PHYTOCHEMICAL ANALYSIS AND CYTOTOXIC EFFECTS OF KELULUT AND ACACIA HONEY ON HUMAN GINGIVAL FIBROBLAST CELLS *IN VITRO*

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by

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LIST OF SYMBOLS

%	Percentage
µL	Microliter
µm	Micrometre
mm	Millimetre
eV	Electron volt
°C	Degree Celsius
kGy	Kilo gray unit
mL	Millilitre
rpm	Revolutions per minute
mL	Millilitre

LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
ELISA	Enzyme Linked Immunosorbent Assay
SD	Standard deviation
SCFA	Short chain fatty acids
PDL	Periodontal Ligament
HGF	Human gingival fibroblasts
HPDLF	Human Periodontal ligament fibroblasts
KH	Kelulut honey
AH	Acacia honey
VOC	Volatile organic compounds
HMF	Hydroxymethyl furfural
TNF-α	Tumour necrosis factor alpha
IL-6	Interleukin 6
IL-1 β	Interleukin 1-beta
IL-8	Interleukin 8
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
GC-MS	Gas chromatography and mass spectrometry
DMSO	Di-methyl sulfoxide
IC_{50}	Inhibitory concentration

ANALISIS FITOKIMIA DAN KESAN SITOTOKSIK MADU KELULUT DAN AKASIA KE ATAS SEL FIBROBLAS GINGIVA MANUSIA IN VITRO

ABSTRAK

Perubatan sintetik adalah lazim digunakan sebagai terapi untuk sebarang penyakit termasuk masalah penyakit oral. Seperti bahan-bahan yang lain, walaupun terdapat manafaat menyembuhkan penyakit, terdapat juga kesan sampingan jika digunakan berterusan dan/atau berlebihan. Oleh itu adalah penting untuk mencari bahan alternatif asli yang dapat mengurangkan kesan sampingan ini. Madu adalah bahan asli yang sejak lama digunakan perubatan alopati. Madu kaya dengan berbagai bahan fitokimia, fenolik, asid-asid dan mineral yang mempunyai efek positif kepada kesihatan. Sehingga sekarang hasil kajian kesan madu keatas tisu periodontium adalah terhad, maka lebih kajian diperlukan di dalam bidang ini. Di dalam kajian ini, sel fibroblas gingiva manusia (HGF) didedahkan kepada beberapa kepekatan dua jenis madu Malaysia; madu kelulut (KH) dari lebah tiada sengat dan madu akasia (AH) dari lebah bersengat. Pemerhatian kesan kepekatan yang berbeza dari paling rendah 0.015% kepada yang paling tinggi 5% pada setiap jenis madu dengan menganalisis 'viability' sel fibroblas gingiva pada ujian MTT pada 24j, 48j dan 72j. Sampel juga dihantar untuk analisis 'GC-MS' untuk menentukan komposisi madu-madu. Hasil analisis GC-MS menunjukkan kehadiran campuran seperti flavonoids, furans, pyrans, levoglucosan dan hydroxymethylfurfural didalam sampel madu. Hasil kajian dari ujian MTT menunjukkan sel fibroblas gingiva mempunyai 'viability' yang baik pada KH dan AH. Nilai IC50 untuk kedua-dua madu pada 24j, 48j, dan 72j konsisten berada diparas 4 untuk KH dan atas dari 4 untuk AH. Ini menunjukkan 'viability' sel HGF yang baik pada madu KH dan AH pada kepekatan 0.015 sehingga 4%.

PHYTOCHEMICAL ANALYSIS AND CYTOTOXIC EFFECTS OF KELULUT AND ACACIA HONEY ON HUMAN GINGIVAL FIBROBLAST CELL LINE

ABSTRACT

Synthetic medicine is the most common form of treatment available for alleviation of various health conditions. As with any other substance, despite the benefits of medication, there is a risk of the adverse side effects. Thus, it is prudent to search for more natural alternatives. Honey is a naturally occurring substance which has a history of being used as an allopathic medication for many years. It is rich in various phytochemical compounds, phenolics, acids and minerals which have a positive effect on health. In this study, both honey samples underwent GC-MS analysis to ascertain their composition. Our report by GC-MS detected various compounds within our samples of KH and AH. Overall, 34 compounds were detected in the sample of KH and 32 compounds in AH. Out of these, 12 compounds were identified in KH and 7 compounds in AH by matching the peaks of their mass spectra after ionization by different online libraries. The remainder of the compounds remained unidentified. The identified compounds included flavonoids including furans, pyrans and furfural, larger percentage of HMF in both honey samples and compounds like diterpenes and furfuryl alcohol; as well as glycols in AH and levoglucosan in KH. The presence of flavonoids indicates possible antimicrobial, anti-inflammatory, and antifungal effects of KH and AH, though further study needs to be done to ascertain the exact effect of each compound on HGF cells. The presence of the identified compounds in both honey samples supplement the popularity of their use in general as both honey varieties show promising medical properties and support the popular claim of KH, and AH being used as herbal medicine in various cultures. Human gingival fibroblasts (HGF) were exposed to various concentrations of two types of Malaysian honeys; kelulut honey

(KH), acquired from the stingless bees and acacia honey (AH) acquired from sting bees. The effects of different concentrations of each honey type from the lowest 0.015%, to the highest 5% was observed by analysing the viability of HGF cells using MTT assay for 24h, 48h and 72h. The results from the MTT assay showed that the HGF cells demonstrated viability in KH from 0.015% to 3.9% and from 0.015% to 4% in AH. The IC_{50} values for both KH and AH were determined at 24h, 48h and 72h, and at all time frames remained consistent around 4% for KH and above 4% for AH. This study gave a range for the viability of HGF cells after exposure to KH and AH. HGF cells within 3% concentration of both KH and AH, appear to proliferate effortlessly. This range of viability in both the honey samples can be used to further examine other medically beneficial effects of KH and AH on HGF and other periodontal cells.

CHAPTER 1 `

INTRODUCTION

1.1 Background

Many artificially produced medicines are available for treatment of different medical conditions. However, as useful as these compounds are, they also carry with them their adverse side effects which cannot be avoided. The use of naturally occurring compounds derived from various organic sources and extracts is useful as these have shown to have all the benefits of giving good therapeutic effects without the added risk of side effects as seen in synthetic medication.

Honey is one of the more popular of the medical alternatives as it has a history of use since ancient times. Nowadays, it is being actively investigated to confirm its effects and uses in various fields of medicine. Honey is a product of various nectars from different flowers. The processing of the nectars is done by the honeybee. It is produced within the beehive and has been in use a source of food and medicine. It is a solution which can be said to be supersaturated with multiple sugars, which include fructose, glucose, maltose, and sucrose. Compounds like minerals, proteins, phenolics, flavonoids, acids, enzymes (catalase and peroxidase), maillard reaction products, carotenoids and acids can also be found (Bakar *et al.*, 2017).

The importance the role of honey in the use of traditional medicine has been highlighted by numerous investigations performed by different researchers throughout several decades (Eteraf-Oskouei and Najafi, 2013). Until now, studies have been conducted to ascertain the properties of honey from different parts of the world as an antibacterial (Allen *et al.*, 1991; Basson and Grobler, 2008; Gomes *et al.*, 2010; Irish *et al.*, 2011; Küçük *et al.*, 2007; Mundo *et al.*, 2004; Sherlock *et al.*, 2010; Tan *et al.*, 2009). Regarding its medicinal properties, honey has the capability to overcome gastrointestinal, cardiovascular and liver problems (El-Arab *et al.*, 2006). Honey also possess properties within its natural composition that prevents bacterial growth and therefore promotes healing (Simon *et al.*, 2009; Zumla and Lulat, 1989).

Further research on honey has shown that it may have the capability to stimulate immune responses and exhibit anti-inflammatory activity in a wound (Medhi *et al.*, 2008; Tonks *et al.*, 2003). In cases of burns, honey use has been known to enable improved wound healing and provide pain relief; The effects of anti-inflammation has been examined by microscopic evaluation of damaged tissues after honey was applied on wounds of animal models which resulted in a reduced number of inflammatory cells (Molan, 1998). A more important use of honey has also been observed in the form of a possible preventive agent in cancer therapies and the anticarcinogenic effects of honey has been observed and reported in various studies (Bansal *et al.*, 2005; Molan, 2001; Sela, 1998). All these properties of honey which have been investigated indicate its massive usefulness in the medical field as a source of alternative medication.

Kelulut honey (KH) is harvested by a species of stingless bee called *Trigona sp.* These bees produce KH, which is a variety of multifloral honey and is stored in small resin pots near kelulut bee nests. Acacia honey (AH), known for its pale-yellow colour, herbaceous and delicate flavour, is produced from the nectar of gathered acacia blossoms (Varga, 2006). It is not produced by stingless bees but from a sting bee variety. Both honey varieties have shown in various studies to have medicinal potential (Alzahrani *et al.*, 2012; Hasali *et al.*, 2015; Iftikhar *et al.*, 2010; Saiful Yazan *et al.*, 2016).

Cytotoxicity is described as the characteristic of a compound or a reagent that is toxic to a specific or multiple type of cells and cytotoxicity levels can help us determine the exact range of concentrations, dilutions or specific amounts of a compound that is medicinally effective and relevant for a certain role (McGaw *et al.*, 2014). In vitro cell toxicity tests are widely used to test various chemicals and for drug screenings (Ishiyama *et al.*, 1996).

This concept of cytotoxicity when applied to our chosen honey samples of KH and AH, will help us determine the exact concentrations at which the honey is most beneficial and the values which will indicate the concentrations that are unsuitable for use. The cytotoxic values that we shall attempt to determine for KH, and AH will be based upon their direct effect on human gingival fibroblast (HGF) cell line. The determination of the inhibitory concentration values (IC_{50}) can help determine the range at which the HGF cells survive and propagate effortlessly. Based on these observations, other beneficial effects of KH, and AH can be safely observed and tested. The phytochemical breakdown of KH and AH can aid in pinpointing the compounds contained within these specific honey samples which show antibacterial or anti-inflammatory action. This observation can be useful for future studies and use in oral conditions involving HGF cells.

1.2 Problem Statement

The beneficial effect of honey in medicine is widely stated and is universally recognised as a promising alternative to artificial medicine. The main issue with the use of medicines are their side effects resulting from long term usage. Medicines used to treat oral conditions like periodontal disorders have their beneficial effects, but they are not free from side effects. Honey can be a good alternative in this case as it is organic and readily available. Honey has the capability of being stored for a long period of time, however, chemical changes over prolonged storage and formation of possible non-beneficial compounds may present a problem in the overall efficacy of honey. The effects of direct exposure of honey to periodontal cells like HGF in this study, needs to be observed and viability range needs to be determined as certain concentrations of raw honey might damage cells.

1.3 Justification of the study

The breakdown of the composition of the honey samples will not only reveal the chemical composition but will also help to point out compounds that might have beneficial effects like reduction of inflammation, and anti-microbial action which can be investigated independently in the future. We can also observe any components present in the honey samples due to factors like extended period of storage or any treatment prior to use like heating or irradiation.

Determination of the benefits of KH and AH honey on the proposed cell line of HGF cells will require the exact concentrations at which the cells proliferate. Therefore, the concentrations of honey, which will allow the HGF cells to to proliferate will be investigated, so that any further work regarding the medicinal effects of the honey samples can be investigated using those parameters.

The observations collected regarding the effects of KH and AH on HGF cell lines will open the way for further research on the possible health benefits of these honey types on periodontal tissue and its effects in cases of conditions like gingival or periodontal disease. As mentioned, acquiring honey is simple and its supply is ample in nature.

Because it is produced in large quantities and is stable in long term storage, honey is a suitable bio agent to be considered for research and testing in medicine. As we shall be using HGF cells to observe the effects of KH and AH, and the fact that it can be consumed orally, the direct application for oral use in dental medicine is an interesting prospect. This will open a pathway for research on further direct application of KH and AH in the oral cavity.

1.3.1 Objectives:

1.3.1(a) General

To analyse and compare the phytochemical compounds of KH and AH and to investigate the cytotoxic effects of KH and AH on HGF cell line.

1.3.1(b) Specific

1. To analyse the phytochemical compounds of KH and AH from GC-MS.
2. To determine the cytotoxic concentrations of KH and AH to HGF cells from its IC_{50} values by performing MTT assay.

CHAPTER 2

LITERATURE REVIEW

2.1 Stingless and Sting bee honey

Bees are insects that visit flowers and take part in the pollination of plants. Honeybees (*Apis* sp.) are the main source of honey production, including both sting and stingless bees (*Meliponini* sp).

The stingless bees are emerging as a good source of honey not only for consumption but also for its potential medicinal uses. In Malaysia, it is known locally as *lebah kelulut*, is a species that has adapted well for tropical climates (Mustafa *et al.*, 2018) and honey has undergone testing and experimentation to determine medicinal properties in numerous studies. Stingless bee Ranneh *et al.* (2019) observed that stingless bee honey provides a good protection against LPS-induced chronic subclinical systemic inflammation in rats which was mediated with enhanced inflammation, oxidative stress, P38 mitogen-activated protein kinases (p38 MAPK), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and leucine zipper protein (Nrf2) signalling.

Honeybees (with stings) are generally well known and are found in many parts of the world naturally and are also domestically or commercially grown. However, the possession of a sting in these types of bees is a naturally occurring defensive and an offensive mechanism. Sting bees are dealt with cautiously as their sting is deadly and can cause great amount of pain and in some cases rapid allergic reactions like anaphylaxis due to presence of honey bee venom (Hymenoptera venom) (Helbling and Müller, 2019).

Figure 2.1 Stingless bee and stingless beehive (Amin *et al.*, 2018)

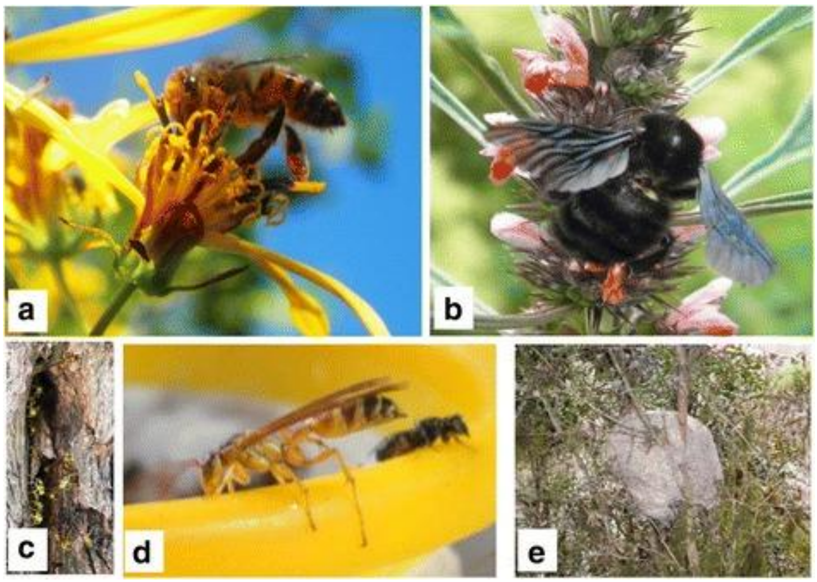

Figure 2.2 Some varieties of sting bees (Flores *et al.*, 2018)

2.2 Honey

Honey is a sweetened viscous substance that bees produce by gathering the nectar from flowers and storing it as food (Ahuja and Ahuja, 2010). The colour and flavour depend on the flowers and the honey is concentrated via dehydration process within the beehive. The chemical composition of honey is complex, usually depending on the botanical source (Eteraf-Oskouei and Najafi, 2013).

There are several bee species found in Malaysia, however, four types of bees are particularly well know known to the Malaysian population; these are the Kelulut bees, Tualang bees, Cerung or Cerang bees and the Jungle bees. Kelulut bees belong to the stingless variety of bees and are the smallest in size (Barakhbah, 2007).

For centuries honey has been known to combat various diseases. The healing of wounds was possibly the initial usage of honey in human healthcare (Jones, 2001). Numerous civilizations throughout the ages saw honey not only as a desirable food source but also as a natural product with medicinal properties. Honey was considered and used as a medicine by ancient Greeks and they believed that its regular use could prolong human life (Bogdanov, 2012). In India during ancient times, honey was used an ayuruvedic medicine for many purposes. Honey, in the old roman pharmacopoeia, was considered one of the most useful substances and was believed to be a good cure for afflictions like pneumonia, pleurisy, mouth diseases and even as a possible remedy for snake bites (Bogdanov, 2012). According to Chinese medicine honey has a balanced character as well as nutritional value. In Chinese culture, honey is believed to be a "neutral" food with medicinal properties (Bogdanov, 2012).

The Holy Qur'an dedicates an entire chapter in the creation of honey by bees. According to a saying of the Prophet Mohammed, honey is considered as the ‘‘remedy for every illness" (Aboelsoud, 2010). The Egyptians also were well advanced in the use of herbal medicine for various ailments and were quite proficient in the use of honey as part of many wound treatments (Aboelsoud, 2010).

The consumption of honey is worldwide and is favoured as a replacement for processed sugars (Phillips *et al.*, 2009). Apart from its sweetening properties (Ischayek and Kern, 2006), the potential therapeutic value of honey is in the treatment of cataracts, heart disease, several inflammatory diseases and cancer (Al-Mamary *et al.*, 2002).

Honey in alternative medicine, is valued by many users for therapeutic purposes (Meo *et al.*, 2017). An exclusive criterion of honey is its antibacterial property for wound healing (Majtan, 2014). Research advances, have stressed that the organic components of honey possess properties which can promote health (Muhammad *et al.*, 2016). Honey is used in the treatment of various ailments including burns, promotes rapid wound healing and offers a broad spectrum of antimicrobial properties (Molan, 2006).

The nutritional value of honey is high, along with affirming anti-oxidation properties (Alvarez-Suarez *et al.*, 2012; Kishore *et al.*, 2011), anti-inflammation (Ahmad *et al.*, 2012; Hadagali and Chua, 2014; Hussein *et al.*, 2012) , anti-microbial actions (Hegazi, 2011; Irish *et al.*, 2011; Ismail *et al.*, 2015) as well as reduction of cough (Cohen *et al.*, 2012; Shadkam *et al.*, 2010)

The capacity of honey being an antioxidant is important with respect to mitigating numerous disease conditions (Eteraf-Oskouei and Najafi, 2013). This is attributable to a range of compounds found in honey itself including organic acids, enzymes, phenolics and peptides, (Eteraf-Oskouei and Najafi, 2013). Honey consists of polyphenols that are beneficial for reduction of dental caries, oral cancer, and periodontal diseases (Ahuja and Ahuja, 2010).

Orally, it has been observed that the chewing-gum infused with honey for flavour substantially reduces the risk of gingivitis and accumulation of plaque and is also useful in the treatment of oral ulcers and stomatitis following radiotherapy (Newadkar, 2016).

2.2.1 Composition of honey

Raw honey is different from commercial honey as it is produced by bee farms and left in their natural state without undergoing processing such as filtration or heat treatment and is collected from the honeycomb, and contains extraneous matter which is later removed to make honey consumable on a larger commercial scale (Blasa *et al.*, 2006).

Raw honey naturally consists of almost 200 compounds, which also include vitamins, amino acids, enzymes, and minerals. Honey primarily consists if various sugars and water and sugar accounts for 95-99% of honey which is responsible for properties like hygroscopy, viscosity, energy value and granulation (Cavia *et al.*, 2002). Primary carbohydrate compounds of honey include glucose (28.54 to 31.3 %) and fructose (32.56 to 38.2%), which are readily absorbed in the gastrointestinal tract (Mundo *et al.*, 2004). Disaccharides such as sucrose, maltose, turanose, isomaltose, nigerose, panose, meli-biose, melezitose, maltotriose and fructooligosaccharides, at around 4-5% which serve as probiotic agents (Alvarez-Suarez *et al.*, 2010; Chow, 2002; Ezz El-Arab *et al.*, 2006).

Organic acids in honey which include gluconic acid that is an of enzymatic by-product of the digestion of glucose are 0.57%. The acidity of honey is due to organic acids, which are responsible for the characteristic taste of the honey (Olaitan *et al.*, 2007).

Mineral compounds in honey consist of 0.1% to 1.0% and the most common metal is potassium, followed by calcium, magnesium, sodium, sulphur, and phosphorus. Certain trace elements like copper, iron, manganese and zinc are also present (Kumar *et al.*, 2010). Honey contains vitamins as well which can include B_1 (thiamine), B_2 complex vitamins and vitamin B_6 like riboflavin. Pantothenic acid and nicotinic acid are present as well.

Proteins in honey are found in only in little amounts (0.1–0.5%) (Lee *et al.*, 1998). The honeybee origin determines any specific protein quantities as observed by Won *et al.* (2009).

2.2.2 Phytochemicals

Phytochemicals generally describe a variety of compounds derived from plants which exhibit beneficial therapeutic activity such as anti-inflammatory, antimutagenic, antioxidant, anticarcinogenic properties and enhancement for re-epithelialization of damaged tissue and collagen formation (Sivamani *et al.*, 2012). They are secondary metabolites that provide colour, flavour and defence against infections (Sivamani *et al.*, 2012). Bioactive compounds/metabolites, are substances that possess the capability for interaction with single or multiple components of a living tissue, giving a range of plausible effects (Guaadaoui *et al.*, 2014).

Phytochemicals in honey can be organised into carbohydrates, volatile compounds and phenolic compounds (flavonoids and non-flavonoid phenolic compounds) (Kaškonienė and Venskutonis, 2010). Copious amounts of these compounds are contained in raw unprocessed honey (Weston, 2000).

Flavonoids can be described as bioactive compounds which are extensively found in foods derived from plants. The use of such food is linked to reduction in the risk of chronic diseases, which include cardiovascular disease, cancer and neurodegenerative disorders (Kozlowska and Szostak-Wegierek, 2014). Pure honey consists of several flavonoids such as pinocembrin, hesperetin, quercetin, apigenin, galangin, chrysin and kaempferol. It also includes phenolic acids like caffeic, ellagic, p-coumaric, and ferulic acids.

The botanical origin (origin based on the type of flowers involved in bee pollination) of honey can be indicated and traced with flavonoids (Yao *et al.*, 2003), Flavonoids also have anti-inflammatory, anti-atherogenic, anti-carcinogenic, analgesic activity, immune modulation, and anti-thrombotic properties (Vinson *et al.*, 1998). A review by Weston *et al.* (1999) indicated that in manuka honey, flavonoids show antibiotic properties, hence its presence within honey have a role in the possible antibiotic activity.

The functions of antioxidation of phenolics are related to several different mechanisms, like metal ion chelation, scavenging of free radicals, hydrogen donation, singlet oxygen quenching, and acting out as a substrate for radicals like hydroxyl and superoxide (Küçük *et al.*, 2007; Pandey and Rizvi, 2009). Researchers have also established that honey with darker colour has total phenolics in a greater percentage, therefore, indicating higher antioxidant activity. Hence, the phenolic content appears to be related to the colour of honey (Bertoncelj *et al.*, 2007; Blasa *et al.*, 2006). Malaysian honey samples have been identified already with phenolics like cinnamic acid, caffeic acid and ferulic acid. (Aljadi and Yusoff, 2003).

Samples of honey, consisting of certain compounds which are usually found in honeys from tropical origins have been observed time and again. A few of these compounds might include groups like furans, pyrans. diterpenes, terpenoids etc. Inorganic contaminants might also be present within the honey due to environmental factors.

Hydroxymethyl furfural (HMF) is a furan group derivative. It is identified as a cyclic aldehyde which is produced by the acidic decomposition of monosaccharides, hence, appearing naturally in all products where monosaccharides and water coexist in an acidic medium (Tomlinson *et al.*, 1993). HMF is chemically identified as a six-carbon heterocyclic organic compound which consists of alcohol and aldehyde (hydroxymethyl) functional groups. Furan molecules are surrounded by the ring structure which are centered on it, and two functional groups, i.e., formyl and hydroxy-methyl groups, are bound at the second and fifth positions of the structure. HMF has a low melting point but has high solubility in water, and is visualised as a solid, yellow substance (Shapla *et al.*, 2018a). HMF formation is dependent on the presence of certain precursors like amino acids, glucose, fructose as well on conditions like temperature, pH, and storage time (Mehta, 2014).

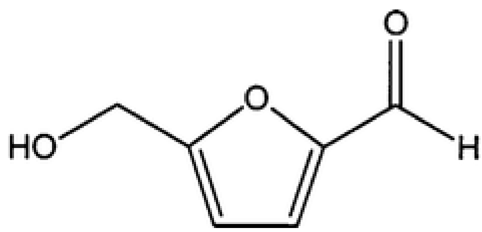

Figure 2.3 Chemical structure of HMF (Shapla *et al.*, 2018a)

HMF presence in honey is caused by prolonged storage or heat treatments, however, its percentage might be higher in honey samples from a tropical origin that are exposed to an increasingly warm atmosphere (Lee *et al.*, 1995). HMF is usually used as a quality indicator in food products. It is produced by way of prolonged storage or any prior thermal treatment and results in general loss of freshness of the honey. In some studies, negative effects on human health by HMF have been reported such as cytotoxicity towards the skin, mucous membranes and the upper respiratory tract (Glatt *et al.*, 2005). Other harmful effect like mutagenicity, carcinogenic effects and chromosomal aberrations toward humans and animals have also been observed (Monien *et al.*, 2012). HMF (hydroxy methyl furfural), was found in a large amount both KH and AH samples. The presence of HMF might influence the general property of the honey samples. The analysis and presence of HMF in various foods was also observed in a study by Teixidó *et al.* (2006).

HMF (2-Furancarboxaldehyde, 5-(hydroxymethyl)) furfural, is a flavonoid which causes darker coloration in honey. It was also found in a previous study of tualang honey (Khalil et al., 2010) and in Melipona beecheii honey of the Yucatan Peninsula (Moo-Huchin et al., (2015).

Moniruzzaman *et al.* (2013) reported in a study that the Malaysian honeys stored for two months at 4–5 °C had lesser concentration of HMF in them. In contrast, when compared to the report by (Khalil *et al.*, 2010) the concentrations of HMF in Malaysian honey stored at 25–30 °C for more than a year had reached high levels. In another study on Malaysian honey samples by (Khalil *et al.*, 2010), the honey stored for 3–6 months had HMF values below the International Honey Commission (IHC) limit for tropical honey, however, samples stored for 12–24 months had HMF higher concentrations than the recommended levels. Therefore, HMF levels can be considered as indicators of not only honey freshness but also as indicators of storage time.

To test the freshness of honey, HMF concentration is used as standard for testing it. The prolonged storage of honey above 27°C or heat treatment, lowers the diastase and increases the HMF (Iftikhar *et al.*, 2014). It was also determined in a study by

Annapoorani *et al.* (2010) that HMF values in honey increased significantly after it was heat treated as compared to regular honey samples.

The formation of HMF in honey could be influenced by other factors like physicochemical properties (pH, total acidity, free acid content, mineral content and lactone content), the use of metallic containers, and thermal and photochemical stress (Spano *et al.*, 2006). The formation rate of HMF in honey is also dependent upon temperature and pH and moisture content (Gökmen *et al.*, 2007; Gökmen *et al.*, 2008).

The study done by Fan and Sommers (2006) observed higher furan presence after irradiation of various food items including honey. As both of our honey samples of KH and AH were irradiated prior to sterilization, the formation of higher HMF and other furan derivatives could have occurred. A study done by Fallico *et al.* (2004) also reported the link between concentration of HMF in relation to the heating time, pH and acidity.

Recent studies have indicated HMF to have certain positive effects, like antioxidation, anti-inflammation, anti-allergic effects and anti-hypoxic actions (Shapla *et al.*, 2018a).

Various types of furans are found within honey as with most food substances. Furans are either naturally present in foods or are produced artificially for the role for flavourants etc.

Furfural, a furan derivative, is a chemical that finds wide applications in oil refining, plastics, pharmaceutical and agrochemical industries. There is no synthetic method to produce furfural as it already pre-exists in a compound, like in naturally produced honey (Mamman *et al.*, 2008). Furfural appears as colourless or reddish-brown mobile liquid with a penetrating odour (Biotechnology, 2020). Furfural found in KH is a precursor of furan, which could be formed naturally (Bakar *et al.*, 2016b). Furfural used as a flavour ingredient in foods and will be significantly diluted during the food preparation or cooking process, prior to consumption and are formed from the acid hydrolysis or heating of polysaccharides which contain pentose and hexose fragments; Furfural has been detected in a broad range of fruits and fruit juices, wines, whiskeys, coffee and tea and is widely used as flavourants in foods, and is considered safe for human consumption in natural or synthesized states (Adams *et al.*, 1997).

The compound, furan-2,5-dicarboxaldehyde is another furan derivative to be naturally found in organic foods such as honeys. The compound 2,5-diformylfuran is a member of the class of furans carrying two formyl substituents at positions 2 and 5 and is also commonly referred as a dialdehyde (PubChem, 2020a). This compound can also be a derivative of HMF and has been tested for its efficacy against microorganisms like Klebsiella sp. (Kaur and Sharma, 2018). The substance furan-2,5-dicarboxylic acid (FDCA) does not raise a safety concern for the consumer when the substance is used as a monomer in the production of polyethylene furanoate (PEF) polymer and the migration of the substance itself does not exceed 5 mg/kg food (EFSA Panel on Food Contact Materials and Aids, 2014), meaning that this compound may be present due to prolonged storage in polymer based storage containers and that its leaching in to food stuff does not pose any credible harm to human beings.

Another derivative of furan called 2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one can also be present naturally in citrus fruits and other food stuffs. This compound may be responsible for the particular aroma generation of the food items as observed in a particular study by Lasekan and Hussein (2018), in which the aroma of different types of pineapple varieties was examined and this compound was one of the few exhibiting a profound effect on the aroma of the pineapple samples. Therefore, if it is present in honey, it might also contribute to the aroma/freshness of the honey.

The furan derivative 5-Formyl-2-furfurylmethanoate is formed in food products after heating or exposure to thermal environments. This was observed in a s study by Ozolina *et al.* (2011) in which the formation of various furans was observed while baking of rye bread. The furan 5-Formyl-2-furfurylmethanoate was also observed to increase after the process of baking, thereby showing that certain furans may increase after thermal exposure. The presence of this furan in naturally in foods like honey, may be due to long storage periods or thermal exposure of some kind.

Furfuryl alcohol is also a furan type found in food materials. Furfuryl alcohol is a renewable material derived from furfural, produced from hydrolysed biomass waste (Sathre and González-García, 2014). The major source of furfuryl alcohol in foods is thermal processing and ageing and the highest content of furfuryl alcohol was found in coffee beans (>100 mg/kg) and in some fish products (about 10 mg/kg), while among beverages, wines contained between 1 and 10 mg/L, with 8 mg/L in pineapple juice (Okaru and Lachenmeier, 2017b). The Joint FAO/WHO Expert Committee on Food Additives (JECFA) set a group acceptable daily intake (ADI) of 0–0.5 mg/kg body weight for furfuryl alcohol, and suggested the compound as being of no safety concern at current levels of intake when used as a flavouring agent (Joint and Additives, 2002). The presence of furfuryl alcohol in natural food products like honey indicate its role as a flavouring agent. It may also be a by-product after prolonged storage or thermal exposure like other furans.

Compounds like pyrans are also found natural foods. For example, the compound of 4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl is a naturally occurring pyran found in honey samples. This compound may possess properties like being an antioxidant as observed in a study by Čechovská *et al.* (2011) in which 4H-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl- (DDMP), was observed as an important chemical which exhibited antifungal activity to inhibit growth or spore germination. Another pyran, 4H-Pyran-4-one,3,5-dihydroxy-2-methyl or also known as 5-maltose is a carbohydrate/sugar derivative which is naturally present in foods like honey. Usually contributing to the unique sweetened flavour of honey. This particular pyran derivative was also found in New Zealand manuka honey (Adams *et al.*, 2015) as well as in blue gum (Eucalyptus leucoxylon) and yellow box (Eucalyptus melliodora) Australian honeys (D'Arcy *et al.*, 1997).

Diterpene groups of compounds may also be present naturally food items as well like honey. The compound of 2-Hydroxy-2-cyclopenten-1-one is most common naturally. This compound is present orange juice, guava fruit, feijoa fruit, blackberries, pineapples, strawberry jams, wines, black tea, passion fruits, pears, wood apple, kiwi fruits and tropical based honeys; It is primarily a flavour inducer and provides a caramel like flavour or coconut notes in food. In short, this compound exhibits flavour enhancing characteristics (Burdock, 1997).

Terpenoids like methyl succinic anhydride, is a tetrahydrofurandione that has a role as a metabolite (Pubchem, 2020b). This compound is thought to be a flavour enhancer alongside maltol and other complex carbohydrates (Khan *et al.*, 2017).

Levoglucosan is an organic compound with a six-carbon ring structure formed from the pyrolysis of carbohydrates, such as starch and cellulose (Aiken *et al.*, 2009; Aiken *et al.*, 2010). Levoglucosan has been described as "an unequivocal biomass burning tracer" in the context of forest and brush fires (Li *et al.*, 2015) the compound is useful as a marker for combustion of various substances such as wood. The hydrolysis of levoglucosan generates the fermentable sugar glucose and its presence in honey samples indicate the region of the honey to be in a warmer climate or in a zone prone to fires or near an urban settlement with air pollution. Contaminant compounds like Tetra ethylene glycol monododecyl ether and decycltetraglycol can be detected in food items at times. This might be due to handling, storage, or pollution etc.

2.3 Therapeutic effects of honey

2.3.1 Antimicrobial properties

It has been reported in various studies that honey has an inhibitory effect on around 60 species of bacteria which include both aerobes and anaerobes, and gram-positive and gram-negative organisms (Olaitan *et al.*, 2007). Antibiotics destroy the bacterial cell wall or inhibit intracellular metabolic pathways. The antimicrobial action of honey is different from antibiotics.

Four properties of honey relate to its antibacterial activity. First, honey removes and drains moisture from the environment thereby, dehydrating the bacteria (Simon *et al.*, 2009). The effect of osmosis by honey is elicited as the strengthened interaction of the water molecules with sugar leave minimal or no water which is needed to support the growth of micro-organisms. Eventually they become dehydrated and die (Halawani and Shohayeb, 2011).

Second, the pH of honey ranges from 3.2 and 4.5. This acidic pH inhibits the growth of most microorganisms. In most instances, the pH value of honey is less than 5, because of the presence of organic acids like acetic, gluconic, propionic, formic and hexadecenoic acid. Honey contains gluconic acid, that emanates mostly from the oxidation of glucose in the presence of water and oxygen (Halawani and Shohayeb, 2011). The overall lower pH is enough to cause inhibition in the growth of most pathogenic organisms which require a pH normally between 7.2 and 7.4 for effective growth (Osmojasola, 2002).

The third and probably the most important antibacterial component is the Hydrogen peroxide produced by the glucose oxidase. The slow release of free radicals such as hydroxyl and superoxide are mild and does no tissue damage, however they exhibit antimicrobial effects. While light and heat have a negative effect on the peroxide generating system, however, certain types of honey still retain their antimicrobial activity.

Alternative factors include a low protein content (a high carbon to nitrogen ratio), low redox potential, viscosity (that opposes convection currents and limits dissolved oxygen), bee defensin-1, and the enhancement of phagocytic and lymphocytic activity are also thought to be responsible for antibacterial effects (Arvanitoyannis *et al.*, 2005; Kwakman *et al.*, 2010).

The variation in the antibacterial activity of the honey is due to honey phenolics, however it may be effective against one strain of bacteria but might have little or no effect on other strains (Aljadi and Yusoff, 2017). Phenolics like flavonoids may render the honey as a good source of antioxidants in addition to its actions as an antibacterial, thereby, increasing its therapeutic effects. The phenolic compounds and the antioxidant activity of honey may also be used as an assessment parameter of their quality (Al-Mamary *et al.*, 2002). Phenolic acids like benzoic, caffeic, and gallic acids are known to have antibacterial effects. The antibacterial properties of honey could be explained due to their presence (Aljadi and Yusoff, 2003).

In the field of dentistry honey has proved to be a good therapeutic agent. It has been studied and is still being further explored for a wide variety of uses in combatting various dental disease. It demonstrated in a study that mouth washes comprising of propolis (present in bee products) in their composition showed antimicrobial activity against *Streptococcus mutans*. Therefore, it can be considered as an alternative treatment option in the prevention of dental caries (Duailibe *et al.*, 2007) including the reduction of polysaccharide formation and plaque accumulation (Koo *et al.*, 2016).

2.3.2 Anti-inflammatory and immune responses

It has been demonstrated that honey has an anti-inflammatory action which is direct and not secondary to the clearance of infection. Honey has the capability to reduce inflammatory response in animal models and cell cultures as seen in the study by Candiracci et al. (2012) where unprocessed multifloral honey was used. The reduction of the activities of cyclooxygenase-1 and cyclooxygenase-2, (Cox-1, Cox-2) thereby exhibiting anti-inflammatory effects (Trushin 2006). These phenolics and flavonoids result in the suppression of the pro-inflammatory activities of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS). Various honey types have been discovered which induce TNF-α, interleukin-1 beta (IL-1β), and IL-6 production as well (Ahmed and Othman, 2013).

The anti-inflammatory action and stimulating effect on tissue repair of honey raises the possibility of it being useful as a therapeutic agent for gingivitis and periodontitis and could possibly be of benefit for the relief of oral conditions resulting from radiotherapy and chemotherapy of cancer. Consumables and confectionaries made with honey may also be useful for prevention of halitosis, as it has been observed that honey accelerates the removal of malodour from infected wounds (Ahuja and Ahuja, 2010). Application of honey on wound sites in various animal models revealed anti-inflammatory effects as such as reduced white blood cell count and reduction of oedematous discharge and exudate at the sites after microscopic examination. This effect also causes reduction pain brought about by the pressure on nerve endings and also causes reduction in the amount of prostaglandin produced in the process of inflammation (Yaghoobi and Kazerouni, 2013).

Honey also demonstrates immunomodulatory, refers to any process in which an immune response is altered to a desired level activities (Al-Waili, 2003). The immunomodulatory activity of honey is complex, as it involves multiple compounds among honeys from different origins. The release of certain cytokines (TNF-α, IL-1β, IL-6) can be either stimulated or inhibited by honey from human monocytes and macrophages in cases of conditions like wound damage. Honey either reduces or activates the formation of reactive oxygen species from neutrophils, which depends on the microenvironment of the wound. The activation of both immune cell types by honey could promote the debridement of a wound and enhance the process of repair. Likewise, fibroblasts, human keratinocytes, and endothelial cell responses are also affected categorically in the presence of honey. In this way honey may accelerate the reepithelization of the wound and speed up closure (Majtan, 2014).

It was indicated in a study that the reduced absorption of honey leads to the production of short-chain fatty acid (SCFA) fermentation agents (Al-Waili and Haq, 2004). SCFA production therefore, may result by the consumption of honey (Kruse *et al.*, 1999). The action of immunomodulation of SCFA have been confirmed in a study by Sanz *et al.* (2005). Nigerooligosaccharides is a sugar found in honey which appears to exhibit immunopotentiation. It is a process directly enhancing specific immune functions, or modifying one or more components of the immunoregulatory network to enable its effects through indirect mechanisms (Chepulis, 2007). The non-sugar ingredients of honey are also responsible for immunomodulation (Schley and Field, 2002).

Manuka, pasture, Nigerian jungle, and royal jelly honeys have been found to enhance IL-1β, IL-6, and TNF-α production. This immunoprotective and immunomodulatory activity of honey is known to be linked to anticancer action as well (Ahmed and Othman, 2013a).

2.3.3 Anti-cancer potential

Cancer is mainly treated by chemotherapy and radiotherapy which are wholly toxic to other viable cells of the body. Honey has been extensively researched to determine its possible use as an anticancer agent. Investigations have indicated that honey might possess anticancer properties as it interferes with multiple cell-signalling pathways, which include apoptosis, antimutagenic, antiproliferative, and anti-inflammatory pathways (Aliyu *et al.*, 2013).

It has been indicated that honey prevents abnormal cell production, causes apoptosis, modifies the cell cycle progression, and cause depolarization of the mitochondrial membrane in several types of cancer such as skin cancer cells (melanoma) (Erejuwa *et al.*, 2014), adenocarcinoma epithelial cells, cervical cancer cells (Pichichero *et al.*, 2010), endometrial cancer cells (Tsiapara *et al.*, 2009; Yaacob *et al.*, 2013), liver cancer cells, colorectal cancer cells, prostate cancer cells (Samarghandian *et al.*, 2010;