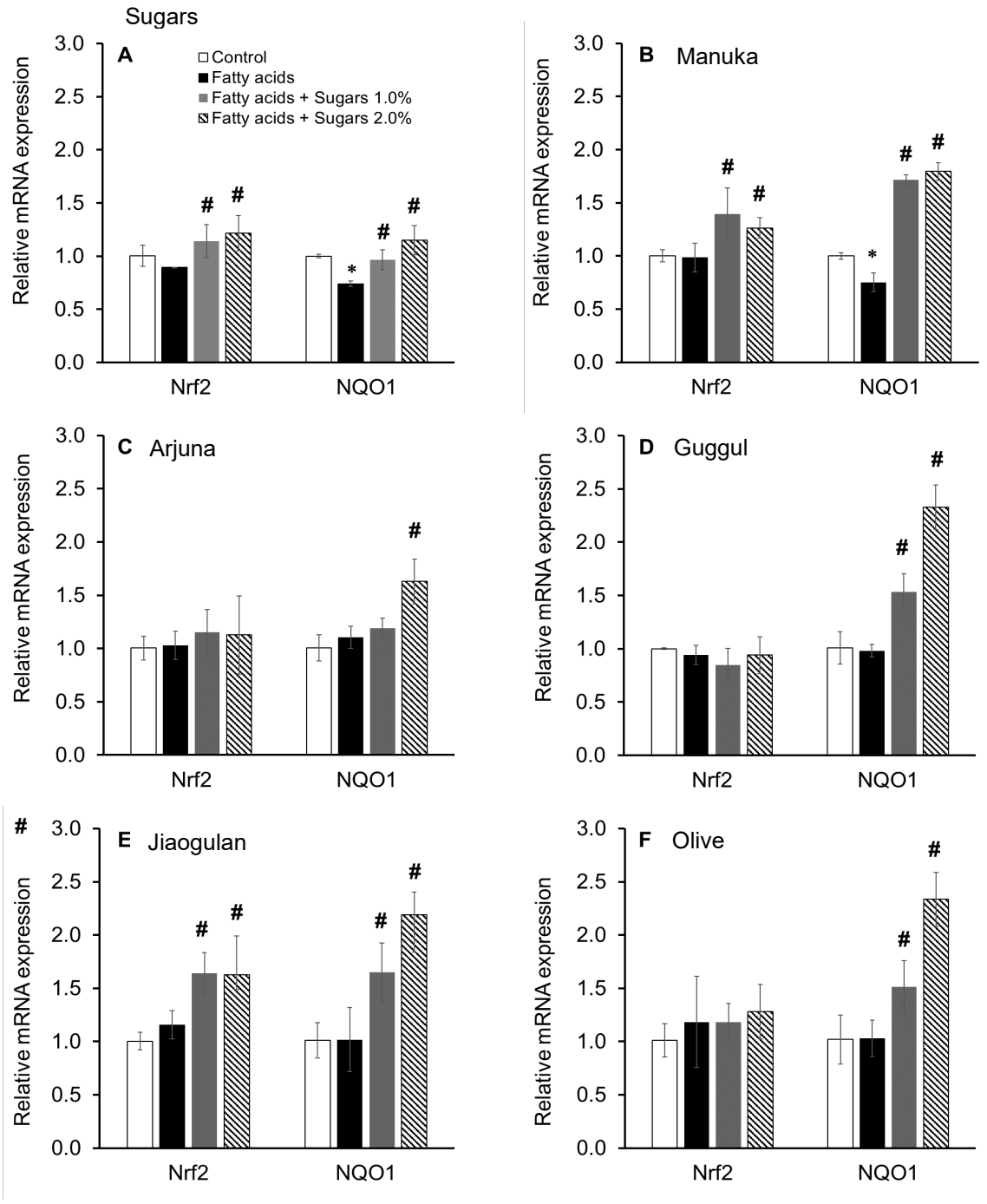

Figure 4. The expression of genes associated with defense responses after 24-hour incubation of HepG2 cells with (A) honey analogue containing sugars, (B) manuka-1 honey containing methylglyoxal 400^{+} mg/kg, (C) arjuna honey, (D) guggul honey, (E) jiaogulan honey, (F) olive honey. Results are mean ± SD of four replicates; ∗: $p < 0.05$ vs. control and #: $p < 0.05$ vs. fatty acid-treated cells, analyzed by one-way ANOVA followed by Duncan's multiple comparison test.

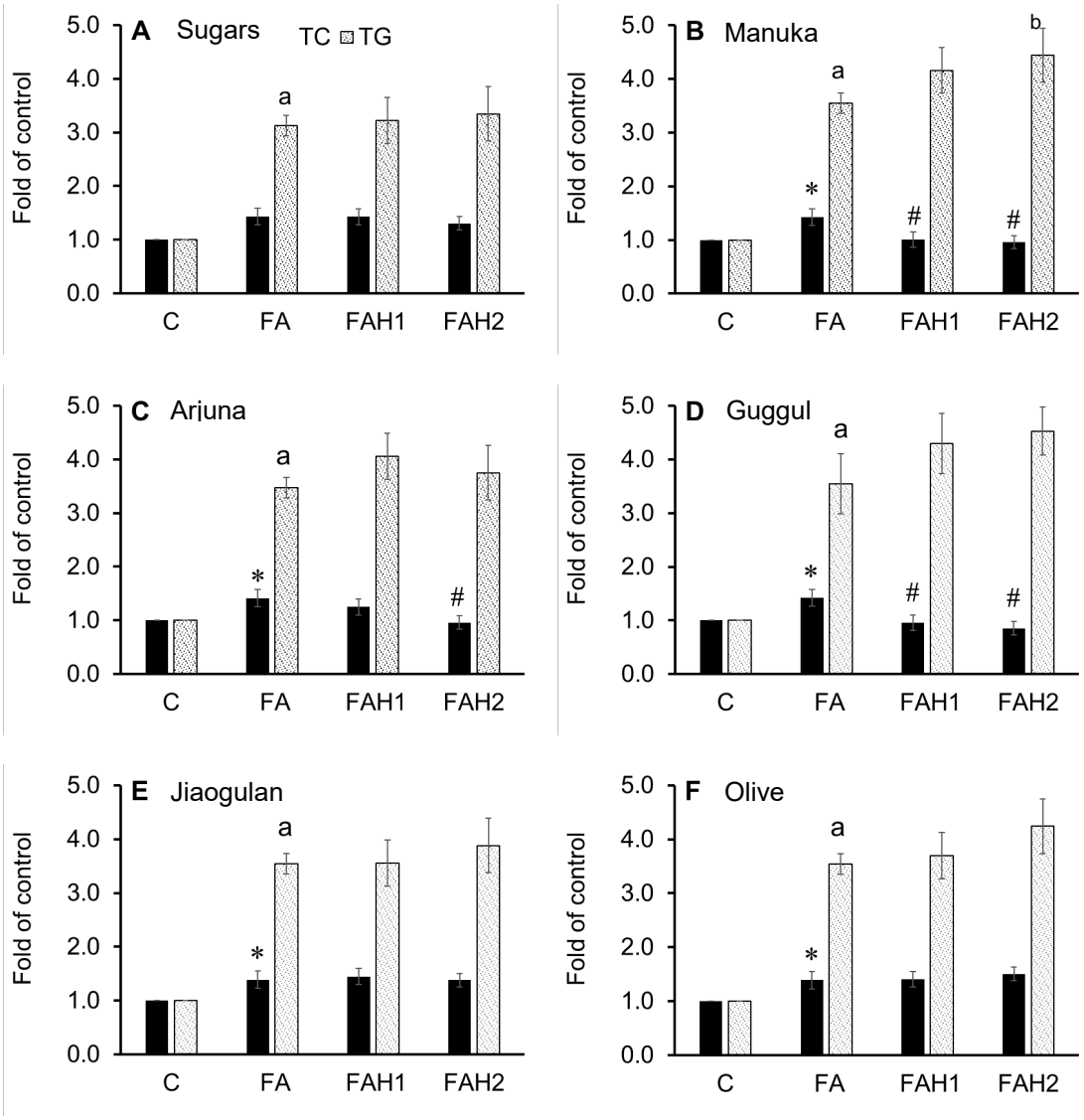

Figure 5. Total cellular cholesterol (TC) and triglyceride (TG) contents after 24-hour incubation of fatty acid-induced HepG2 cells with (A) honey analogue containing sugars, (B) manuka-1 honey containing methylglyoxal 400^{+} mg/kg, (C) arjuna honey, (D) guggul honey, (E) jiaogulan honey and (F) olive honey. C: control cells, FA: fatty acid treatment, FAH1: fatty acids and honey 1.0%, FAH2: fatty acids and honey 2.0%. Results are mean ± SD of four replicates; ∗: $p < 0.05$ vs. control and #: $p < 0.05$ vs. fatty acid-treated cells for TC; a: $p < 0.05$ vs. control and b: $p < 0.05$ vs. fatty acid-treated cells for TG. Data was analyzed by one-way ANOVA followed by Duncan's multiple comparison test.

3.3.6. Effect of medicinal honeys on key biomarkers of cholesterol homeostasis

To understand the effect of honey on lipid parameters at molecular levels, we explored the expression of selected genes (*AMPKα*, *SREBP*-2, *HMGCR*, *LDLR*, *LXRα* and *PPARα*) associated with lipid metabolism pathways using RT qPCR analysis (Figure 6). Firstly, we examined the effect of honey analogue, a solution that represents sugar components of honey (40% fructose, 35% glucose, 5% sucrose, w/w) on the genes to establish a baseline for the medicinal honeys (Figure 6A). The analogue (1.0 and 2.0%) stimulated *SREBP2* through the activation of *AMPKα*, however the upregulation of *SREBP2* downstream genes, *HMGCR* and *LDLR* was observed at 1.0% but not at 2.0% concentration (3.9 and 1.2 fold, respectively). The results indicate that 1.0% honey analogue stimulated the cholesterol synthesis pathway in the fatty acid-induced HepG2 cells, likely through the SCAP-SREBP2 pathway by either or both lowering free cholesterol in the cells and/or desensitizing SCAP to cholesterol (Oteng, Loregger, van Weeghel, Zelcer, & Kersten, 2019) . The results are consistent with an increased TC content in fatty acid-induced HepG2 cells treated 1.0% honey analogue (Figure 5A) and in line with previous studies (Oteng et al., 2019; Zhao et al., 2016).

We found 1.0% honey analogue up-regulated the *LXRα* and *PPARα* (Figure 6A). *LXRs* function in cholesterol absorption, transport, efflux, and excretion process. The upregulation of *LXRs* has been reported to improve reverse cholesterol transport and circulation of high-density-lipoprotein (Jia, Hoang, Jun, Lee, & Lee, 2013). It was reported that glucose binds to LXR and forms a heterodimer with 9-*cis* retinoic acid receptor. Subsequently, the heterodimer binds to *SREBP-1c* and *LXR* response element in the promoter region to induce the transcription of target genes associated with cholesterol and glucose metabolism pathways (Lagu, Lebedev, Pio, Yang, & Pelton, 2007). A recent study reported that either glucose alone (25 mM) or a mixture of glucose and fructose upregulated *LXRα* in the presence of 1.0 mM fatty acids (Zhao et al., 2016). As honey analogue contains 40% fructose and 35% glucose

(w/v), the sugars could attribute to the activation of *LXRα* gene and enhanced *PPARα* transcript level.

In contrast, 2.0% honey analogue did not change the transcript level of any target genes even though high cellular cholesterol was recorded. It is extensively known that SREBP2 works in a negative feedback mechanism, particularly, increased cellular cholesterol leads to the suppression of *SREBP2* and downregulation of its target genes (Goedeke & Fernández-Hernando, 2012). In the present study, the elevation in *SREBP2* mRNA abundance at 2.0% honey analogue may result from the activation of *AMPK* but its level may be either insufficient to induce the target genes or suppressed by the high sugar treatment (2.0%) leading high cellular cholesterol content (Figure 5A and 6A).

The medicinal honeys (except for olive honey) at both 1.0 and 2.0% (w/v) activated *AMPKα* (>1.5 fold) compared to honey analogue (< 1.5-fold to control). Interestingly, all 1.0% medicinal honeys modulated *HMGCR* gene expression close to the level of controls (1.1 fold), much lower than the level upregulated by 1.0% honey analogue (3.9 fold), indicating the tested honeys modulated *HMGCR* gene expression against the effect of sugars. However, honeys showed different patterns in influencing other tested genes (Figure 6B–F).

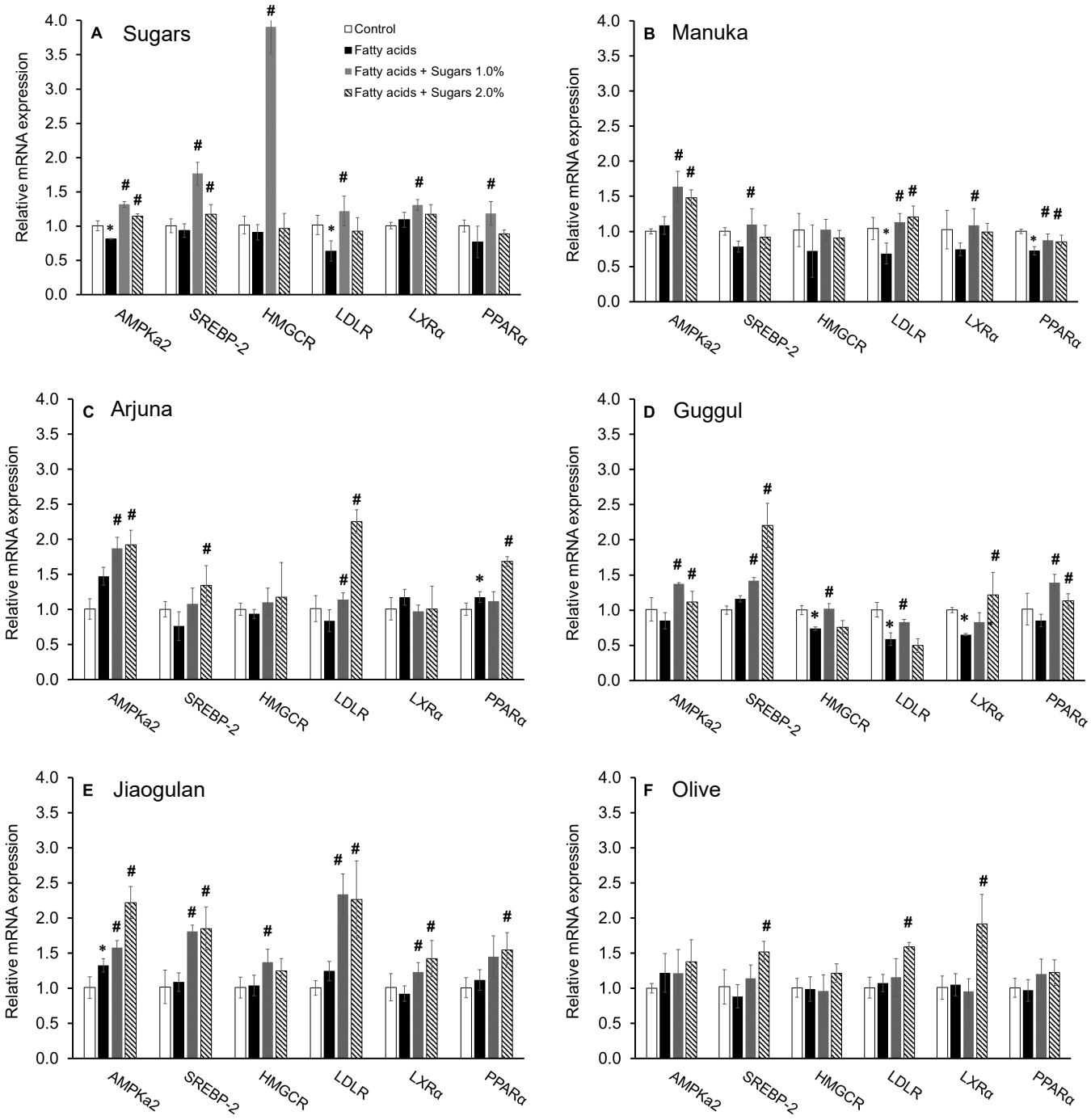

Figure 6. The expression of genes associated with cholesterol homeostasis after 24-hour incubation of fatty acid-induced HepG2 cells with (A) honey analogue containing sugars, (B) manuka-1 honey containing methylglyoxal 400^{+} mg/kg, (C) arjuna honey, (D) guggul honey, (E) jiaogulan honey, (F) olive honey. Results are mean ± SD of four replicates; ∗: $p < 0.05$ vs. control and #: $p < 0.05$ vs. fatty acid-treated cells, analyzed by one-way ANOVA followed by Duncan's multiple comparison test.

Manuka-1 honey (Figure 6B) at 1.0 and 2.0% did not affect *HMGCR*, but upregulated *LDLR* gene expression. As the transcriptional level of *LDLR* gene is highly controlled by cellular sterol levels though the activation of *SREBP2*, an elevation in cellular sterol level suppresses *LDLR* gene transcription and in contrast a depletion of cellular sterols causes to an increased *LDLR* mRNA abundance (Goedeke & Fernández-Hernando, 2012). This leads to an increased clearance of plasma LDL particles (Goldstein, DeBose-Boyd, & Brown, 2006). The results agreed with the reduction in cellular cholesterol content when treated with manuka-1 honey (Figure 5B), suggesting that this honey is effective in lowering total cellular cholesterol. Manuka-1 honey contains high amounts of phenolic compounds (72.1 mg/100g) that originated from manuka-1 floral nectar (Nguyen et al., 2018). These phytochemicals may attribute to its cholesterol-lowering effect.

Arjuna honey (Figure 6C) at 2.0% induced *SREBP2* and highly upregulated LDLR gene expression (2.3 fold) but did not stimulate *HMGCR* gene transcription. As discussed in honey analogue, the observation suggested that 2.0% arjuna honey is effective in lowering cellular cholesterol and possibly leads to improved uptake of plasma LDL-cholesterol. This honey did not vary the transcriptional levels of *LXRα* gene indicating it had no effect on this gene. These observations agreed with the decreased TC content in HepG2 cells treated with arjuna honey (Figure 5C). The results are supported by previous studies demonstrating that *T. arjuna* extracts decreased plasma lipids and increased HDL to reduce the severity of atherosclerotic lesion in aorta (Subramaniam et al., 2011) and favourably modify lipid profile in human subjects showing coronary artery disease (Priya et al., 2019).

Guggul honey (Figure 6D) at 2.0% significantly induced *SREBP2* compared to honey analogue at the same concentration (2.2 and 0.9 fold change relative to control, respectively). This honey also induced *LDLR* mRNA transcription at 1.0% as honey analogue did, but not at 2.0% as expected. Instead, it upregulated *LXRα* gene expression at 2.0% concentration. As

indicated in earlier investigations, activation of *LXRα* leads to the upregulation of its direct targets (ATP-binding cassette transporter A1 and G1) that transfer cellular cholesterol to small high density lipoproteins to prevent the formation of foam cells (Wójcicka, Jamroz-Wiśniewska, Horoszewicz, & Bełtowski, 2007). Several studies confirmed elevated expression levels of transporters genes and proteins were obtained the through the activation of *LXRα* in HepG2 cells (Peschel, Koerting, & Nass, 2007; Xu et al., 2011). Thus, guggul honey through the activation of *LXRα* likely increased the transcriptional levels of transporter genes and proteins, leading to a reduction in cellular cholesterol (Figure 5D). Our current investigation did not include the transporters, so we could not confirm a clear correlation between the transcriptional levels of tested genes associated with cholesterol homeostasis and the reduced cellular cholesterol content by guggul honey treatment, but indeed guggul honey induced the tested genes and modulated cellular cholesterol level in HepG2 cells. Guggul resin (guggulu) has been widely used as a cholesterol reducing remedy in Ayurveda medicine which is native to India. Shah, Gulati, & Palombo (2012) reported that guggul extracts have been approved to use as a dietary supplement by The US Food and Drug Administration since 1994. Guggulsterones (Z and E) have been demonstrated to reduce plasma cholesterol level and function as antagonists of the bile acid and farnesoid X receptors (FXR) in liver and intestine (Francis, Raja, & Nair, 2004; Owsley & Chiang, 2003; Passeri et al., 2019). Thus, further study focusing on FXR, transporter genes and proteins may support the role of guggul honey in hypocholesterolemia. In addition, based on the responsive trend of the genes, we hypothesize that clearer effects of guggul honey on the tested genes could be obtained at latter terminating time of the experiment.

Jiaogulan honey (Figure 6E) induced the expression of the aforementioned genes in a pattern that should lead to a lower TC content, but this reduction was not recorded. Our finding is contradictory with previous findings demonstrating jiaogulan extracts (0.1–0.3 mg/ml)

reduce TC content in primary hepatocytes (Müller et al., 2012), whereas total saponin extract of jiaogulan leaves activates AMPK, LXRα and its target transporters (ABCG5 and ABCG8) leading to a TC reduction in hepatocytes of animal models (Liu et al., 2016). Similarly, olive honey (Figure 6F) stimulated *SREBP2*, *LDLR* and *LXRα* genes at 2.0% concentration which could be a good basis to argue for the reduction of cellular cholesterol, but this expectation did not happen at 24 hours after honey treatment. Olive honey was developed in the current study using olive retentate, a by-product from olive oil production. As olive retentate contains an enriched and purified source of low molecular weight polyphenols that are usable for food and pharmaceutical industries (Russo, 2007), it is supposed to confer protective effect on oxidative stress and cholesterol homeostasis. Hence, further investigations are required to confirm if jiaogulan and olive honeys reduce cholesterol at 48 and 72 hours after treatment.

It has been evidenced that cholesterol exits as free cholesterol and cholesteryl esters in hepatocytes (Chang, Chang, Ohgami, & Yamauchi, 2006). An excess of cellular free cholesterol causes cytotoxicity and the cells have different mechanisms to maintaining cholesterol homeostasis including (i) cholesterol efflux (reverse cholesterol transport) and (ii) conversion of cholesterol into cholesteryl esters in the form of lipid droplets that are an inert storage pool (Drevon, Engelhorn, & Steinberg, 1980; Yamauchi & Rogers, 2018). In the current investigation, although jiaogulan and olive honey treatments could not lead to a reduction in total cellular cholesterol content after 24 hours, they possibly induced a conversion of free cholesterol to cholesteryl esters through the esterification of cholesterol with fatty acids in order to avoid free cholesterol toxicity in HepG2 cells through a certain mechanism, while still modulating the tested genes in the pattern of AMPK-SREBP2 pathway responsive to reduce cellular free cholesterol content (Oteng et al., 2019).

Manuka-1 and guggul honeys upregulated the *PPARα* gene expression at both 1.0 and 2.0% concentration, whereas arjuna and jiaogulan honeys increased the gene transcription at

2.0%. Olive honey did not show its effect on this gene (Figure 6A–F). Upregulation of *PPARα* gene expression is an indicator for an increased *β*-oxidation of fatty acids into smaller molecules, leading to a decreased cellular triglyceride content, essential for inhibiting atherosclerotic events (Lefebvre, Chinetti, Fruchart, & Staels, 2006). In the current study, although *PPARα* gene was stimulated, cellular triglyceride content was not reduced the honey treatments. This observation is likely supported by a previous finding which reported that the activation of *PPARα* suppresses the availability of triglycerides to for the assembly of very low-density lipoprotein (VLDL) and increases hepatic triglyceride accumulation (Edvardsson et al., 2006). However, in the current study the mechanism through which the tested honey types activated *PPARα*, its direct targets and the links to the increased triglyceride accumulation remain unclear.

3.4. Conclusions

The current investigation demonstrated that the physicochemical properties of the medicinal honeys complied with the international quality regulations (EEC, 110/2001), except for olive honey which showed low monosaccharide content (49.2%). FTIR analysis confirmed their functional groups are as of manuka-1 and other natural honeys. The phenolics and flavonoid content, radical scavenging activity and ascorbic acid equivalent antioxidant content recorded for arjuna and guggul honeys are higher than those for the commercial manuka-1 honey. Guggul honey is the best honey among the tested honeys for its highest phenolic content (108.2 mg GAE/100 g), moderate flavonoid content (4.1 mg CAE/100 g), radical scavenging activity (91.7%) and ascorbic acid equivalent content (81.9 mg/100 g). In contrast, jiaogulan honey showed the lowest values in the antioxidant tests. The medicinal honeys were used at 1.0 and 2.0% concentrations in the subsequent analysis after the cytotoxicity test using PrestoBlue reagent.

The study provided preliminary results for the protective effects of the medicinal honeys on lipid metabolism and support the antioxidant activity data. Manuka-1 and guggul honeys (1.0 and 2.0%) and arjuna honey (2.0%) reduced total cellular cholesterol, whereas honey analogue, jiaogulan and olive honeys did not change total cellular cholesterol content in fatty acid–induced HepG2 cells. The transcriptional levels of the tested genes associated with cholesterol homeostasis varied according to honey-type and concentration. Manuka-1 and arjuna honeys showed a good agreement between the modulation of the transcription of the tested genes and the reduced cellular cholesterol content. Guggul honey modulated the tested genes to reduce cellular cholesterol but further research at various time points is required to elucidate the mechanism. Jiaogulan and olive honeys did not reduce total cellular cholesterol content in the fatty acid-induced HepG2 cells, but their modulation pattern of gene expression suggested they could esterify free cholesterol into storage form. However, this hypothesis required further study. Except for olive honey, other medicinal honeys (1.0 and 2.0% for manuka-1 and guggul honey; 2.0% for arjuna and jiaogulan honeys) activated *PPARα,* but all the honeys did not reduce cellular triglyceride content in fatty acid-induced HepG2 cells.

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CHAPTER 3. PHYSICOCHEMICAL AND VISCOELASTIC PROPERTIES OF HONEY FROM MEDICINAL PLANTS

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Statement of authorship

Huong Thi Lan Nguyen (candidate) planned the experiment, carried out all research, analysed and interpreted data. Nguyen also reviewed literature, executed the writing and responded to reviewers.

Naksit Panyoyai shared the fundamental knowledge and experience in viscoelastic analysis of food high-solid materials.

Vilia Paramita shared the fundamental knowledge of viscoelasticity and contributed to editing the manuscript following the format of Food Chemistry journal.

Nitin Mantri supervised and supported the development of tulsi honey.

Stefan Kasapis was corresponding author. He supervised, planned the experimental design, edited the final version of the manuscript and submitted it for review and publication.

All the co-authors give full consent to Huong Thi Lan Nguyen to present the paper for examination towards the Degree of Doctor of Philosophy.

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Physicochemical and viscoelastic properties of honey from medicinal plants

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ABSTRACT

The present work investigated the physicochemical and structural properties of Tulsi, Alfalfa and two varieties of Manuka honey derived from medicinal plants. Chemical analysis yielded data on the content of reducing sugars (glucose and fructose) that dominate the honey matrix, and of the minor constituents of protein, phenols and flavonoids. Standard chemical assays were used to develop a database of water content, electrical conductivity, pH, ash content, visual appearance and colour intensity. Physicochemical characteristics were related to structural behaviour of the four honey types, as recorded by small-deformation dynamic oscillation in shear, micro- and modulated differential scanning calorimetry, wide angle X-ray diffraction and infrared spectroscopy. The preponderance of hydrogen bonds in intermolecular associations amongst monosaccharides in honey yields a semi-amorphous or semi-crystalline system. That allowed prediction of the calorimetric and mechanical glass transition temperatures that demarcate the passage from liquid-like to solid-like consistency at subzero temperatures.

1. Introduction

Honey is a natural sugar-saturated material used as food sweetener, complete food or medicinal supplement. Epidemiological studies reported protective and therapeutic effects of honey on overall health and well-being by improving the immune, antibacterial and antioxidation response extending to cardiovascular protection (Alvarez-Suarez, Gasparrini, Forbes-Hernández, Mazzoni, & Giampieri, 2014). It is made of sugar, mainly glucose and fructose up to 80% (w/w), and over 180 other components including proteins, free amino acids, essential minerals, vitamins, enzymes and phenolic phytochemicals (Alvarez-Suarez, Giampieri, & Battino, 2013). Phytochemicals are the main source of bioactivity and medicinal properties are transferred to honey through floral nectar and pollen collection by the bees (Alvarez-Suarez et al., 2010). They vary amongst plants to influence the level and diversity of bioactive compounds in honey but phenolic acids and flavonoids are the most abundant.

Tulsi plant (*Ocinum tenuiflorum* L.), Manuka tree (*Leptosperma scoparium*) and Alfalfa plant (*Medicago sativa*) have long been used in Indian traditional medicine as a source of bioactive molecules with therapeutic potential (Bora & Sharma, 2011). Literature reports that their pharmacological effects relate to anticancer, antiinflammation, hypolipidemia and cardioprotection, with the combination of nutritional and prophylactic properties promising long-term health benefits (Upadhyay et al., 2015).

To maximise health benefits and establish a solid platform of analytical information leading to application, there is a need to identify primarily the phenol and flavonoid contents in various types of commercially available honey (Meda, Lamien, Romito, Millogo, & Nacoulma, 2005). Physicochemical characterization including water content, electrical conductivity, ash content, pH, visual colour and colour intensity, reducing sugar, and total protein can facilitate standardisation of honey bee products (Saxena, Gautam, & Sharma, 2010). Furthermore, quality attributes that relate to palatability *via* oral administration and topical treatment for infected wounds require fundamental understanding of the physical state of honey and its thermodynamic transition from liquid to solid-like behaviour as a function of environmental temperature (LeBail et al., 2003).

Honey is a high-solid material and should possess a characteristic glass transition temperature (T_g), which is a parameter widely used to predict, hence optimise the quality and stability of products during processing and subsequent storage. Above the respective T_g value, food products develop a rubbery and/or a melt state, with a considerable decrease in viscosity allowing for greater mobility. This outcome results in structural changes of the condensed matrix including collapse, stickiness, caking and fusion (Santivarangkna, Aschenbrenner, Kulozik, & Foerst, 2011). Below the glass transition temperature, systems enter the glassy region where molecular diffusion, leading to chemical, enzymatic and biological reactions, is limited (Roos, 2010). Recently, the concept of mechanical or network glass transition temperature has been introduced to complement estimates of the calorimetric T_g in glass forming matrices like honey as a function of

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0308-8146/

temperature or timescale of observation (Kasapis, 2006).

Given the above, this study aims to examine the physicochemical and structural properties of various types of honey obtained from different medicinal plants known for antiinflammation and lowering cholesterol properties. Making available information from fundamental studies can facilitate development of product concepts, with honey being the main component, showing an increasingly likelihood of acceptance by the consumer.

2. Materials and methods

2.1. Materials

Four different types of honey, i.e. Tulsi (TUL), Alfafa (ALF) and Manuka (MH1 and MH2), were used in this study. The former is a monofloral honey produced from the nectar of Tulsi plants grown in the green house of RMIT University, Australia. Varieties ALF, MH1 and MH2 are from Pennsylvania (USA), New South Wales (Australia) and Warrandyte (New Zealand), respectively. They were stored in air-tight jars under dark ambient conditions and subjected to 40 °C heating for 5 min to provide a common baseline for all systems prior to experimentation. Chemicals and reagents used in this study were AR standard. Folin–Ciocalteu's phenol reagents (2 N), sodium carbonate (> 99.5%), absolute ethanol (> 99.8%) and gallic acid (> 97.9%) were purchased from Sigma-Aldrich Co (Sydney, Australia).

2.2. Methods

2.2.1. Standard physicochemical analyses

Triplicate measurements were taken from distinct batches for each of the four types of honey studied following the protocol of International Honey Commission (Bogdanov, Martin, & Lullmann, 2002). In doing so, water content and total soluble solids were measured with a Refracto 30GS (Mettler Toledo, Australia) and converted accordingly using the Chetaway Table. Electrical conductivity was measured on SevenCompact Conductivity Meter S230 (Mettler Toledo) at 20 °C in 20% (w/v) honey solution in Milli-Q water. Ash content was obtained by placing 5 g of honey in a crucible (Labec, Australia) and heating at 600 °C overnight in a muffle furnace. pH measurement of 10% (w/v) honey solution was performed following the method of Moniruzzaman, Khalil, Sulaiman, and Gan (2013).

Visual colour was assessed following a method described by Bertoncelj, Doberšek, Jamnik, and Golob (2007). A chromameter CR-400/410 (Konica Minolta, Australia) was used for CIE L^*, a^*, b^* measurements of our samples, where L^*: lightness, $-a^*$: greenness, a^*: redness and b^*: yellowness, as compared with the white tile background. Colour intensity of 50% (w/v) honey solution, which was filtered at 0.45 μm to remove any coarse particles, was measured as described by Beretta, Granata, Ferrero, Orioli, and Maffei Facino (2005). Spectrophotometric absorbance was taken at 450 nm using a Lambda 35 UV–vis spectrophotometer from Perkin Elmer (Waltham, USA).

2.2.2. Reducing sugars

Amounts of D-glucose and D-fructose in honey were determined with Megazyme's Assay Kit (K-SUFRG 06/14). D-glucose was determined by utilising hexokinase and glucose-6-phosphate without hydrolysing sucrose. D-fructose was determined subsequent to the determination of D-glucose following isomerisation with phosphoglucose isomerase. Samples were analysed in triplicate and the mean is expressed as g/100 g honey.

2.2.3. Protein content

This was determined with Thermo Scientific™ Coomassie (Bradford) Protein Assay Kit (23200). Twenty μl of 10% (w/v) honey solution were pipetted into a microplate. Then, 250 μl of Coomassie reagent were added and the plate was put in a shaker to incubate at ambient temperature for 10 min. Absorbance was measured at 595 nm with a Polar microplate reader, against a standard solution of bovine serum albumin (0–100 μg/ml) that reached linearity of $R^2 = 0.994$. Milli-Q water was used as blank, and each sample was analysed in triplicate, with the mean being expressed in mg/100 g honey.

2.2.4. Total phenolic content

This was determined with the Folin-Ciocalteu method (Singleton, Orthofer, & Lamuela-Raventós, 1999). Honey sample (5 g) was diluted to 50 ml with Milli-Q water and filtered through Whatman No. 1 paper. Solution (0.5 ml) was mixed with 2.5 ml of 0.2 N Folin-Ciocalteu reagent (Sigma-Aldrich, Australia) for 5 min and 2 ml of 75 g/l sodium carbonate was then added. Mixture was incubated at ambient temperature for 2 h before reading with a Lambda 35 UV–vis spectrophotometer. Absorbance was measured at 760 nm against an ethanol blank. Gallic acid was used to produce a standard curve from 0 to 100 mg/l and obtained linearity was $R^2 = 0.999$. All analyses were carried out in triplicate and the mean was expressed in mg of gallic acid equivalents (GAE)/100 g honey.

2.2.5. Fourier transform infrared spectroscopy

A spectrometer equipped with a MIRacleTM ZnSe single reflection ATR plate (Perkin-Elmer, Norwalk, USA) was used to record FTIR spectra for honey. In doing so, 0.5 g was placed onto the measuring plate and scanned forty times from 4000 to 650 cm^{-1} at a resolution of 4 cm^{-1} at ambient temperature.

2.2.6. X-ray diffraction analysis

Presence of crystal nuclei in honey was examined using a D4 Advanced Bruker AXS (Karlsruhe, Germany) attached with a Cu-Kα radiation source (l = 1.54 A°). Triplicate samples were loaded onto the measuring holder and covered with an X-ray film. Raw data were obtained within a 2θ range of 5–90° in the interval of 0.1° and subsequently analysed using Diffract.EVA version 4.1.1.

2.2.7. Modulated and microdifferential scanning calorimetry

First-order thermodynamic transitions of honey were detected with a Setaram VII microdifferential scanning calorimeter (Seturau, France). Hundred mg of honey were loaded into a standard Hastelloy cell and an identical-weight water sample was used as reference. They were equilibrated for 20 min at 20 °C, heated to 90 °C, and then cooled to 20 °C at 1 °C/min. Heat capacity measurements to determine the calorimetric glass transition temperature were conducted on Q2000 calorimeter (TA instruments, New Castle, USA), with nitrogen purge gas at a flow rate of 50 ml/min. Ten mg of honey were loaded into a hermetic aluminium pan and equilibrated for 20 min at 20 °C. Samples were cooled to −90 °C and heated up to 30 °C at 1 °C/min. Triplicate measurements were performed at modulation amplitude of 0.53 °C every 40 s.

2.2.8. Viscoelastic analysis

This was performed with ARG-2 controlled strain rheometer using a magnetic-thrust bearing technology (TA Instruments, New Castle, USA). The rheometer was connected to a liquid nitrogen system to achieve rapid and uniform cooling. Parallel plates of 5 mm diameter and 1 mm gap were used, with samples (0.5 g) being loaded at 15 °C and covered with silicone oil (BDH; 50 cS) to minimise moisture loss. They were equilibrated at 15 °C for 10 min, then cooled deeply within the subzero regime at 1 °C/min with a frequency of 1 rad/s and strain of 0.01%. Frequency sweeps of 0.1 to 100 rad/s were taken from −60 to −20 °C at intervals of three degree centigrade. Time-temperature superposition principle was implemented to generate the master curve of viscoelasticity for honey.

2.2.9. Statistical analysis

Statistical differences in Table 1, represented by letters in the same row for the physicochemical properties of all samples, were obtained

Table 1
Characteristic parameters of honey.

	TUL	ALF	MH1	MH2
Physicochemical parameters				
Water content (%)	18.7 ± 0.3b	18.2 ± 0.3a	18.5 ± 0.1ab	19.1 ± 0.2c
Electrical conductivity (μS/cm)	0.80 ± 0.01d	0.16 ± 0.01a	0.54 ± 0.01c	0.44 ± 0.01b
Ash content (%)	0.16 ± 0.01c	0.04 ± 0.01a	0.11 ± 0.05b	0.07 ± 0.01ab
pH	4.1 ± 0.1b	3.9 ± 0.1a	4.1 ± 0.1b	4.0 ± 0.1a
Visual colour				
L^*	66.9 ± 0.4c	44.2 ± 1.6b	47.8 ± 0.5b	32.5 ± 2.50a
a^*	−4.1 ± 0.1a	−2.0 ± 0.1b	12.9 ± 0.4d	3.1 ± 1.2c
b^*	26.8 ± 1.2c	7.7 ± 0.3a	36.3 ± 1.3d	16.9 ± 1.6b
Colour intensity (mAU)	743 ± 3b	174 ± 1a	2201 ± 60d	1574 ± 8c
Reducing sugars (g/100 g honey)				
Glucose	34.9 ± 3.4b	30.9 ± 3ab	28.9 ± 2a	29.9 ± 0.3ab
Fructose	38.3 ± 3a	36.4 ± 2a	44.2 ± 2b	37.4 ± 0.6a
Total protein (mg/100 g honey)	147.1 ± 5d	102.9 ± 3a	128.5 ± 1c	117.6 ± 2b
Total phenol (mg GAE/100 g honey)	50.6 ± 2.7b	18.3 ± 0.3a	72.1 ± 2c	75.4 ± 0.8c
Total flavonoid (mg QE/100 g honey)	3.74 ± 0.08b	1.92 ± 0.12a	9.57 ± 0.16d	6.46 ± 0.09c
Viscoelastic parameters				
T_g (°C)	−47	−45	−44	−46
$C_1{}^g$	10.70	10.85	11.43	11.13
$C_2{}^g$ (deg)	50	50	50	50
f_g	0.040	0.040	0.038	0.038
α_f (deg^{-1})	8.0×10^{-4}	8.0×10^{-4}	7.6×10^{-4}	7.6×10^{-4}
E_a (kJ/mol)	108	86	81	99

Note: 1. Data are mean ± STD of triplicate measurements taken from distinct batches for each of the four types of honey studied, with different letters in the same row (a–d) indicating significant differences ($p < 0.05$) using Duncan's multiple-range test.
2. TUL = Tulsi honey, ALF = Alfafa honey, MH1 = Manuka honey 1 having unique Manuka factor 20+, and MH2 = Manuka honey 2 having methylglyoxal 400+.

through one-way analysis of variance (ANOVA). This was followed by Duncan's multiple-range test with $p < 0.05$, and it was run on SPSS version 22 (IBM Australia Limited, NSW).

3. Results and discussion

3.1. Quality parameters characterising honey

A plethora of physicochemical and viscoelastic parameters have been obtained for the various types of honey in this investigation and these have been summarised in Table 1. To start with, water content for all samples remained within the range of 18.6 ± 0.5%, i.e. below 20% (w/w), which is the limit approved by the European Union (number 110; EEC, 2001) and similar to that found earlier (Habib, Al Meqbali, Kamal, Souka, & Ibrahim, 2014). This is an important quality criterion indicating shelf-life and ripeness, with high water content promoting fermentation of honey by osmotolerant yeasts. Electrical conductivity varies from about 0.16 to 0.80 μS/cm. Honey contains organic acids and mineral salts, which are chemically ionizable and conduct electric current in solution, and this is affected by botanical origin. Based on European Union guidelines, it should not exceed 0.8 μS/cm for nectar flower honey, which is a determinant of good quality with the exception of some plant species such as strawberry tree (*Arbutus unedo*), bell heather (*Erica*), etc.

Ash content analysis represents the level of minerals and trace elements, and yields values between 0.07 and 0.16%, with TUL being at the upper end of the range (Table 1). Correlation coefficient between electrical conductivity and ash content is very good ($R^2 = 0.810$) in the present study. Ash values are within the limit allowed for blossom honey (≤0.6%) indicating clearness and no adulteration with molasses (EEC, 2001). Nectar honey contains minerals from 0.1 to 0.3% while honeydew honey can reach 1.0% of the total (Belay et al., 2017; Bogdanov, 2009). pH measurements indicate that our honey samples are acidic within normal expectations being comparable to the previous findings of 3.5–4.7 (Saxena et al., 2010). Visual appearance of honey was characterised by a set of tristimulus values arguing that the Tulsi honey possesses the lightest colour ($L^* = 66.9$) with a blend of yellowish and greenish hues. Its colour intensity in the form of absorbance at 450 nm was intermediate (743.2 mAU) between ALF (174.5 mAU) and MH1 (2201.5 mAU), an outcome which is congruent with earlier observations for several types of honey ranging from 125 to 3400 mAU (Beretta et al., 2005; Habib et al., 2014).

The overall content of reducing sugar (glucose and fructose) shown in Table 1 is similar amongst the honey varieties examined presently demarcating a range of 70 ± 3% solids, an outcome which is in line with previous observations (Gomes, Dias, Moreira, Rodrigues, & Estevinho, 2010; Habib et al., 2014). However, the individual amounts of D-glucose and D-fructose in TUL (34.9 and 38.3 g, respectively) is statistically distinct from those in MH1 (28.9 and 44.2 g, respectively). The ratio of fructose-to-glucose indicates the physical state of the honey matrix, and when this is less than 1.0, it crystallises rapidly (Draiaia et al., 2015). Total protein content was determined with bovine serum albumin as the standard and, accordingly, it makes our samples fall into the category of "high protein" honey compared to the range of 41 to 79 mg/100 g reported in the literature (Kishore, Halim, Syazana, & Sirajudeen, 2011). Protein in honey is mainly composed of enzymes or amino acids varying with floral source, enzymes from the honeybees and nectar, and length of storage (Saxena et al., 2010).

Total phenol and flavonoid content in TUL was higher than ALF but lower than MH1 and MH2. Results match those of visual appearance reported in Table 1, where honey with a stronger colour intensity exhibits a higher phytochemical content. Since phenolic content is strongly correlated with antioxidant activity (Mahmoodi-Khaledi et al., 2017; Meda et al., 2005), this parameter is a good criterion for a quick assessment of honey bioavailability.

3.2. Physicochemical characterization of honey

Building a database of quality parameters allowed us to further examine the samples of this investigation by subjecting them to a chemometric analysis based on Fourier transform infrared spectroscopy, wide-angle X-ray diffraction, and microdifferential scanning calorimetry. Fig. 1 illustrates vibrational assignments corresponding to specific chemical linkages obtained using FTIR. Broad bands in 3700–3000 cm^{-1} and 3000–2800 cm^{-1} regions correspond to O–H stretching, indicating the presence of bound and free water molecules,

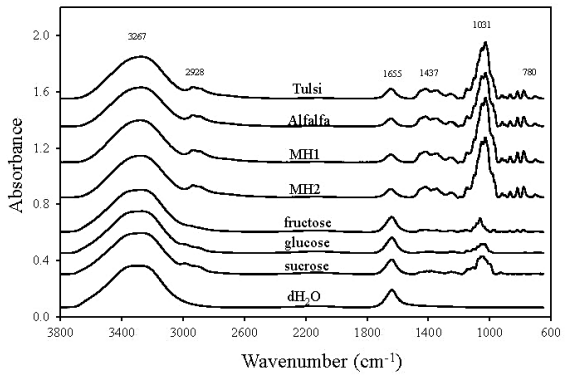

Fig. 1. FTIR spectra of Tulsi, Alfalfa, MH1 and MH2 honey, condensed fructose, glucose and sucrose systems at a moisture content of 19% (w/w), and distilled water.

and C–H stretching of sugars present in honey (Tewari & Irudayaraj, 2004). Infrared vibrations appearing in 1540–1175 cm^{-1} are specific of bending modes for C–C–H, C–O–H and O–C–H groups (Pataca, Neto, Marcucci, & Poppi, 2007).

There is a significant absorbance band in 1175–940 cm^{-1} primarily from C–O and C–C stretching modes, which makes this region a suitable fingerprint for assessment of honey's sugars (Anjos, Campos, Ruiz, & Antunes, 2015). Bands in 900–750 cm^{-1} arose primarily from specific saccharide configurations of C–O and C–C stretching modes being relatively intense in the various types of honey examined here but not in fructose, glucose or sucrose molecules. Finally, a peak at 1655 cm^{-1} appearing in all samples (including distilled water) is due to bending of H–O–H molecules.

Next, we assessed the morphological characteristics of our samples using WAXD (Fig. 2). TUL and MH1 exhibit an amorphous structure, as for the concentrated fructose preparations, with a typical broad peak occurring at ~20°. From MH2 to ALF there is an increasing number of peaks, which indicate crystallinity in honey similar to that recorded for pure sucrose and glucose systems. A peak at 44° being specific to all samples but MH1 is suggestive of aliphatic carbon chains (Paramita, Bannikova, & Kasapis, 2015).

Thermal analysis was conducted on micro-DSC at a scan rate of 1 °C/min for the samples of this investigation (Fig. 3). Upon heating, maxima in endothermic peaks were observed at about 47 and 50 °C for MH2 and ALF, respectively, which were similar in overall shape arguing

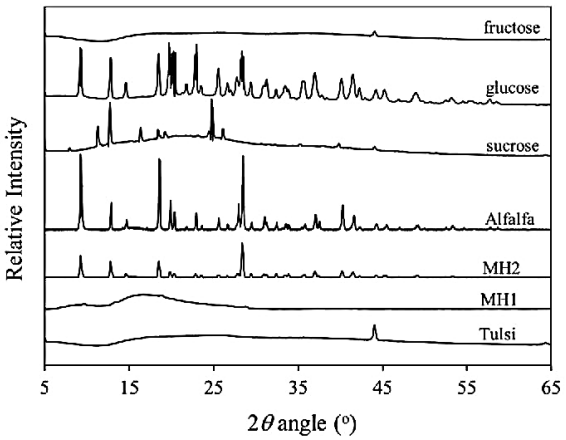

Fig. 2. X-ray diffractograms of fructose, glucose and sucrose systems at a moisture content of 21% (w/w), and Alfalfa, MH2, MH1 and Tulsi honey.

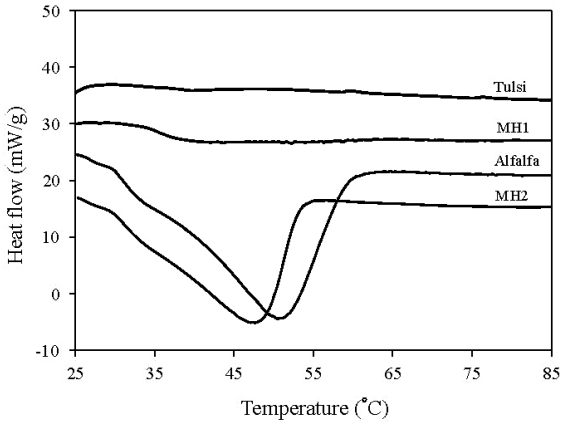

Fig. 3. MicroDSC heating profiles for Tulsi, MH1, Alfalfa and MH2 honey at a scan rate of 1 °C/min.

for a single molecular process. In contrast, TUL and MH1 appeared in essence as featureless thermograms. Endothermic events should be due to the melting of sugar crystals in the honey matrix, an event that depends on sample composition and storage conditions. Results are congruent with the X-ray diffractograms in Fig. 2, which produced largely flat baselines in TUL and MH1, but multiple peak spectra in MH2 and ALF.

3.3. Determination of the calorimetric glass transition temperature for honey

Besides the first-order thermodynamic transition recorded for some honey samples in the preceding section, high-solid systems should also exhibit an element of vitrification upon controlled cooling. This is best related to the glass transition region, which has been monitored presently well into the subzero regime (Fig. 4). A step change of heat capacity is observed for all systems with heating from −75 to −15 °C, an outcome which indicates a devitrification process. The onset, midpoint and endset temperatures have been considered as empirical indicators of the calorimetric glass transition temperature and, in general, this depends on parameters including solids level, sample annealing and scan rate to mention but a few. The four types of honey in our study exhibited a midpoint T_g of −47 ± 2 °C being in good agreement with previous estimates that range from −47 to −51 °C (Ahmed, Prabhu,

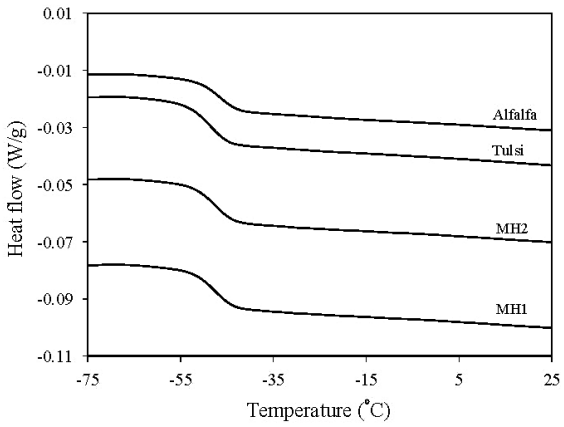

Fig. 4. Modulated DSC heating profiles of Alfalfa, Tulsi, MH2 and MH1 honey at a scan rate of 1 °C/min.

Raghavan, & Ngadi, 2007).

The magnitude and temperature band of the thermal transition for honey is mainly influenced by the plasticizing effect of water content, which is about 18.5% as highlighted in Table 1. That should also be affected by the chemical composition of glucose, fructose, protein, pigments and other solid constituents (Cordella et al., 2002). A previous study on polysaccharide/sugar systems reported a fifty centigrade depression of the calorimetric T_g values as a result of water plasticization from 10 to 20%, w/w (Roos & Karel, 1991). The nature of calorimetric measurements reflects primarily the sugar content, as opposed to molecular interactions between structural components of the high-solid matrix. Mechanical spectroscopy, on the other hand, is ideally posed to examine such interactions supporting long range effects in relation to predictions of the glass transition temperature, and this will be dealt with next.

3.4. Determination of the mechanical glass transition temperature for honey

Viscoelastic properties were evaluated with dynamic oscillatory measurements in-shear yielding values of storage modulus (G') and loss modulus (G'') that reflect the relative solid-like and liquid-like properties of materials (Panyoyai, Bannikova, Small, & Kasapis, 2015). Fig. 5a depicts the variation in values of G' and G'' for Tulsi honey with controlled cooling at 1 °C/min. There is a sharp increase in moduli of four orders of magnitude from 10^5 to 10^9 Pa with cooling at subzero temperatures. This is known as the glass transition region where the viscous component dominates over the elastic component of the system. At the low end of the temperature range, modulus traces crossover to yield a dominant elastic response, and remain essentially constant. This is known as the glassy state where long-range molecular movements, hence physicochemical reactions are limited.

To advance the discussion from a qualitative to a quantitative treatment that deconvolutes the temperature and time contributions to the viscoelastic spectrum, we employed the time-temperature superposition principle (Kasapis, Al-Marhoobi, & Mitchell, 2003). In doing so, we implemented a series of frequency sweeps from 0.1 to 100 rad/s at constant temperature intervals of three degree centigrade. Fig. 5b reproduces data for storage modulus in Tulsi, which remain unchanged in the glassy state (e.g. −54 °C) but show a strong frequency dependence in the glass transition region (e.g. −24 °C); G'' data for Tulsi is not plotted here.

Mechanical spectra of storage and loss modulus were superposed horizontally by selecting an arbitrary reference temperature in the glass transition region ($T_o = -42$°C) and shifting the remainder left or right in relation to T_o in order to generate the master curve of viscoelasticity for Tulsi honey (Fig. 5c). This reproduced the passage of viscoelastic functions from the glass transition region to the glassy state over a wide frequency range, i.e. $10^{-3.5}$–$10^{3.5}$ rad/s, and represents the time (or frequency) analogue of the temperature profile discussed in Fig. 5a.

Horizontal superposition of frequency sweeps to construct the viscoelastic master curve generates a set of shift factors, a_T, which constitute a fundamental mechanism in honey vitrification. These are plotted against experimental temperature in Fig. 5d for TUL (and also MH1) honey. Within the temperature range of the glassy state, there is a linear correlation between temperature and log a_T suggesting that solidified honey follows the predictions of the reaction rate theory, as

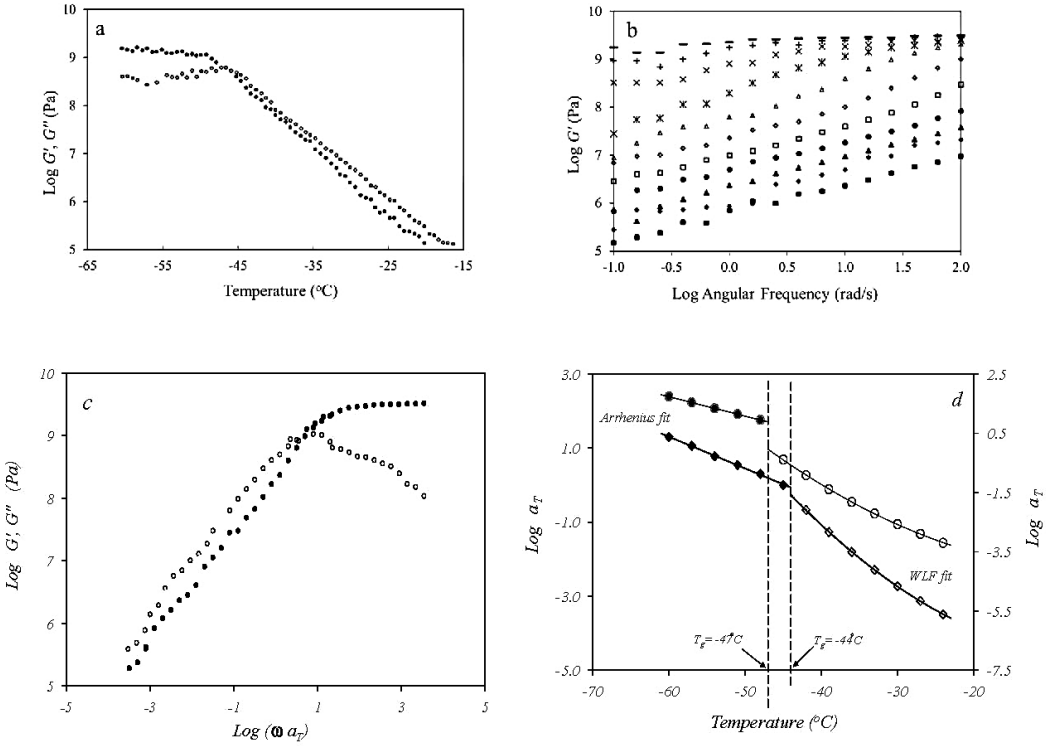

Fig. 5. (a) Heating profiles of G' (●) and G'' (○) for Tulsi honey at a rate of 1 °C/min, frequency of 1 rad/s and strain of 0.01%, (b) Frequency variation of G' for Tulsi honey at −54(–), −51(+), −48(×), −45(*), −42(△), −39(◇), −36(□), −33(●), −30(▲), −27 (♦) and −24(■) °C arranged successively downwards, (c) Master curve of viscoelasticity for reduced shear modulus (G'_p, ● and G''_p, ○) for Tulsi honey, and (d) Shift factors as a function of experimental temperature for Tulsi (●,○; right y-axis) and MH1 (♦,◇; left y-axis) honey with the Arrhenius fit (close symbols) and WLF fit (open symbols); dashed lines pinpoint predictions of the mechanical T_g.

described by the modified Arrhenius equation (Kasapis, 2008):

$$\log a_T = \frac{E_a}{2.303R}\left(\frac{1}{T} - \frac{1}{T_o}\right) \quad (1)$$

where, E_a is the activation energy for permitted vibrations from one conformational state to another and R is the universal gas constant. E_a values are calculated to be ~108 and 81 kJ/mol for TUL and MH1, respectively, and similar estimates are cited for ALF and MH2 in Table 1. The above energy of activation values afford direct comparison of molecular dynamics between carbohydrate matrices with glassy consistency. They are comparable to glucose syrup and glucose syrup/K-carrageenan systems at 80% (w/w) solids (98 and 140 kJ/mol) used as excipients for bioactive compound encapsulation (Kasapis & Shrinivas, 2010). However, the energy of activation of bioactive compounds (e.g. caffeine) is considerably lower (0.23–0.47 kJ/mol) reflecting the freedom of caffeine to diffuse within the glassy carbohydrate matrix.

At the upper temperature range of the glass transition, progress in viscoelasticity for TUL and MH1 is better described in Fig. 5d by the theoretical framework of free volume, as modelled by the Williams, Landel and Ferry equation (Ferry, 1980):

$$\log a_T = -\frac{C_1^0(T-T_0)}{C_2^0 + T - T_0} = -\frac{(B/2.303f_0)(T-T_0)}{(f_0/\alpha_f) + T - T_0} \quad (2)$$

where C_1^0 and C_2^0 are the WLF constants at T, (C_1^g and C_2^g at T_g), f, is the fractional free volume at T_o (f_g at T_g), α_f is the thermal expansion coefficient (deg^{-1}) above T_g, and B is usually set to 1.

Data fitting to the WLF model was also implemented for ALF and MH2 and results are summarised in Table 1. As far as we are aware, values of WLF constants, fractional free volume, thermal expansion coefficient and mechanical T_g, based on free volume theory, are given for the first time in honey systems. These are according to experience for amorphous synthetic polymers and condensed carbohydrate matrices undergoing glass transition (Kasapis, 2008). Estimates of the fractional free volume at the glass transition temperature are about 0.040 indicating that the honey matrix has reached a state of kinetically trapped equilibrium in molecular relaxation (Roos, 2010).

Values of the mechanical glass transition temperature range between −44 and −47 °C for the various types of honey in this investigation. They are comparable to the estimates from calorimetric T_g in Fig. 4 recorded at about −47 °C from the midpoint of the sigmoidal thermograms. In both cases, honey samples were identical in terms of composition and preparation history, and in the absence of three-dimensional polymeric structures, it is the sugar molecules that determine the vitrification patterns for both analytical techniques.

4. Conclusions

The present investigation develops a detailed picture of the physicochemical and viscoelastic properties of four types of honey, i.e. Tulsi, Alfalfa and two types of Manuka, from medicinal plants. Quality criteria are in accordance with the international regulations from the European Union for good quality honey. This includes the relatively high total phenolic and flavonoid content that supports a potential for natural remedy. Results indicate that at ambient temperature honey is a supercooled liquid with glucose crystals being precipitated into a solution of fructose and other minor ingredients. Therefore, MH1 and TUL honey with a relatively-high fructose content produce amorphous diffractograms (as for the pure fructose sample) shown in Fig. 2, and flat microDSC thermograms shown in Fig. 3. In contrast, ALF and MH2 honey exhibit multiple WAXD peaks whose glucose crystals liquefy in an endothermic transition recorded calorimetrically. Condensed systems of polysaccharide/sugar or protein/sugar yield different predictions for the mechanical and DSC T_g, due to the distinct property and distance scale being probed by the two techniques. In the absence of polymeric network formation in honey, however, thermomechanical predictions of glass transition temperature coincide, hence emphasizing the predominant role of bulky sugars in the metastable state of honey matrix. Finally, successful utilisation of the WLF equation in this work allowed estimation of the free volume parameters for honey vitrification.

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CHAPTER 4. STRUCTURAL VARIATION IN GELATIN NETWORKS FROM LOW TO HIGH-SOLID SYSTEMS EFFECTED BY HONEY ADDITION

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Statement of authorship

Huong Thi Lan Nguyen (candidate) planned the experiment, carried out all research, analysed and interpreted data. Nguyen also reviewed literature and executed the writing of the manuscript.

Lita Katopo examined English, format and presentation of the manuscript.

Eddie Pang sponsored travel grant for presenting parts of this research in a Food Hydrocolloids Conference.

Nitin Mantri supervised and evaluated the research.

Stefan Kasapis was corresponding author. He supervised, planned the experimental design, edited the manuscript, submitted it for review and publication.

All the co-authors give full consent to Huong Thi Lan Nguyen to present the paper for examination towards the Degree of Doctor of Philosophy.

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Structural variation in gelatin networks from low to high-solid systems effected by honey addition

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ABSTRACT

Honey is a biologically active material functioning antibacterial, anti-inflammation and immune responses that enhance wellbeing. This research aims to record and rationalise the structural properties of honey as part of a convenient delivery system in the presence of gelatin that provides the structuring matrix. In doing so, we employ dynamic oscillation in-shear, micro and modulated DSC, WAXD, FTIR and ESEM. A wide range of solids was employed from 10% (*w/w*) gelatin to mixtures with up to 75% (w/w) honey. Increasing addition of co-solute created thermally stable gelatin networks, which at high levels of total solids undergo a glass transition. This allows deconvolution of the total heat flow into the reversing and non-reversing thermograms. In addition, mechanical spectra can be treated by the combined free volume/reaction rate theory to predict the molecular dynamics of the gelatin-honey system. Molecular interactions between the two components and the relative contribution of honey to the crystalline or amorphous part of the binary preparation are elucidated guiding future applications for orally and topically treated ailments.

1. Introduction

Honey is a natural aromatic sweetener containing mainly sugar and non-sugar components. With water content typically < 20%, honey is a sugar saturated material, comprising up to 70% monosaccharides, particularly fructose and glucose, 10% disaccharides, higher sugars, and a minor proportion of vitamins, minerals, proteins and amino acids. It is also an abundant source of phenolic acids and flavonoids originated from flower pollen and nectar (Bogdanov, Jurendic, Sieber, & Gallmann, 2008). Numerous honey phenolics are in aglycon form, as a result of hydrolysis of glycosylated flavonoids by the enzyme glycosidase in the bee salivary glands. These are bioavailable being easily absorbed through the gut barrier compared with their corresponding glycosides (Alvarez-Suarez, Giampieri, & Battino, 2013). Thus, the diverse secondary metabolites obtained from floral nectar and pollen are the main factors for the unique phytochemical profile of honey (Nicolson, Nepi, & Pacini, 2007). Apparently, honey antioxidant activity is more potent than vitamin E, C and carotenoids due to the synergistic effect of its various constituents displaying distinct structural architectures and functional groups (Gheldof & Engeseth, 2002).

There is increasing evidence from *in-vitro* and *in-vivo* studies that honey exhibits a wide range of health benefits including antioxidant, antimicrobial, anti-inflammatory, immunological and wound healing (Alvarez-Suarez et al., 2013). Functional food and biomedical industries are interested in developing convenient delivery vehicles of honey's bio-functionality but in doing so need to overcome certain physical limitations. Crystal formation during prolonged storage at ambient temperatures affects adversely its organoleptic quality for oral administration. (Escuredo, Dobre, Fernández-González, & Seijo, 2014). In addition, honey liquifies readily at skin temperature limiting its delivery to most body regions *via* an accurate therapeutic dose over the required time (Nguyen, Panyoyai, Kasapis, Pang, & Mantri, 2019). A recent attempt utilised cabopol 934, a cross-linked polyacrylic acid polymer, to formulate a pharmaceutical-grade topical product for healing and antibacterial action (El-Kased, Amer, Attia, & Elmazar, 2017; Sarhan, Azzazy, & El-Sherbiny, 2016).

Gelatin is a water soluble and high molecular weight protein extracted from cattle bone or pigskin collagen using alkaline or acid hydrolysis. Cooling solutions below ambient temperature allows rearrangement of disordered coils to a triple helix, which above the minimum critical gelling concentration (about 1%) facilitates the formation of a gel (Gómez-Guillén et al., 2002). OH groups of amino acids (mainly hydroxyproline) and the peptide C=O groups form a multitude of hydrogen bonds with water molecules to stabilise thermodynamically the triple helix and account for the protein's conformation and structural properties (Xing et al., 2014). Collagen is abundant in

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nature hence gelatin is inexpensive, easily processed, biocompatible and is used extensively in food and pharmaceutical industries (Dash, Foston, & Ragauskas, 2013).

Addition of sugars (sucrose and lactose) up to 40% to mammalian gelatin increased the gel strength of the protein and maintained its elastic nature (Kuan, Nafchi, Huda, Ariffin, & Karim, 2016; Shimizu & Matubayasi, 2014). Incorporation of a sugar replacer, polydextrose, to a gelatin preparation at levels that reached 65% showed considerable enhancement of thermal stability and prevention of ice formation at subzero temperatures (Almrhag et al., 2012b). It is expected that sugars will interfere with the hydration cylinder that coats gelatin's triple helix thus altering the water-molecule filled grooves of its network. Honey is a relatively complex mixture of sugars that are able to crystallise at ambient or subzero temperatures. The present work utilises honey to reach concentrations up to 85% in preparations in order to identify and discuss unexpected structural properties and phase behaviour of the gelatin network during the transition from hydro- to high solid gels.

2. Materials and methods

2.1. Materials

Type A gelatin from pigskin, bloom 300 (G2500-1KG), was supplied from Sigma-Aldrich (Sydney, Australia). Medicinal Manuka honey (MH) is a commercial product from New South Wales, Australia with MGO index 400^{+}. MH has water activity 0.54, contains total soluble solids of 80% including glucose 28.9% (*w*/w), fructose 44.2% (w/w), total phenolic content 0.72% (g GAE/100 g honey), total protein content 1.28% (w/w), other sugars and minor components.

2.2. Methods

2.2.1. Sample preparation

Systems containing 10% gelatin and different levels of honey based on dry mass ranging from 0, 10, 20, 30, 40, 50, 60, 70, 75% (w/w) were prepared to cover the entire range of low-solid gelatin-honey hydrogels to high-solid glassy matrices. Briefly, gelatin was hydrated in Milli-Q water overnight at 4 °C and then dispersions were heated at 55 °C for 30 min to yield clear solutions. Honey was incubated at 45 °C for 30 min before adding to the gelatin solution at 55 °C followed by mixing for 10 min to ensure formation of a homogeneous mixture.

2.2.2. Rheological measurements

Viscoelasticity of low and intermediate solid mixtures (≤ 60% honey) was assessed using rheometry from 50 to 0 °C to prevent ice formation (AR-G2; TA Instruments, New Castle, DE, USA). A constant strain of 0.1% within the linear viscoelastic region (LVR), angular frequency of 1 rad/s, scan rate of 1 °C/min, 20 mm parallel plate geometry and 1 mm gap were applied throughout. Values of elastic (storage) modulus, G', and viscous (loss) modulus, G'', were recorded using the technique of small-deformation dynamic oscillation in-shear. Samples were loaded onto the Peltier plate of the rheometer at 45 °C, exposed edges were covered with silicon oil (dimethylpolysiloxane, 50 cP) to minimize water loss and were equilibrated at this temperature for 5 min prior to subsequent examination.

Viscoelasticity of high solid mixtures (> 60% honey) was also assessed at subzero temperatures. The rheometer was connected to a liquid nitrogen source for rapid and uniform cooling. A constant strain of 0.01% within LVR, angular frequency of 1 rad/s, scan rate of 1 °C/min, 5 mm diameter parallel plate geometry, and 1 mm gap were applied throughout. Samples were cooled well within the subzero regime (*e.g.* -50 °C), and this was followed by a heating run that was interrupted every three-degree centigrade to obtain frequency sweeps from 0.1 to 100 rad/s. This facilitated application of the time-temperature superposition principle to generate the master curve of the sample's viscoelasticity.

2.2.3. Thermal analysis

A Setaram VII micro differential scanning calorimeter (Seturau, France) was used to detect first-order thermodynamic transitions due to the melting of gelatin helices in mixture with a wide range of honey concentrations. Five hundred and fifty mg of sample was loaded into a standard cell with an equal weight of water serving as reference. They were heated to 55 °C to erase thermal history from preparation and loading, cooled to 5 °C, equilibrated at that temperature for 30 min and then heated for a second time to 80 °C at 1 °C/min to record the endothermic thermograms.

A Q2000 modulated differential scanning calorimeter (TA instruments, New Castle, USA) was used to record changes in heat flow of the gelatin-honey mixtures at subzero temperatures. Ten mg of the sample were loaded into a hermetic aluminium pan and equilibrated for 30 min at 5 °C against an empty pan. They were then cooled to −90 °C, left there for another 30 min, and heated up to 0 °C at the scan rate of 1 °C/min with nitrogen purge gas at a flow rate of 50 ml/min, and a modulation amplitude of 0.53 °C every 40 s. Thermal runs for both micro and modulated DSC were carried out in triplicate generating overlapping thermograms.

2.2.4. Fourier-transform infrared spectroscopy (FTIR) of gelatin-honey systems

A MIRacleTM ZnSe single reflection ATR plate-linked spectrometer (Perkin-Elmer, Norwalk, USA) was used to obtain interferograms for our systems. 0.2 g sample were placed onto the measuring plate and scanned sixty-four times from 4000 to 500 cm^{-1} with a resolution of 4 cm^{-1} at ambient temperature. Final spectra were obtained by subtracting the water spectrum. Analysis was performed in triplicate yielding identical results.

2.2.5. Wide angle X-ray diffraction (WAXD) of gelatin-honey matrices

The crystalline component of the gelatin-honey matrix was evaluated using a D4 Advanced Bruker AXS (Karlsruhe, Germany) attached to a Cu-Kα radiation source (λ = 1.54 A°). Samples were loaded onto the measuring holder and raw data were recorded within a 2θ range of 5 to 90° in an interval of 0.1°. Subsequent analysis was implemented with Diffract.EVA version 4.1.1. Triplicate samples were measured at ambient temperature returning consistent results.

2.2.6. Scanning electron microscopy (SEM) of freeze-dried gelatin-honey samples

To obtain SEM images, the binary systems of gelatin with honey were freeze dried and platinum coated. They were then examined using FEI Quanta 200 SEM (Hillsboro, OR, USA). Topography images were taken under low vacuum condition (0.3 Torr) with an accelerated voltage of 30 kV and spot size of 3.0. Each micrograph was set at 10 mm working distance and 1600 times magnification.

3. Results and discussion

3.1. Effect of honey addition on the rheology of gelatin hydrogels

The procedure utilised to determine the mechanical properties of gelatin gels at low and intermediate levels of honey addition (≤ 60%) involved a cooling run from 55 to 0 °C, which allowed observation of the elastic contribution (G') of the developing network. The measured values of strain amplitude and phase angle of the dynamic oscillatory trace in Fig. 1 shows a typical behaviour for a 10% gelatin preparation that exhibits structure formation at temperatures below 27 °C. The temperature dependent nature of this phase transition results in a smooth increase in the resolved values of storage modulus reaching just over 10^{4} Pa at 0 °C. The role of hydrogen bonding, being reinforced with decreasing temperature, in stabilising gelatin's triple helix is well documented in the literature (Kuan et al., 2016; Tau & Gunasekaran, 2016).

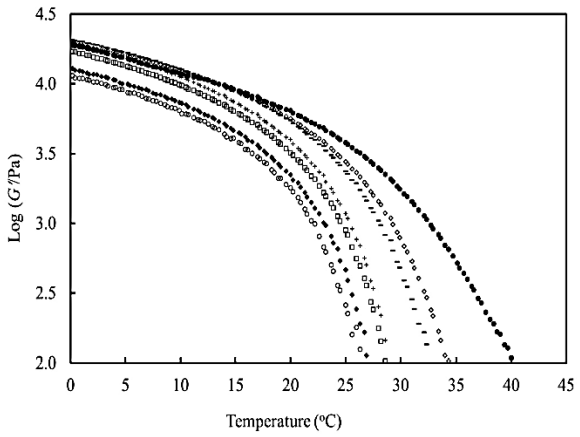

Fig. 1. Cooling profiles of storage modulus (G') for samples containing 10% gelatin in the presence of 0 (○), 10 (♦), 20 (□), 30 (+), 40 (−), 50 (◊), 60 (●) honey (%, w/w); scan rate of 1 °C/min, 0.1% strain and frequency of 1 rad/s.

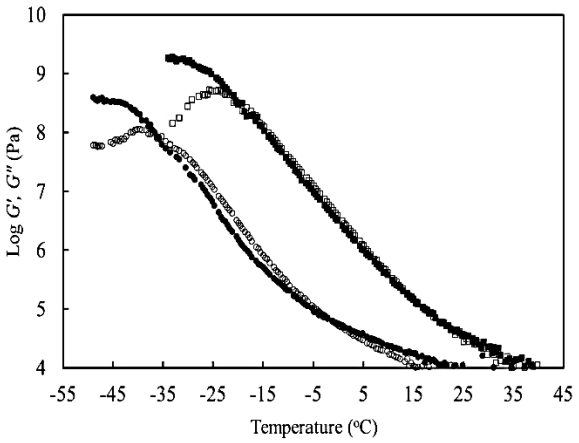

Fig. 2. Cooling profiles of storage (G') and loss (G'') modulus for samples containing 10% gelatin in the presence of 70 (●,○) and 75 (■, □) honey (%, w/w); scan rate of 1 °C/min, 0.01% strain and frequency of 1 rad/s.

The plot of log G' *versus* temperature also shows the full range of system responses effected by gradual removal of water molecules and replacing with honey. Thus, a 60% co-solute addition generates thermally stable structures, well above the deconvolution temperature of the aqueous gelatin helix, with the onset of gelation being recorded at 41 °C. In addition, cooling of the honey incorporating systems shows a smooth progression of storage modulus, which at the end of the cooling run reaches values in excess of 21 kPa. Outcomes are congruent with earlier investigations on polysaccharide coil-to-helix transitions, where stronger and thermally stable networks were reported with the addition of glucose syrup or mixtures of sucrose and glucose syrup to agarose, deacylated gellan and κ-carrageenan preparations (Kasapis, Al-Marhoobi, Deszczynski, Mitchell, & Abeysekera, 2003; Oakenfull & Scott, 1986; Stenner, Matubayasi, & Shimizu, 2016; Tau & Gunasekaran, 2016). It has been argued that such addition of co-solute in "soft solid" hydrocolloids creates an overriding thermodynamic drive for the formation of numerous and extended junction zones that alter their thermomechanical profile, as observed presently in Fig. 1.

3.2. Effect of honey addition on the rheology of high solid gelatin gels

In this section of the study, the development of viscoelastic structure is monitored as a function of temperature in high solid preparations; these are made by mixing 10% gelatin with 70 and 75% (*w*/w) honey. Log G' and log G'' are plotted against temperature in Fig. 2, which this time extends well within the subzero regime creating an extensive window of observation. At the high temperature end, both samples exhibit a dominant elastic component ($G' > G''$) due to the formation of a gel with rubbery consistency. The relatively flat temperature dependence of shear modulus is the outcome of an enthalpic network where rapid conformational changes of chain segments occur but there is no rearrangement of adjacent cross-links (Hutchinson, 1995).

Upon cooling to subzero temperatures, there is a clear progression in viscoelasticity of five orders of magnitude, with both systems achieving extraordinarily high values in the vicinity of 10^9 Pa. There is also a clear crossover of the modulus traces with the viscous component becoming dominant (*e.g.* $G'' > G'$ between −5 and −36 °C for the preparation of 80% total solids). This is known as the glass transition region and the dominance of the loss modulus in this region reflects the formation of an entropic network from the vibrations of molecular segments that are shorter than the distance of neighbouring cross-links or points of entanglement (Kasapis & Sablani, 2005). The dynamics of Rouse and sub-Rouse motions in the glass transition region result in a bigger separation between the loss and storage modulus traces, *i.e.* higher tan δ (G''/G') values are recorded at lower levels (70%) of honey addition to the gelatin network.

There is yet another development at the low temperature end of our experimental window where the two modulus traces cross over once more and the elastic contribution to the network becomes dominant. This is known as the glassy state where the values of G' level off around 10^9 Pa and those of G'' diminish rapidly leading to tan δ values well below one. It commences at −36 and −21 °C for the 80 and 85% (*w*/w) total solid preparations, respectively, hence documenting the anti-plasticising effect of increasing solids content to the glass transition of our systems. Within the glassy state, rapid local segmental motions, which are enthalpic in nature, involve the relaxation of β transitions or bending and stretching of chemical bonds generating a solid-like mechanical response (Panyoyai, Bannikova, Small, & Kasapis, 2015). Fig. 2 constitutes the temperature analogue of the master curve of viscoelasticity, and mechanical glass transitions have been recorded for amorphous synthetic polymers and high solid polysaccharide-glucose syrup preparations (Kasapis & Sablani, 2008; Yan, Saiani, Gough, & Miller, 2006).

A principal characteristic of the glass transition is its time-temperature equivalence, which allows quantitative treatment of experimental observations (Paramita & Kasapis, 2018). This approach has been adopted presently by generating an expanded frequency window that follows the progression of shear modulus at subzero temperatures. In doing so, we implemented the time-temperature superposition principle (TTS) by obtaining frequency sweeps of G' and G'' from 0.1 to 100 rad/s at constant temperature intervals of 3 °C (Supplementary Fig. 1 illustrates the frequency variation of storage modulus for the preparations with 80 and 85% total solids). Then, a reference temperature (T_o) was chosen arbitrarily within the glass transition region and the remaining mechanical spectra were shifted horizontally on either side to create a continuous and smooth trace.

Fig. 3a depicts the outcome of the horizontal superposition for both moduli and high solid preparations, which is the time analogue of the viscoelastic master curve. It is based on the notion that the shear modulus values obtained from a single frequency sweep at a temperature T are equivalent to those at T_o, provided the frequency (ω) is multiplied by a shift factor (a_T) (Kasapis, 2008). Fig. 3a depicts the reduced values of shear modulus (G'_p and G''_p) following shifting of the experimental data over a seven-decade frequency range ($10^{-3.3}$ to $10^{3.7}$ rad/s), i.e. well beyond the instrumentally accessible frequency range (typically between 10^{-1} and 10^2 rad/s). For both preparations, this

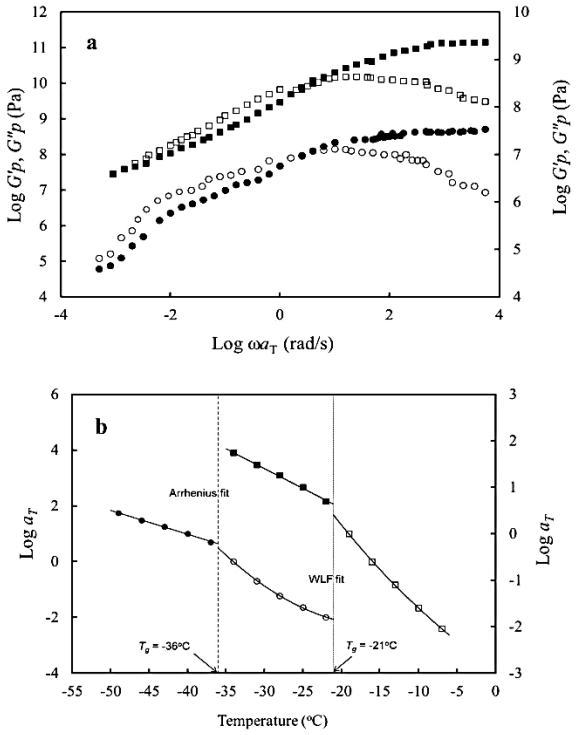

Fig. 3. (a) Master curves of viscoelasticity for gelatin-honey systems containing 10% gelatin in the presence of 70% (*G'p*, ●; *G"p*, ○; plotted on the left Y-axis) and 75% honey (*G'p*, ■; *G"p*, □; plotted on the right Y-axis), and (b) Logarithmic shift factors a_T as a function of temperature for systems containing 10% gelatin with 70% (●,○; plotted on the left Y-axis) and 75% honey (■, □; plotted on the right Y-axis); arrows indicate the predictions of the mechanical glass transition temperature.

covers the passage from the glass transition region ($G'' > G'$) to the glassy state ($G' > G''$) with increasing frequency, in analogy with the temperature induced transformation in Fig. 2.

Superposition of the mechanical spectra creates a set of shift factors that possess fundamental value in elucidating the molecular process governing vitrification phenomena. Values of factor a_T are plotted against experimental temperature (in Celsius for convenience) in Fig. 3b for the high solid gelatin-honey samples. Two distinct profiles are obtained, and data in the high temperature end can be modelled with the theoretical framework of Williams, Landel and Ferry (WLF equation in Ferry, 1980):

$$\log a_T = -\frac{C_1^o(T - T_o)}{C_2^o + T - T_o} = -\frac{(B/2.303f_o)(T - T_o)}{f_o/\alpha_f + T - T_o}$$

where, C_1^o and C_2^o are the WLF parameters, f_o is the fractional free volume at T_o, α_f is the thermal expansion coefficient (deg^{-1}), and B is usually set to one.

This non-exponential dependence of shift factor is superseded at the low temperature end with a linear semi-log profile that is described better by the predictions of the reaction rate theory (Paramita, Bannikova, & Kasapis, 2015):

$$\log a_T = \frac{E_a}{2.303R}\left(\frac{1}{T} - \frac{1}{T_o}\right)$$

The modified Arrhenius equation with a set of two experimental temperatures utilises the universal gas constant (R is equal to 8.31 kJ/mol) to yield a value for the activation energy (E_a). Progression in viscoelasticity from free volume governed processes to a reaction rate, required to overcome an energetic barrier for the molecular rearrangements from one state to the other, has been utilised earlier to offer a fundamental definition of the mechanical glass transition temperature (T_g) (Kasapis, 2008). These were found to be −21 and −36 °C for samples of 85 and 80% total solids, respectively, an outcome that coincides with the second crossover of shear modulus traces in Fig. 2.

Table 1
Viscoelastic parameters of single honey samples and gelatin-honey mixtures.

Parameters	Gelatin-honey mixture (w/w)		Honey (w/w)[a]
	80%	85%	(80%)
T_g (°C)	−36	−21	−44
C_1^g	11.43	10.86	11.43
C_2^g (deg)	50	50	50
f_g	0.038	0.040	0.038
α_f (deg^{-1})	7.6×10^{-4}	8.0×10^{-4}	7.6×10^{-4}
E_a (kJ/mol)	119	135	81

[a] Parameters for Manuka honey are taken from Nguyen et al. (2018)

Modelling outcomes are presented in Table 1 and these are compared with data from the literature for single preparations of Manuka honey at 80% (*w/w*) concentration (Nguyen, Panyoyai, Paramita, Mantri, & Kasapis, 2018). Working protocol appears to be internally consistent producing values of WLF parameters, fractional free volume and thermal expansion coefficient according to experience. Interestingly, values of the activation energy are lower for the single honey system reflecting the increasing difficulty for configurational rearrangements to occur within the glassy state of the gelatin matrix. This outcome is consistent with previous work reporting an increase in E_a from 97.8 to 140.2 kJ/mol at 65 and 70% glucose syrup supported by a gellified κ-carrageenan (0.5%) matrix (Kasapis, 2001). Similarly, the glass transition temperature of Manuka honey is considerably lower than its mixture with gelatin at the same level of solids (80%), which also supports the concept of cohesive intermolecular interactions limiting segmental mobility in the vitrified protein/co-solute system.

3.3. Thermal properties of the gelatin-honey system

Macromolecular characterisation of the gelatin-honey blend using rheology is complimented in this section with micromolecular aspects of the system *via* calorimetry. Fig. 4 depicts thermograms from micro DSC obtained for the protein in the presence of a wide range of co-

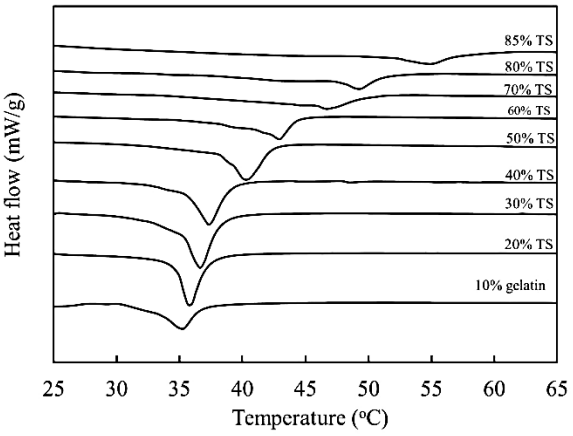

Fig. 4. Heat flow profiles of 10% gelatin with increasing levels of honey creating mixtures of 20, 30, 40, 50, 60, 70, 80, 85% (w/w) total solids; heating rate of 1 °C/min.

solute following controlled heating. A highly cooperative endothermic peak is observed for the single gelatin preparation (10% w/w) that corresponds to a helix-to-coil transition in the vicinity of the collagen deconvolution temperature (about 35 °C). Raising the content of honey in formulations generates a sequence of increasingly thermally stable materials. For example, values of the midpoint transition temperature (T_m) increase from 36 and 42.5 to 54.9 °C at 20, 60 and 85% total solids, respectively. Simultaneously, thermal events become less cooperative giving rise to very broad melting peaks of reduced enthalpic content. For the above three solid levels, values of enthalpy change (ΔH) were found to be 3.0, 2.3 and 0.9 J/g, respectively. It appears that the increasing shortage of water molecules and their relatively stable hydrogen bonding with honey molecules deprives gradually the gelatin helix of the hydration layer necessary for the formation of a three-dimensional structure. Eventually, the threshold of thermodynamic stability for network formation is exceeded, and considerable parts of the protein chains "dissolve" in the saturated sugar environment with concomitant collapse in the values of ΔH.

As for small-deformation rheology, work was extended to the sub-zero regime utilising modulated DSC where the sample temperature is modulated sinusoidally with a constant heating rate and the total heat flow is deconvoluted into reversing and non-reversing signals (Otte, Zhang, Carvajal, & Pinal, 2012). Separation into two thermal signals enhances the reproducibility and accuracy in determining the glass transition temperature, a thermally reversible event that is presented in the reversing component of the total heat flow (Cuq & Icard-Vernière, 2001).

As shown in Fig. 5, we were able to follow the reversing heat flow for a number of preparations at temperatures as low as −90 °C. Fifty percent was the lowest level of total solids (10% gelatin plus 40% honey) that produced realistically meaningful results, with a massive trough at about −20 °C indicating the reorganisation of molecular species into considerable ordered/crystalline assemblies following sample devitrification (Venir, Spaziani, & Maltini, 2010). This type of thermal event is substantially reduced at 60% (w/w) total solids and absent at higher levels of honey addition, since the increasingly dense structure of the matrix is able to bind effectively water molecules within the experimental timecale of obsevation. In the latter, there is a continuous and sigmoidal reduction in the heat flow signal giving rise to a step change in heat capacity, which is defined as the calorimetric manifestation of the glass transition region. The onset and endset temperatures of the heat capacity change define the empirical boundaries of the glass transition region, thus the midpoint T_g is located at the centre of the step change (Altay & Gunasekaran, 2013; Bell & Touma, 1996). Using this approach, we find the lowest recorded glass transition temperature of −76 °C at 60% total solids.

Remaining heating cycles see a gradual increase in the values of T_g, which becomes −63, −38 and −20.3 °C at 70, 80 and 85% total solids, while single honey systems (80% w/w) yield T_g estimates of −45 °C. The antiplasticising effect of honey addition is clearly seen in calorimetric data, as reported for the rheological counterparts in Table 1. Results are congruent with the midpoint T_g reported for other 80% solid biomaterials including pectin-glucose syrup (T_g = −41 °C) and individual polydextrose (T_g = −45 °C) or glucose syrup (T_g = −41 °C) preparations (Almrhag et al., 2012a, 2012b). There is a good overlap between rheological and calorimetric prediction of T_g in gelatin-honey blends of 80% solids (−36 and −38 °C, respectively), which emphasizes the contribution of the co-solute in the vitrification of these systems.

3.4. Other structural aspects of the gelatin-honey system

This section probes further into the physicochemical characteristics of our mixture by employing X-ray diffraction and Fourier transform infrared spectroscopy (FTIR). Fig. 6 presents the diffractograms of Manuka honey, 10% (w/w) gelatin and mixtures up to 85% total solids using wide angle (2-θ) between 5° and 90°. Gelatin exhibits a broad peak at 28°, which is characteristic of an amorphous material due to the lack of aggregation of its triple helix. A second broad peak appearing at about 40° with tailing up to 53° in both protein and protein/co-solute samples is typical of a dense amorphous morphology as a result of the freeze-drying conditions utilised presently to prepare samples (Paramita et al., 2015). There is a characteristic shift of the main peak to lower angles in mixtures, which indicates the considerable effect of co-solute addition to their morphology; 17° peak is obtained in pure honey samples. Diffractograms are devoid of sharp peaks arguing against the presence of substantial random order or crystallinity in the components and mixtures of our investigation. This largely amorphous character is congruent with the vitrification process discussed in thermomechanical analyses of the preceding sections (Figs. 2, 3 and 5).

Fig. 7 depicts the FTIR spectra of constituents and mixtures recorded in the mid-infrared range from 4000 to 500 cm^{-1} at ambient temperature. Absorption is specific to the linkages present in molecules, thus providing information on their identity and interactions. Gelatin exhibits five main spectral regions of 3300–3250, 3000–2800, 1680–1620, 1565–1475 and 1280–1180 cm^{-1}, corresponding to assignments of amides A, B, I, II and III, respectively (Kuan et al., 2016; Shurvell, 2006). Manuka honey shows particularly frequency vibrations

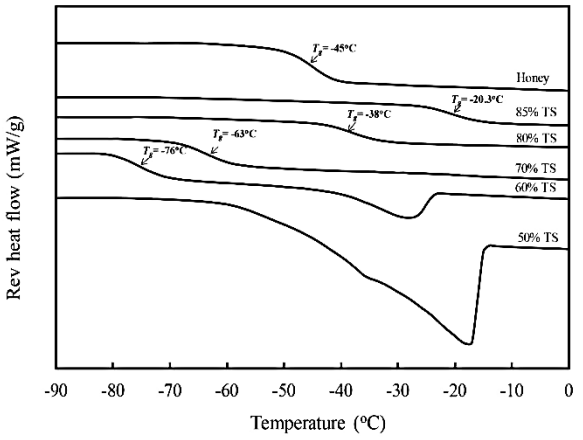

Fig. 5. Reversing heat flow of a single honey system and 10% gelatin with increasing levels of honey creating mixtures of 50, 60, 70, 80, 85% (w/w) total solids; heating rate of 1 °C/min.

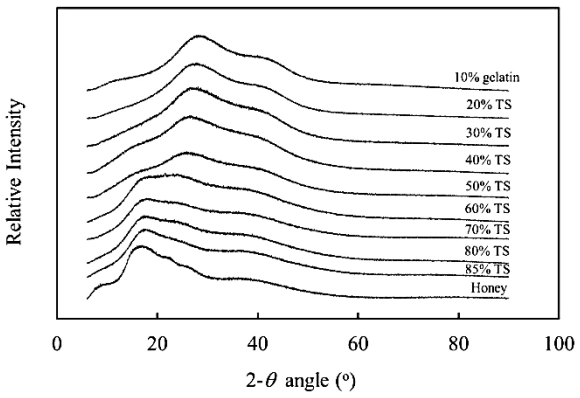

Fig. 6. X-ray diffractograms for single honey and gelatin preparations, and 10% gelatin with increasing levels of honey creating mixtures of 20, 30, 40, 50, 60, 70, 80, 85% (w/w) total solids.

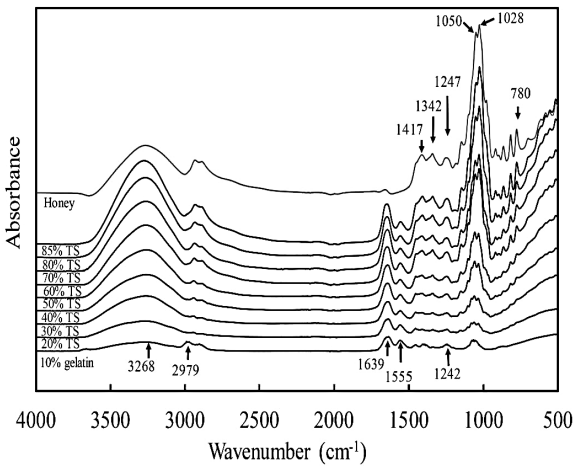

Fig. 7. FTIR spectra of systems containing 10% gelatin in the presence of honey at 0, 10, 20, 30, 40, 50, 60, 70, 75% (w/w) and a single honey system arranged successively upwards.

at 1500–1200 cm^{-1}, which are specific to C—C—H, C—O—H and O—C—H bending modes determining the carbohydrate contribution (Pataca, Neto, Marcucci, & Poppi, 2007). The range of 1200–900 cm^{-1} contains an intensive peak assigned to C—O and C—C stretching modes being identified as the fingerprint region of honey. Further bands at 900–750 cm^{-1} originate from the specific saccharide configurations of C—H bending modes (Gok, Severcan, Goormaghtigh, Kandemir, & Severcan, 2015).

The main IR peak assignments of the binary mixtures also show the vibrational regions of 3300–3250 and 3000–2800 cm^{-1} contributed by the NH stretching and CH stretching modes of amides A and B, respectively. Frequency range of 1680–1620 cm^{-1} (amide I), originating from the absorption of C = 0 stretching (Garidel & Schott, 2006), and region 1565–1475 cm^{-1} (amide II), derived from C—N stretching coupled with N—H bending modes (Benjakul, Oungbho, Visessanguan, Thiansilakul, & Roytrakul, 2009), remain unaltered in the blend. Collectively, the wavenumber bands characterising the vibrations of gelatin amides remain visible in the spectrum of the blend. In addition, intensifying or appearance of honey moiety vibrations reflects the overweight effect of carbohydrates in the mixture. Increasing inclusion of Manuka honey tends to shift the amide B peak to lower wavenumbers (from 2979 to 2929 cm^{-1}). Variation in peak wavenumbers of gelatin amide B in the presence of co-solute suggests some changes in the secondary conformation of the protein, most probably due to molecular interactions between O—H groups of sugars and C═O groups of gelatin amides (Farhat, Orset, Moreau, & Blanshard, 1998; Garidel & Schott, 2006).

Finally, scanning electron microscopy was employed to obtain tangible evidence of the phase morphology in gelatin-honey blends. Typical images of the binary systems containing 10% (*w*/w) gelatin and increasing amounts of honey (0, 60 and 75%) were selected to visualise three-dimensional structure. Individual gelatin gels in Fig. 8a form oriented features of super-helical and "phase separated" structures. Introduction of honey at intermediate levels in Fig. 8b tends to "dilute" the density of the helical strands of the protein. Formation of a high solid system in Fig. 8c leads to the development of a featureless background of a distinctly amorphous nature. This uniformly spread assembly following enrichment of the mixture with honey molecules argues for glass transition phenomena, as rationalised using thermomechanical analyses.

4. Conclusions

We have dealt with the molecular interactions and vitrification properties of gelatin-honey blends by changing the solvent quality of its physicochemical environment. That was achieved by keeping the protein content constant and increasing gradually that of honey, as the co-solute, thus cutting across conditions from low and intermediate to high solid systems. This allows monitoring the enthalpic behaviour of standard gelatin hydrogels, which is gradually superseded by the entropic viscoelasticity of high-solid honey-incorporating networks. Results argue for the formation of bioglasses that exhibit antiplasticising effects with increasing incorporation of the co-solute in mixtures. Cooling to subzero temperatures prevents ice formation to unveil a dramatic glass transition region in thermomechanical analyses. Implementation of the "sophisticated synthetic polymer approach" allows estimation of the mechanical glass transition temperature for the mixture in comparison to the experimentally derived DSC T_g.

Partial replacing of water molecules reinforces the interactions between gelatin and honey moieties creating a homogeneous system whose amorphicity provides the basis for niche applications in the functional foods and biomedical industries. For example, accurate determination of the mechanical strength in the gelatin-honey matrix, as advised in Fig. 1, can guide applications for burn wound dressing or oral administration. The latter can further benefit by considering the extent of crystalline consistency, as monitored by diffractograms in Fig. 6, in medicated lozenge tablets where mouthfeel is important given their slow solvation in the mouth. Finally, understanding the transition from the glassy state to the rubbery plateau, as recorded in Fig. 2 and rationalised in Fig. 3, can provide the basis for kinetically controlled and targeted delivery of bioactive compounds from a gelatin-honey

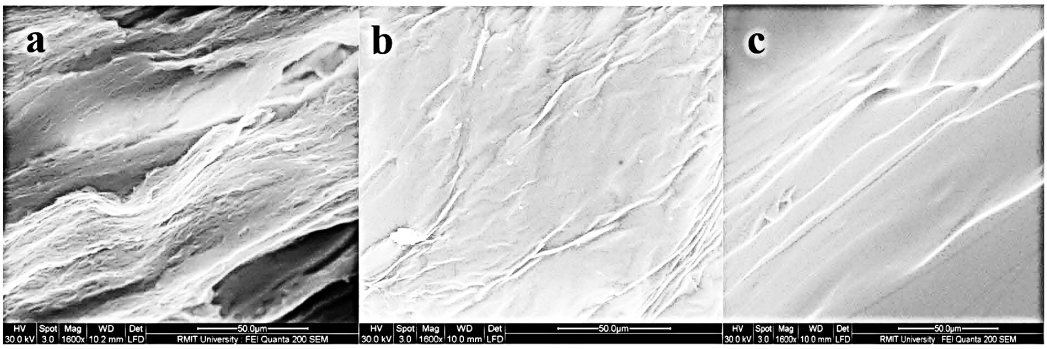

Fig. 8. Scanning electron microscopy images for representative systems containing 10% gelatin with (a) 0, (b) 60 and (c) 75% (w/w) honey; each micrograph was set at 10 mm working distance and 1600 times magnification.

based tablet in the way that has been demonstrated to a great advantage for drug delivery in the pharmaceutical research.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2019.03.048.

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CHAPTER 5: SUMMARY, CONCLUSIONS AND FUTURE WORK

5. 1. Summary and conclusions

Honey has been used throughout human civilization. Several studies reported its macro- and micro- components synergistically work to confer powerful antioxidant activities (Beretta, Orioli, & Facino, 2007; Gheldof & Engeseth, 2002; Özcan & Juhaimi, 2015). Our comprehensive literature review emphasizes the advantages of honey for the reduction of oxidative stress and hypercholesterolemia, two leading causes of atherosclerosis (Nguyen, Panyoyai, Kasapis, Pang, & Mantri, 2019). Recent studies indicate that numerous bioactive compounds are transferred from the plant tissues to floral nectar and honey, such as hesperetin, kaemferol, quercetin (Anand, Pang, Livanos, & Mantri, 2018; Yamani, Mantri, Morrison, & Pang, 2014). Moreover, honey contains higher number of secondary metabolites than its original floral nectar (Gismondi et al., 2018). The accumulated evidence solidifies the concept that honey can be an excellent delivery vehicle of dietary antioxidants and other plant medicinal compounds.

5.1.1. Physicochemical and biochemical characteristics of medicinal honeys and their effect on key biomarkers of oxidative stress and cholesterol homeostasis

Four medicinal honeys were developed using plant extracts from *T. arjuna* bark (arjuna honey), *C. mukul* stem (guggul honey), *G. pentaphyllum* leaves (jiaogulan honey) and *O. europaea* fruit retentate (olive honey) in The Pangenomics Laboratory. Plant species were selected based on scientific literature reporting their phytochemical profiles and biofunctions linked to either or both, antioxidant activities and hypocholesterolemia. In addition, a commercial manuka honey containing methylglyoxal 400^{+} mg/kg (manuka-1) from *L.*

scoparium floral nectar was included in the study as manuka honey is a "gold standard" for its high antioxidant capacity and health benefits (Alvarez-Suarez, Gasparrini, Forbes-Hernández, Mazzoni, & Giampieri, 2014). The main aims of the study in **Chapter 2** were to (i) first assess the physicochemical, and biochemical properties for the four medicinal honeys and (ii) elucidate their effect on cholesterol homeostasis in fatty acid-induced HepG2 cells.

The moisture content, electrical conductivity, and pH values of our medicinal honeys comply with international standards (EEC, 110/2001) and are consistent with other studies (Anand et al., 2018; Saxena, Gautam, & Sharma, 2010). However, manuka-1 honey showed its colour intensity 3−10 times higher than four medicinal honeys, possibly due to differences in the composition of *L. scoparium* floral nectar and the nectars made from medicinal plant extracts. Regarding viscosity of honeys which is inversely proportional to moisture content, the newly developed medicinal honeys were more viscous than manuka-1 honey and they all showed Newtonian liquid behavior. Manuka-1 and four medicinal honeys presented typical infrared spectra regions as reported for several honey varieties (Kasprzyk, Depciuch, Grabek-Lejko, & Parlinska-Wojtan, 2018), indicating high similarities in their main components.

The total monosaccharide content is over 60% for the honeys excluding olive honey (49.2%) as regulated by EEC (110/2001). Arjuna and guggul honeys were superior to manuka-1 honey in their phenolic and flavonoid contents, radical scavenging activity (RSA) and ascorbic acid equivalent antioxidant content (AEAC). Jiaogulan honey showed lowest phenolic and flavonoid compounds, corresponding to their least RSA and AEAC, whereas olive honey exhibited the highest flavonoid content but modest RSA and AEAC values. The RSA and AEAC data indicated synergistic effect of both phenolic and flavonoid compounds.

Cytotoxicity tests are extensively used to evaluate the safety thresholds of novel drugs/compounds. The current study indicated jiaogulan honey significantly reduced HepG2 cell population (93.0−95.0%) at 5.0% after 72 hours and 10.0% after 24 hours, suggesting this

honey either has anticancer effect or may contain more toxic compounds than other honeys, probably saponins abundant in *G. pentaphylum* leaves (Liu et al., 2016; Xie et al., 2012). The cytotoxic data also indicate no toxicity of honeys at 1.0 and 2.0% (w/v) for 24 hours. This is consistent with several findings for other natural honeys (Abel & Baird, 2018; Tonks et al., 2003).

Several studies reported antioxidant capacity of honey is contributed by antioxidant enzymes (such as catalase), phenolics that chelate metal elements, trap or scavenge free radical species (e.g. $O_2^{-\cdot}$, •OH, H_2O_2) and induce cellular enzymatic (superoxide dismutase, catalase, glutathione peroxidase), and non-enzymatic antioxidants (e.g. vitamins C and E, β-carotene, glutathione) (Procházková, Boušová, & Wilhelmová, 2011; Saxena et al., 2010). Antioxidant ability is tested in several biological systems including cell metabolites, tissue homogenates, aqueous portions of blood (plasma) where honey dissolves. In this study, the antioxidative effect of honeys was assessed through the expression of *Nrf2* and *NQO1*, the central genes associated with cellular defense pathway that eliminate oxidative and xenobiotic stress (Heiss, Schachner, Zimmermann, & Dirsch, 2013).

The expression analysis indicated that manuka-1 and jiaogulan honeys (1.0 and 2.0%) activate Nrf2 and significantly upregulate *NQO1* (>1.5 fold) compared to honey analogue (<1.5 fold). Other honeys at 2.0% concentration (arjuna, guggul and olive) considerably increase *NQO1* mRNA levels compared to the honey analogue. The possible reason is phenolic and flavonoid compounds in honey modulate Keap1/Nrf2/ARE gene pathway that ameliorates cellular oxidative stress (Gu et al., 2017; Roubalová et al., 2017; Sharath Babu, Anand, Ilaiyaraja, Khanum, & Gopalan, 2017; Vigliante, Mannino, & Maffei, 2019). However, the activation of *Nrf2* was not observed in arjuna, jiaogulan and olive honeys at 2.0%. This may be due to expression of this transcription factor before 24 hours which was not captured in this study (Gu et al., 2017).

Manuka-1 (1.0 and 2.0%), arjuna (2.0%) and guggul (1.0 and 2.0%) honeys modulate the transcription of *AMPKα*, *SREBP*-2, *HMGCR*, *LDLR* and *LXRα* genes depending on concentration and honey-type and reduced cellular cholesterol. The results are congruent with their outstanding phenolics, flavonoids, RSA and AEAC values. However, a clear mechanism for guggul honey to work was not established in this study. Jiaogulan and olive honeys expression patterns suggested a reduction in cholesterol content however this did not reflect in the cellular cholesterol assay, suggesting probable esterification of free cholesterol into storage form (Drevon, Engelhorn, & Steinberg, 1980; Yamauchi & Rogers, 2018). Except for olive honey, the medicinal honeys at 2.0% activated *PPARα* gene transcription, and they may therefore suppress the availability of triglycerides for the assembly of VLDL; although cellular triglyceride content did not reduce upon combined treatment of fatty acids and honey to HepG2 cells (Edvardsson et al., 2006).

5.1.2. Physicochemical and viscoelastic properties of honey from medicinal plants

As the scope of this project focused not only on the bio-functions of honey in antioxidative stress and hypocholesterolemia, but also on its technological functions, the next study in **Chapter 3** was conducted to set up a fundamental platform for developing food and pharmaceutical products that contain honey as a main component and enhance the acceptance by consumers. The study examined four natural honeys originated from floral nectars of medicinal plants namely *L. scoparium* (manuka-1 and -2 honeys), *O. tenuiflorum* (tulsi honey), *M. sativa* (alfalfa honey) for their quality attributes and physicochemical properties.

The data of moisture content, electrical conductivity, pH, ash content, monosaccharides, visual appearance and colour intensity was obtained for these blossom honey varieties and complied with the EEC (110/2000) standards. Two manuka honeys contained highest phenolic and flavonoid contents among the natural honeys tested. The physicochemical

properties associated with structural behavior of the honeys were recorded by infrared spectroscopy, wide angle X-ray diffraction (WAXD), micro-and modulated differential scanning calorimetry and small-deformation dynamic oscillation in shear.

The natural honeys exhibited typical FTIR spectra regions as aforementioned (Kasprzyk et al., 2018). Their morphological characteristics were classified into amorphous (tulsi and manuka-1) and semi-crystalline (alfalfa and manuka-2) systems based on the absence or presence of sucrose and glucose peaks in X-ray diffractograms. The WAXD data were supported by thermal analysis showing featureless thermograms for tulsi and manuka-1 honeys, opposed to large endothermic peaks caused by the melting of sugar crystals for alfalfa and manuka-2 honeys.

The physicochemical properties supported the concept of glass transition temperature (T_g) for the honeys. Determination of T_g is critical for optimization of product quality and stability during processing and storage. Above respective T_g value, food products develop a rubbery or a melt state with greater molecular mobility. Below T_g, food systems experience a glassy state with limited molecular diffusion, so chemical and biological reactions are inhibited (Li, Lin, Roos, & Miao, 2019; Roos, 2010). The four natural honeys exhibited calorimetric T_g of −47 ± 2 °C, suggesting the effect of moisture plasticizer which accounts for about 18.5% in honey composition (Nguyen, Panyoyai, Paramita, Mantri, & Kasapis, 2018). These were in line with earlier findings ranging from −47 to −51 °C (Ahmed, Prabhu, Raghavan, & Ngadi, 2007). The calorimetric measurement well reflected the sugar content in honey composition, but it did not elucidate the molecular interactions of structural components in honey matrix.

In contrast, mechanical spectroscopy is an ideal tool to investigate such interactions and predict mechanical T_g. This was performed through monitoring viscoelastic properties expressed in the progression of storage (elastic) modulus (G') and loss (viscous) modulus (G'') upon cooling to subzero temperatures. Based on individual viscoelastic spectrum of the tested

honeys, the current study employed time-temperature superposition principle to obtain a master curve that exhibited the viscoelastic behaviour of honey in a larger window of eight-decade frequency range, far beyond the capacity of any instruments. The work simultaneously generated data of shift factors which were then used to plot against temperatures to elucidate a molecular mechanism in the phenomenon of honey vitrification. This firstly demonstrated a linear correlation of temperature and a_T values within glassy state range, indicating honey follows the predictions of the reaction rate theory, as described by the modified Arrhenius equation. The fit of the data to the equation allowed the calculation of activation energy for vibrational changes from one conformational state to another (108 and 81 kJ/mol for tulsi and manuka-1, respectively, and similar values for alfalfa and manuka-2 honeys). However, the viscoelastic pattern then deviated from this model within the glass transition region, but it fit well with the free-volume theory modelled in WLF equation (Ferry, 1980). This allows the calculation of the free volume estimates for honey vitrification, including WLF constants, fractional free volume, thermal expansion coefficient and mechanical T_g (Table 1, Chapter 3). The estimates were in good agreement with those for amorphous polymers and high-solid carbohydrate systems (Kasapis, 2008). Interestingly, the values of the fractional free volumes were around 0.04 suggesting a kinetically trapped equilibrium in molecular relaxation. The mechanical glass transition temperatures were determined between –44 to –47 ^{o}C that are comparable to the calorimetric T_g of the tested honeys confirming the predominant effect of sugars in honey matrix. Taken together, the biophysical study provides fundamental data of the stability and consistency for using honey as a bioactive material in added-value products.

5.1.3. Structural variation in gelatin networks from low to high-solid systems affected by honey addition

Recently, efforts have been made in the development of convenient delivery systems

of honey's functionality mostly in the fields of wound healing and tissue engineering (El-Kased, Amer, Attia, & Elmazar, 2017; Minden-Birkenmaier & Bowlin, 2018; Wang, Zhu, Xue, & Wu, 2012). Although the incorporation of honey into templates of cryogels and hydrogels has reached certain achievements, it is not well understood how honey affects the structural transformation and phase behavior of these templates when it is used at concentration ranges. Gelatin is commonly used in food and biomedical industries as it chemically resembles collagen but is more readily available, much less expensive and reduces immunological risk (Elzoghby, 2013). The study in **Chapter 4** aims at elucidating the effect of manuka-1 honey addition (10 to 75%) on the structural variations and phase transition of gelatin hydro- to high solid gels to accommodate its application to industries.

The current study found that honey addition gradually removed water molecules in the hydrogels and generated stronger and thermally stable structures due to the generation of various and extended junction zones that alter their thermomechanical characteristics. This is evidenced in the onset temperature of gelation that was recorded at 41 °C for the 60% co-solute addition, well above that recorded at 27 °C for the 10% gelatin preparation. In addition, storage modulus values reached over 21 kPa at the end of cooling run for the former, compared to around 10 kPa for the latter (Fig. 1, chapter 4). Outcomes are in good agreement with earlier studies that added glucose syrup or mixtures of sucrose and glucose syrup to biopolymers such as agarose, deacylated gellan and κ-carrageenan (Kasapis, Al-Marhoobi, Deszczynski, Mitchell, & Abeysekera, 2003; Stenner, Matubayasi, & Shimizu, 2016; Tau & Gunasekaran, 2016).

The observation of viscoelastic responses was extended to subzero regime for high solid preparations containing 10% gelatin mixed with 70 and 75% (w/w) manuka-1 honey. At high temperature end, storage modulus is dominant ($G' > G''$) due to the formation of a gel with rubbery consistency in both systems. Upon cooling to subzero temperatures, viscoelasticity

progressed five orders of magnitude reaching values around 10^9 Pa for both samples and the dominant viscous component was recorded within glass transition region ($G'' > G'$ between −5 and −36 °C for the 80% total solid sample). The results reflect the formation of an entropic network from the vibrations of molecular segments that are shorter than the distance of neighboring cross-links (Kasapis & Sablani, 2005). A clearer separation of loss from storage modulus in the glass transition temperature range (higher G''/G' value), possibly due to the dynamics of Rouse and sub-Rouse motions. At the low temperature end, the elastic component becomes dominant, commencing at −36 and −21 °C for the 80 and 85% (*w*/w) blends and defining glassy state which involves the relaxation of β transitions (Panyoyai, Bannikova, Small, & Kasapis, 2015). Successful implementation of synthetic polymer approach generated viscoelastic master curves that cover a seven-decade frequency window exceeding the instrumentally accessible capacity which typically expands from 10^{-1} to 10^2 rad/s. Significantly, the high fit of data to WLF and Arrhenius mathematic models elucidates the molecular process governing vitrification phenomena and confirmed the predicted mechanical glass transition at -36 and -21 °C for 80 and 85% total solid samples, respectively. The obtained viscoelastic parameters and values of activation energy (Table 1, chapter 4) agree with previous studies reporting for glucose syrup (65 to 70%) supported by 0.5% carrageenan network or polydextrose (80%) within gellan matrix (Chaudhary, Small, & Kasapis, 2013; Kasapis, 2001). In addition, value of activation energy for manuka-1 honey (81 kJ/mol) was lower than this for the gelatin-honey systems (119 and 135 kJ/mol) reflecting the elevated difficulty for the molecular motions within the gelatin matrix in the glassy state. Moreover, T_g value was also lower for manuka-1 honey (–44 °C, 80% total solids) than this for honey-gelatin blend at the same solid content (–36 °C), suggesting the occurrence of cohesive intermolecular interactions.

The rheological characteristics of the gelatin-honey mixtures are complimented by calorimetry exhibiting micromolecular interactions. It was found that increased honey content

in the gelatin system enhances the thermal stability. This was demonstrated in the increased midpoint transition temperatures from 36 and 42.5 to 54.9 °C for the systems with 20, 60 and 85% total solids. This phenomenon is supported by a decrease in enthalpy values found at 3.0, 2.3 and 0.9 J/g, respectively.

The thermal behavior was also monitored at extended subzero regime using modulated DSC and deconvolution of thermal signals into reverse and non-reverse heat flows to enhance the reliability in determining T_g. A large trough was found at −20 °C at 50% (w/w) total solids indicating the molecular species are organized into ordered assemblies. The trough was significantly narrowed at 60% total solids and diminished in higher honey levels. The lowest T_g was recorded at −76 °C for 60% total solids and gradually increased T_g values were obtained at −63, −38 and −20.3 °C for samples with 70, 80 and 85% total solids, compared to T_g value of −45 °C for single honey at 80% total solids. The results clearly emphasize the anti-plasticizing effect of honey on gelatin matrix, support the rheological data (Table 1, chapter 4) and agree with other biomaterials (Almrhag et al., 2012a, 2012b).

Other structural considerations confirmed the amorphous characteristic of honey-gelatin blends through WAXD and SEM analysis. FTIR spectra analysis suggested molecular interactions between O–H groups of sugars and C–O groups of gelatin amides. These complementary data argue for the phenomena of glass transition in honey-gelatin high solid hydrogels rationalized via thermomechanical analyses.

5.2. Limitations of this study

Chapter 2 validated manuka-1 and the four medicinal honeys according to international regulations for honey (EEC, 110/2001) and presented their physicochemical and biochemical characteristics. Further, the effect of honeys on cellular cholesterol and cholesterol associated genes was assessed for the first time. The results suggest probably similar compounds are

associated with reduced cellular cholesterol content and antioxidant activity of manuka-1, arjuna and guggul honey. However, there are some limitations that need additional considerations to reach sufficient evidence supporting these honeys as therapeutic agents for antioxidative stress and reducing cholesterol levels as follows:

- The physicochemical analysis of quality focused on the main parameters but not all those regulated by (EEC, 110/2001). For example, the study did not determine sucrose content.
- Phytochemical analysis of honeys that had better antioxidant and cholesterol lowering ability will help understand which compounds are associated with these activities.
- The study evaluated phenolics, flavonoids content, RSA and AEAC in honeys and effect of honey in inducing antioxidative genes (e.g. *NQO1*), but it did not show the levels of cellular reactive oxygen species by honeys. Moreover, gene expression study involving more genes and a detailed time point study is required to confirm the antioxidative effects.
- The relation of cellular cholesterol content and genes associated with cholesterol homeostasis was unclear for guggul honey and contradictory for jiaogulan and olive honeys. These require further investigations at transcriptional and translational levels as honeys contain varying phytochemical compounds that may stimulate other pathways, rather than those assessed in the study.
- Animal studies and clinical trials will further corroborate the findings from this study.

Chapter 3 produced fundamental information of physicochemical properties, molecular interactions and vitrification characteristics of four natural honeys to facilitate the development of honey-containing products. Chapter 4 elucidated structural variation in gelatin networks

from low to high-solid systems affected by manuka-1 honey. However, some other aspects remained unclear in this study:

- Although FTIR spectra analysis indicated the typical regions for honey-gelatin blends and suggested chemical interactions between O–H groups of sugars and C–O groups of gelatin amides, this could be better described by deconvolution of the FTIR spectra.
- Swelling capacity is an important parameter affecting area exposed to the gels, but this is missing the current study. Moreover, the release of either honey or its active compounds from hydrogel preparations has not yet been investigated.
- Visual parameters such as their colour, consistency and homogeneity were not assessed to ensure the repeatable and reliable sensory quality. pH values of the gels should be determined because the addition of honey which is an acidic sample may alter the pH of hydrogels in a concentration-dependent manner.

5.3. Future directions

Due to the differing types of phytochemicals present in the selected plant materials, it is likely that medicinal honeys (manuka-1, arjuna, guggul, jiaogulan and olive) will affect different pathways of cholesterol homeostasis, including intestinal cholesterol absorption, the regulation of *de novo* cholesterol synthesis and uptake, cholesterol efflux. It is thus suggested that the determination of chemical profiles of plant extracts and honeys should be conducted for inferring the bioactivity of a specific compound or groups of compounds and their action modes. In addition, molecular investigations should be extended to more genes associated with a specific pathway and followed by post-transcriptional and translational evidence to confirm protective effects of honey in reducing oxidative stress and cholesterol content. Moreover, animal or human trials using the best honeys should be conducted to monitor cholesterol homeostasis and lipid profiles and validate the clinical benefits of these medicinal honeys in

the management of the two leading risks in atherosclerotic pathogenesis.

On the other hand, to make honey hydrogels closer to clinical availability for antioxidative stress (for example, anti-aging using topical treatments and oral administrations) and hypocholesterolemia, swelling and release study should be conducted for the kinetic control and targeted delivery of honey and/or its bioactive compounds. In addition, visual parameters of the hydrogels such as colour, consistency, homogeneity and pH values should be assessed to obtain reliable and repeatable quality hydrogels. Moreover, the honey-gelatin hydrogels should be directedly assessed for some biofunctions such as antimicrobials, cell protection and regeneration among the others.

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