Abstract

The physicochemical parameters of eight Iranian honey samples from different botanical origins were characterized in this study. Differences in pH, moisture, sugar profiles and phenolic profiles were observed. Iranian honeys contain different carbohydrate structures. The relationship between different parameters such as phenolic concentration, colour, antioxidant properties, moisture content and hydroxymethylfurfural (HMF) content were established. High correlations between total phenolic content and radical scavenging properties, moisture content and antioxidant properties were also observed for all the samples. The correlation between HMF content and honey colour was observed within the eight types of honeys. Some antioxidants are potential floral origin markers of honey and Determination of polyphenolics find to be suitable for quality control and biochemistry analysis of natural honey samples. They contribute to physicochemical properties of honey and fulfill human health.

A high positive correlation was found between the two assays for antioxidant activities of honeys, and their phenolic content, indicating the contribution of phenolics in the antioxidant activities of honey although there may be other minor factors contributing to the antioxidant effects of honey. There is linear correlation between water content and antioxidant activities. The antioxidant activities and phenolic content differed amongst the honey samples possible due to their different floral sources.

The Iranian honey also showed potential anti-proliferative effects against the breast cancer cells, MCF-7 and MDA MB 231. Saffron honey showed strong anti-proliferative effects and its high phenolic content could be a contributing factor. When the honey sample was added to broilers' feed in a feeding study, there was no significant difference in feed intake, weight gain, feed to gain ratio and weight of gastrointestinal tract, abdominal

fat, liver, spleen and pancreas in the animal model.

Several chromatographic techniques were applied to separate and detect the presence of polyphenols in the honey samples. The most important classes of antioxidant polyphenols are flavanone, flavone, flavan, flavonols and phenolic acids. The characterization of polyphenols depends on the method of identification. These polyphenols can be considered as a botanical marker and was tentatively characterised by HPLC-DAD, HPLCMS/MS, GCMS and LC-MSTOF. This study shows that ITTOF is the best instrument for analysis of polyphenols in the honeys.

The results obtained indicated possible markers for honey type authentication by different analysis. Apigenin (41.74 ng/100 µl) and quercetin (3.38 ng/100 µl) as potential bioactive markers present in saffron honey are found in high quantities. Available literature indicates that no previous studies on antioxidant property have been done on Iranian honeys. This is the first study to evaluate the antioxidant properties of selected Iranian honeys and may provide useful data for their potential medicinal uses.

Abstrak

Kajian ini menganalisa parameter fizikokimia lapan sampel madu Iran dengan asalan botani yang berlainan. Perbezaan pH, lembapan, profil gula dan profil fenolik daripada sampel madu dianalisa. Madu Iran mempunyai struktur karbohidrat yang berbeza. Hubungan di antara parameter berlainan, iaitu kepekatan fenolik, warna, sifat antioksida, lembapan dan kandungan hidroksimetilfurfural (HMF) di analisa. Korelasi tinggi di antara jumlah kandungan fenolik dan sifat penghapus radikal, kadungan lembapan dan aktiviti antioksidan daripada semua sampel dilihat. Korelasi antara kandungan HMF dan warna daripada lapan sampel madu juga didapati.

Korelasi positif yang tinggi didapati antara dua asei pa aktiviti antioksidan dan kandungan fenolik madu, menunjukkan adanya sumbangan daripada kandungan fenolik terhadap aktiviti antioksidan madu. Meskipun demikian, faktor lain pun dijangka berperanan terhadap kesan antioksidan madu. Korelasi linear didapati antara kandungan air dan aktiviti antioksidan. Aktiviti antioksidan dan kandungan fenolik daripada setiap sampel madu diandaikan berlainan oleh sebab asal botani yang berbeza.

Madu Iran juga menunjukkan kesan anti-proliferatif terhadap sel kanser payu dara, MCF-7 dan MDA MB 231. Madu saffron menunjukkan kesan anti-proliferatif yang kuat dan kandungan fenoliknya yang tinggi mungkin menyumbang kepada kesan ini. Apabila sampel madu ditambah kepada makanan ayam dalam suatu kajian pemakanan, tiada perubahan signifikan dilihat dari segi pengambilan makanan, penambahan berat badan, nisbah pemakanan terhadap kenaikan berat badan dan juga berat usus, lemak abdomen, hati, limpa dan pankreas haiwan-haiwan tersebut.

Beberapa teknik kromatografi digunakan untuk mengasing dan mengesan kehadiran polifenol didalam sampel madu. Kelas polifenol antioksidan yang terpenting ialah flavanon, flavon, flavan, flavonol dan asid fenolik. Pencirian polifenol bergantung kepada

kaedah yang digunakan. Sebatian fenolik ini boleh dianggap sebagai penanda botani dan telah dicirikan dengan menggunakan HPLC-DAD, HPLCMS/MS, GCMS dan LC-MSTOF. Kajian ini menunjukkan bahawa ITTOF ialah instrumen paling baik bagi analisa polifenolik madu.

Keputusan kajian ini mengesahkan pengesahihan kemungkinan penanda jenis madu dari pelbagai analisis. Penanda bioaktif yang berpotensi iaituapigenin (41.74 ng/100µl dan quercetin (3.38 ng/100 µl), ditemui dalam kuantiti yang besar didalam madu saffron. Sorotan kajian menunjukkan bahawa pencarian aktiviti antioksidan ke atas madu Iran belum lagi dilakukan. Kajian ini merupakan kajian pertama yang menganalisa kandungan antioksidan beberapa jenis madu Iran dan diharapkan dapat menyumbangkan data yang berguna bagi penggunaannya sebagai ubat.

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The kind support of the Iranian Apitherapy Society is gratefully acknowledged. This project without IAS collaboration and my father financial support wouldn't have been possible.

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ACN: Acetonitrile

APCI: Atmospheric pressure chemical ionization

DAD: Diode array detector

DPPH: 2, 2 –Dipheny l-1-picrylhydrazyl

ESI: Electrospray ionization

FRAP: Ferric reducing antioxidant potential

GC: Gas chromatography

GC-MS: Gas chromatography mass spectrometer

HMF: 5-Hydroxymethylfurfural

HPLC: High performance liquid chromatography

LC-MS: Liquid chromatography mass spectrometer

LLE: Liquid–liquid extraction

m/z: Mass-to-charge ratio

Me OH: Methanol

MIC: Multiple ion chromatograms

MW: Molecular weight

ND: Not detected

SPE: Solid-phase extraction

TAP: Total Antioxidant Power

TIC: Total ion current

TPC: Total Phenolic Content

TOF/MS: Time of flight mass spectrometer

CHAPTER ONE

General Introduction and Review of literature

Honey has been historically used as traditional medicine for the treatment of various ailments and diseases. It is believed that antioxidants and carbohydrates, which are among the bioactive components, can provide the beneficial effects. Hence, the analysis of antioxidants and carbohydrates in honey are important to evaluate the potential use of honey in medicine.

The role of free radicals, such as superoxide radical and hydroxyl radical has been emphasised in a number of diseases, including cancer , cardiovascular disease , cataracts , macular degeneration , impaired wound healing , gastrointestinal inflammatory diseases and other inflammatory processes (Almahdi & Kamaruddin, 2004). The antioxidant activities of honeys can have the potential to be utilised to provide protection against these diseases.

The main objective of this research project was to investigate the identification of phenolic compounds in selected Iranian honeys using various chromatographic approaches. The broad varieties of floral honeys provided a wide number of matrices with differing composition of their precursors. To meet the main objective, the following specific objectives were targeted:

1) Characterization of the physicochemical parameters of different honey samples.

2) Determination of phenolic content and antioxidant activities of selected Iranian honeys.

3) Determination of the biological activities of selected Iranian honeys.

4) Identification and determination of the phenolic compounds in selected Iranian honeys.

1.1 Free radicals and Antioxidants

A free radical is defined as any species able of independent existence that contains

one or more unpaired electrons . To stabilize itself, the free radical abstracts an electron from a stable compound, which in turn, is transformed into a new free radical. This chain reaction will continue until the free radical containing the lone electron pairs up with another molecule with an unpaired electron .

Some of these reactive species for example hydrogen peroxide (H_2O_2) have no unpaired electrons but can still act as free radicals. Radicals that contain oxygen are known as reactive oxygen species (ROS) . Other radicals such as nitrogen oxide ($NO^{\cdot}$) contain nitrogen and they are termed as reactive nitrogen species (RNS). All these reactive species are grouped as "oxidants" . Reactive oxygen and nitrogen species are commonly created as a consequence of normal metabolic pathways of the human body and are vital parts of its roles . They function in signaling cascade in cellular functions such as proliferation, inflammation, and adhesion .

The roles of ROS and RNS have been emphasised in the development of a number of diseases including asthma, cancer, cardiovascular diseases, cataracts, macular degeneration, impaired wound healing, rheumatoid arthritis, diabetes, gastrointestinal inflammatory diseases, liver diseases and periodontal diseases .

The term "oxidative stress" describes the lack of equilibrium in the organism between the production of free radicals and the antioxidant protective activity. The protection against oxidation is thought to prevent some chronic diseases. The oxidative modification of the lipoproteins is considered to be an important factor for the pathogenesis of arteriosclerosis.

Antioxidants are a group of substances, which when present at low concentrations in relation to oxidisable substrates, significantly delay or inhibit the oxidation of lipids or

other molecules by inhibiting the initiation or propagation of oxidizing chain reactions . In recent years there has been an increased interest in the application of antioxidants in medical treatment as information is constantly gathered linking the development of human diseases to oxidative stress . Many studies have been undertaken on the role antioxidants play in delaying or preventing the onset of oxidative damage-related diseases. The most likely and practical way to fight against these diseases is to improve the body's antioxidant status, which could be achieved by administration of exogenous antioxidants. Many epidemiological studies have linked diets containing moderate to high proportions of fruit and vegetables to lower mortality and to reduced risk of developing cardiovascular diseases, cancers, cataracts and macular degeneration .

Many antioxidants are used in food and food products to prevent oxidation and prolong the shelf life of food. However, most of these antioxidants are synthetic and may cause adverse health effect if consumed in large quantities. With today's consumer trends of demanding 'natural food', there is strong interest in the development of natural antioxidants . Numerous studies have been performed in the search for potent natural sources of antioxidants. One of these widely researched natural sources is honey.

Honeys from various parts of the world have been reported to contain substantial antioxidant compounds and antioxidant activities . Due to its antioxidant activities, honey has potential to be used as a feed additive, feed flavoring, acidifier, antibiotic, prebiotic, diuretic feed ingredients and improve poultry performance. Honey can also prevent deteriorative oxidation reactions in foods such as enzymatic browning of fruit and vegetables , lipid oxidation in meat and inhibit the growth of foodborne pathogens and food spoilage organisms . The good antioxidant effect, sterility and easy availability of honey make it an ideal source of natural antioxidants.

1.1.1 History of honey

Throughout the literature, honey has been an admired folk remedy around the world for a variety of ailments since the ancient times. Cave painting in Europe dating from 8,000 years BC depicted honey hunter (Figure 1.1), indicating that honey had been accepted from ancient times as a good product for the treatment of illnesses .

The Ebers papyrus of ancient Egypt (1700 BC) contained recommendations on the medicinal uses of hive products . Similar observations were seen in ancient Assyrians, Greeks, Romans and Persia. Honey was also mentioned in religious books including Torah and Bible. The Holy Qur'an specifically mentioned honey as having 'shifak' or healing properties to mankind.

In the works by Ibn sina (Avicenna), Aristotle, Pliny, Dioscorides, Galen,

Hippocrates, Varro and other ancient scholars there are traces and backgrounds on Apitherapy, the therapy based on bee products .

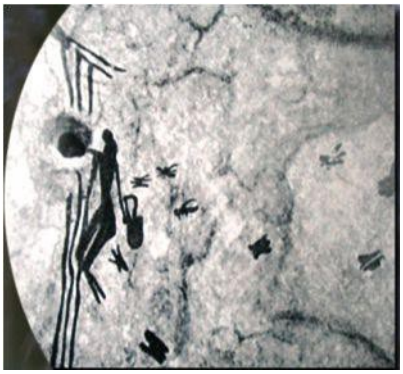

Figure 1.1 Cave painting more than 8,000 years old

1.1.2 Honey composition

Honey is technologically produced by bees from nectar of flowering plants. The Codex Alimentarius Commission (1981) defines honey as the natural sweet substance produced by bees from the nectar of blossoms or from secretions of living parts of plants or excretions of plant-sucking insects on the living part of plants that the bees collect, transformed, ripened and stored in honeycombs. It is the most complex natural tonic ever synthesised by the bees. Honey contains natural chemicals which vary due to differences in geographical, climatic or seasons as well as processing and storage conditions. These variations may also affect the known biological or pharmacological activities of honey even if they are from the same origin.

Honey is a substance that consists of over 300 compounds. In general, sugars represent the major composition of honey (95-99% solids of honey) , whereby levulose

(38.19%), dextrose (31.28%) and water constitute the main portion of the honey mass . As can be seen in Figure 1.2, the remaining sugars are sucrose (1.3%) and maltose (7.3%). Minor components of honey are acids (0.57%), protein (0.27%), nitrogen (0.04%), amino acids (0.1%), a small amount of minerals (0.17%) , and a number of other minor compounds such as pigments, flavorings and aromatics, phenolic compounds, colloids, sugar alcohols and vitamins, which together represent 2.1% of the composition of honey .

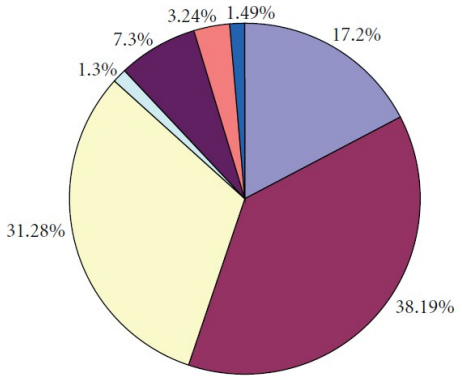

Figure 1.2: Honey composition; Water 17.2%, levulose 38.19%, dextrose 31.28%, sucrose 1.3%, maltose 7.3%, complex sugars 1.49% and 3.24% others.

The diversity and concentration of these components are dependent on the botanical, geographical, processing, storage and origins of honey. Moreover, it is influenced by the variation of climate and availability of plant sources for the honeybees to harvest nectar within a specific region . Therefore, honeys originating from different floral sources differ in their chemical compositions. The honey quality is variable and quality can be assessed largely based on color, flavor and density. Fructose to glucose ratio is also assessed to provide characterization of honey samples from different origins, as it may indicate the tendency of honey to crystallize. Heating and ageing can also affect the quality of honey .

Honey has been reported to have various biological effects including anti-bacterial, anti-inflammatory, angiogenic properties, and is shown to generate granulation tissue, facilitate epithelialization, prevent and reduce scars and alleviate pain . The wound healing properties of honey is especially well established, potentially contributed by its anti-bacterial properties and its high viscosity providing a protective barrier to prevent infection .

1.1.3 Antioxidants in Honey

In recent years, researchers have identified a number of phytochemicals in different foods, including honey. Phytochemicals are substances in plants. Many phytochemicals promote health activities. Antioxidants, a large group of phytochemicals, reduce the risk of oxidative damage to tissues. Honey is rich in enzymatic and non-enzymatic antioxidants, including catalase, ascorbic acid, flavonoids and alkaloids.

Various studies have been conducted on the antioxidant properties of honeys. Honey has been found to contain significant antioxidant compounds including glucose oxidase, catalase, ascorbic acid, flavonoids, phenolic acids, carotenoid derivatives, organic acids, Maillard reaction products, amino acids and proteins . The main antioxidants seem to be the phenolics and the Maillard products named melanoidins.

Flavonoids are phytochemicals that cannot be synthesized by animals and human beings. Flavonoids found in animals originated from food . Therefore, the flavonoids are present in most vegetables including fruits, cereals, grains, nuts, herbs and honey. In addition, any drink containing natural flavors and colors made with plant material also contains flavonoids, such as white wine, red wine, tea, coffee, fruit juice, cider, and cocoa. Many flavonoids have been reported to contain potent antioxidant activities .

Several flavonoids have been reported to be present in honey. These included pinocembrin, kaempferol, quercetrin, naringenin, pinocembrin, galangin and chrysin . Flavonoids in honey can have different profiles depending on the floral nectar source. To date, data on the antioxidant components and antioxidant activities of Iranian honey are still lacking and merit further research.

The dependence of the antibacterial activity on the botanical origin is less clear cut than the antioxidant properties of honey and there is a relation between antibacterial activity and antioxidant activity of honey . The antioxidant effect of honey prevents oxidation of food during storage. Honey acts against lipid oxidation of meat and is thus an efficient additive for preventing oxidation spoilage, e.g. to poultry or to meat.

The effect of heat on the antioxidant capacity of clover and buckwheat honey during storage was analysed recently . Processing clover honey did not significantly impact antioxidant capacity. Storage for 6 months reduced the antioxidant capacity of honeys by about 30%, with no impact of storage temperature or container type detected at the end point of the storage period. Antioxidant capacity of processed and raw honeys was similar after storage.

Different methods have been applied for determination of antioxidant activities. The different methods for the determination of the antioxidant activity have been reviewed. There is a significant correlation between the antioxidant activity, the phenolic content of honey and the inhibition of the *in vitro* lipoprotein oxidation of human serum. A study utilizing 14 commercial honey reported significant positive correlations between several antioxidant assays with the phenolic content and colour intensity of the honey samples, implying the importance of phenolic compounds in honey in providing antioxidant activities (Beretta et al., 2005).

Iran is a country that is rich in honey production and due to its geographical location and climatic conditions; different types of honeys are produced. However, not much data is available on the composition of Iranian honey especially their antioxidant content and antioxidant activities.

1.1.4 Honey from Iran

Some parts in Iran are an ideal source of medicinal honey due to different climates, high mountains and herbs. Honey is considered an important food and for use in traditional Persian medicine, either individually or as an ingredient in medicinal compounds. There are evidences that show the production of honey and apitherapy have been active in Iran since ancient times. One of the most famous Iranian physicians who have a broad therapeutic use of bee products was Avicenna who wrote dozens of recipes for honey-based drugs. He used the word "ASAL" (Honey) over 1400 times in his book (Canon), and cited several internal and external treatments of honey in the circulatory system and visual aids, cosmetics, superficial wounds, deep wound infections, eczema, inflammations, infections of the tonsils, throat and some digestive system disorders .

Iran extends north to the Caspian Sea, Tajikistan, Armenia, Azerbaijan and Turkmenistan and south to Oman Sea and the Persian Gulf. With these wide limits, its climate varies a lot with the existence of nearly four seasons. The sites for beekeeping, located by satellite (GPS navigator) shows a large number of beehives in Iran with more than five million beehive colonies with the number of beekeepers of 61,704 and annual production of honey of 45,023 tons .

The physicochemical properties of the honeys are carried out based on the Institute

of Standards and Industrial Research of Iran . The standardization of honey is possible if the analytical analysis is controlled. Iranian honey standard organization decides the standard of Persian honeys. In this research, the Iranian honeys were selected based on Iranian honey standards .

1.2 Determination of Antioxidant Properties

It is reported that part of the therapeutic function of honey is due to its antioxidant activities . Hence, studying the antioxidative components and antioxidant activities of honeys are important to further discover their potential.

The main characteristic of an antioxidant is its ability to trap free radicals. Different types of free radicals can be generated through different reactions. The more common radicals are hydroxyl radicals, the peroxyl radicals and the superoxide anion radicals.

Numerous *in vitro* antioxidant analyses are used to evaluate antioxidant activities of natural compounds in foods or biological systems. Almahdi and Kamaruddin (2004) recommended a quick, reliable, cost effective and simple test to study the antioxidant compounds and antioxidant activities. These included the Folin-Ciocalteu assay for the total phenolic content (TPC), the ferric reducing antioxidant power (FRAP) assay for total antioxidant activity, the DPPH assay to analyze radical scavenging capacity .

Many authors in different countries (Malaysia, Sudan, Saudi Arabia, Turkey, and many other countries) have shown that honey is a natural source of antioxidants . The antioxidant activity of honey varies widely depending on the floral source, geographical origin and climatic characteristics of the place. However, there is little knowledge on the

profiles of antioxidants in Iranian honey.

Studies on the antioxidant properties of Iranian honey are severely lacking. Thus, the main objective of this study was to evaluate the antioxidant capacity of Iranian honeys and to evaluate their relationship with total phenolic content.

In addition to their antioxidant activities, phenolic compounds such as phenolic acids and polyphenols have been recently suggested as potential markers for the determination of the botanical origin of honey. Studies have reported differences in the composition and amounts of phenolics in different unifloral honeys. Heather honeys for instance contained more phenolic acids whereas citrus and rosemary honeys contained more flavonoids . Hence, determination of phenolic compounds in the Iranian honeys can potentially be useful as biomarkers for their botanical origin.

1.3 Chromatography Methods of Analysis

1.3.1 Carbohydrate analysis

Several in depth reviews, covering carbohydrates' analyses have been published so far . All the different methods have their strengths and weaknesses, and some of them are more appropriate when it comes to the analysis of food commodities. The high performance liquid chromatography (HPLC) and gas chromatography (GC) techniques are the most commonly used methods when it comes to the determination of carbohydrates.

1.3.2 High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) is the most used instrument for the analysis of carbohydrates. The HPLC methods are precise and much faster than GC-based methods . There are different approaches for the HPLC determination of sugars. They can be analyzed with or without derivatization. The separation can be carried out using two types of columns; silica-based amino propyl columns or ion-exchange columns . The detection of underivatised carbohydrates can be achieved with a refractive index detector (RID), an evaporative light-scattering detector (ELSD), an electrochemical detector or by mass spectrometric determination (MS). The UV detection is inappropriate as carbohydrates have no suitable chromophores. The refraction index detector is one of the most common detectors for sugar analysis. The detection principle involves measuring the change in refractive index of the column effluent passing through the flow-cell. The sensitivity will be higher for samples having higher difference in refractive index relative to the mobile phase. The RID is a non specific detector, and it cannot be used for analyses requiring a gradient elution because it is a differential instrument. In several published methods, sugars were monitored by RID. The analysis of sugar composition of 53 Spanish honey samples and different commercially processed foods like cereals, coffee creamer, ice cream, maple syrup and peanut butter (Iverson and Bueno, 1981) were reported. The

evaporative light scattering detector (ELSD) is one alternative to RID. It has a better sensitivity than RID, excellent baseline stability, and it is fully compatible with all solvents and gradient elution. However, like RID, it has a low selectivity because of its universal detection mode: detect all solutes that are less volatile than the mobile phase. The drawback of the ELSD is that it generates a non-linear response, so several concentrations must be used to do quantitative work. In a review (Molnar-Perl, 2000), the use of HPLC-ELSD method for the determination of the carbohydrate content of beet juice and beer has been reported. The electrochemical detector is more selective and sensitive than RID and ELSD. However, the major problem with the electrochemical detector is the passivation of the electrode. Different types of electrode have been investigated to avoid this drawback.

1.3.3 Gas Chromatography

Most of the methods for the determination of sugars by gas chromatography (GC) were published in the 1980 or before. Since the 1990's, the GC has been largely replaced by HPLC for the analysis of sugars. Nonetheless, it is possible to analyze sugars with GC. However, the direct analysis of sugars using GC is not possible. In order to quantify carbohydrates by CG, the carbohydrates must go through a derivatization step. Sugars must be derivatized to stable trifluoroacetates, trimethylsilyl (TMS) or tert-butyldimefhylsilyl (TBDMS) derivatives to be volatilized in the GC. The sugar composition of the sample and the type of food matrix are the determining factors for the choice of derivatizing agents. If the main difference between the analytes is the orientation of hydroxyl groups, the acylated derivatives are a better option. For the saccharides with a higher molecular weight or with glycosidic linkages, it is preferable to use TMS or TBDMS derivatives. Nonetheless, several problems can arise in the GC procedures (Molnar-Perl, 2000). One of the main

problems is the formation of interfering compounds. Sometimes two or three interfering compounds are generated for the same sugar due to incomplete derivatization. The resulting chromatogram is more complicated to analyse. Particular attention must be given to the derivatization conditions to ensure maximum yield. The inappropriate derivatization process can lead to difficulties in interpretation of the chromatograms.

1.4 Analytical Methods for the Analysis of Phenolics

Apart from the GC and HPLC methods in phenolics analysis, other alternative methods have been developed for determination of honey phenolics.

ESI-LCMSMS-TOF is the best method to analyze honey phenolics in comparison to the other methods which were used in this study. Flavonoids are not analyzed by GC-MS because they do not hold nitrogen atoms and include low basicity in the liquid phase. Atmospheric pressure chemical ionization (APCI) LCMS works better on non-polar molecules but provide the highest sensitivity for phenolics. The analysis of polyphenols in complex matrices includes a procedure of sample preparation, which can cover phenolics isolation and cleanup further to remove impurities.

CHAPTER TWO

2.1MATERIALS

2.1.1 Honey samples

In this study, 40 honey samples (Table 2.1) were initially harvested from Khorasan

province in Iran (Figure 2.1). Subsequently, eight samples were selected based on Malaysian honey standards, using pH value and moisture content as the criteria. The physicochemical properties of these eight Iranian honeys were studied. These unpasteurized honeys were obtained from the Iranian Apitherapy Society (IAS), and were kept at room temperature, away from direct light before use.

Figure 2.1: Honey harvest in Khorasan province in Iran (taken by Gilles Ratia .

Table 2.1: 40 honey samples that were initially tested in this study.

Honey Sample	Floral origin	Honey Sample	Floral origin
1	Crocus sativus	21	Mentha piperita
2	Berberis	22	Polyfloral
3	Foeniculum vulgare	23	Malus domestica
4	Citrus	24	Polyfloral
5	Thyme (Astragallus,Thistle)	25	Vitex agnus castus
6	Tamarix(Rosmarinusofficinalis)	26	cotton
7	Polyfloral	27	helianthus
8	Sunflower (alfalfa, Lotus)	28	Polyfloral
9	polyfloral	29	Polyfloral
10	polyfloral	30	eucalypyus
11	Lavandula angustifolia	31	Ferula gumosa
12	polyfloral	32	Centaurea cyanus
13	polyfloral	32	Polyfloral
14	Prunus	34	Robinia pseudoacacia
15	polyfloral	35	Polyfloral
16	polyfloral	36	Prunus domestica
17	Rosmarinus officinalis	37	Prunus persica
18	polyfloral	38	polyfloral
19	Foeniculum vulgare	39	Pyrus communis
20	polyfloral	40	Citrus limon

2.1.2 Chemicals

All chemicals used were either of analytical or HPLC grade and were obtained from the following sources:

Ajax Chemicals Ltd., Sydney Australia:

Sodium metabisulfite ($Na_2S_2O_5$).

Fluka Chemicals, Switzerland:

2, 4, 6-Tripyridyl-s-Triazine (TPTZ), Kaempferol and Caffeic acid.

J.T. Baker Chemicals Ltd. England:

Hydrochloric Acid.

Merck, Darmstadt,Germany:

Ethyl acetate, Acetic acid, Iron(II) sulfate heptahydrate($FeSO_4.\ 7H_2O$), Iron (III) chloride hexahydrate ($Fe\ Cl_3.6H_2O$), Methanol, Acetonitrile, Sodium hydroxide (NaOH), sodium carbonate (Na_2CO_3) Sodium acetate trihydrate ($C_2H_3NaO_2.3H_2O$).

Sigma Chemicals Company, St. Louis USA:

Ascorbic acid , 2-2- Diphenyl-1-Picrylhydrazyl (DPPH), Folin- Ciocalteau Reagent, Levulose, Dextrose, Saccharose, Maltose, Benzoic acid, Chlorogenic acid, Gallic acid, P-coumaric acid, Pinocemberin, Apigenin, Galangin, Chrysin, Hesperetin, Naringenin, Eriodictyol, Morin hydrate, Epicatechin, Catechin, Ellagic acid, Rutin, Trolox, BHT, Quercetin, Ferulic acid and Fumaric acid.

2.1.3 Instruments

Centrifuge (KHT-420B, Gemmy Industrial Corp., Taiwan)

Rotary evaporator (EYELA, Tokyo Rikakikai Co., Ltd., Tokyo, Japan)

UV-VIS Spectrophotometer (Hitachi, U-2000 Japan)

Vortex-mixer (Vm-2000 YIH Instruments Co Ltd, Taiwan)

Water bath (Schutzart DIN 40050-memmert, Germany)

Milli Q water (Millipore, Milford, MA USA)

Isolute SPE columns & VacMaster vacuum manifolds (International Sorbent Technology Ltd Hengoed, Mid Glamorgan.UK)

GC (GC-14A, Shimadzu Corporation, Kyoto, Japan).

GC-MS (QP 5050, Shimadzu Corporation, Kyoto, Japan).

HPLC (LC-10A series, Shimadzu Corporation, Kyoto, Japan).

LC-MS-APCI (LC-20AD series, Shimadzu Corporation, Kyoto, Japan).

LC-MS-ESI (LC-20AD series, Shimadzu Corporation, Kyoto, Japan).

LC-MS-MS-IT-TOF (LC-20AD series, Shimadzu Corporation, Kyoto, Japan).

Ultrasonic bath.

Volumetric flask, 100 mL.

Membrane filter for aqueous solutions, pore size 0.45 μm.

0.2 mm filter (Satorius-Minister® NML).

Filter holder for membrane filters with suitable syringe.

HPLC consisting of pump, sample applicator, temperature-regulated RI-detector thermostated at 30˚C, temperature regulated column oven at 30˚C and integrator.

Analytical stainless steel column, e.g. 4.6 mm in diameter, 250 mm length, containing

amine-modified silica gel with 5 μm -7 μm particle size.

Analytical balance, accurate to 0.001 g.

Refractive index detector (RI, Waters 2410).

Microplate reader (Power wave x 340, Bio-Tek Instruments, INC.,Winooski, Vermont, USA).

Analysis was performed from SPSS 17.0 and also lab solution software.

2.2 METHODS

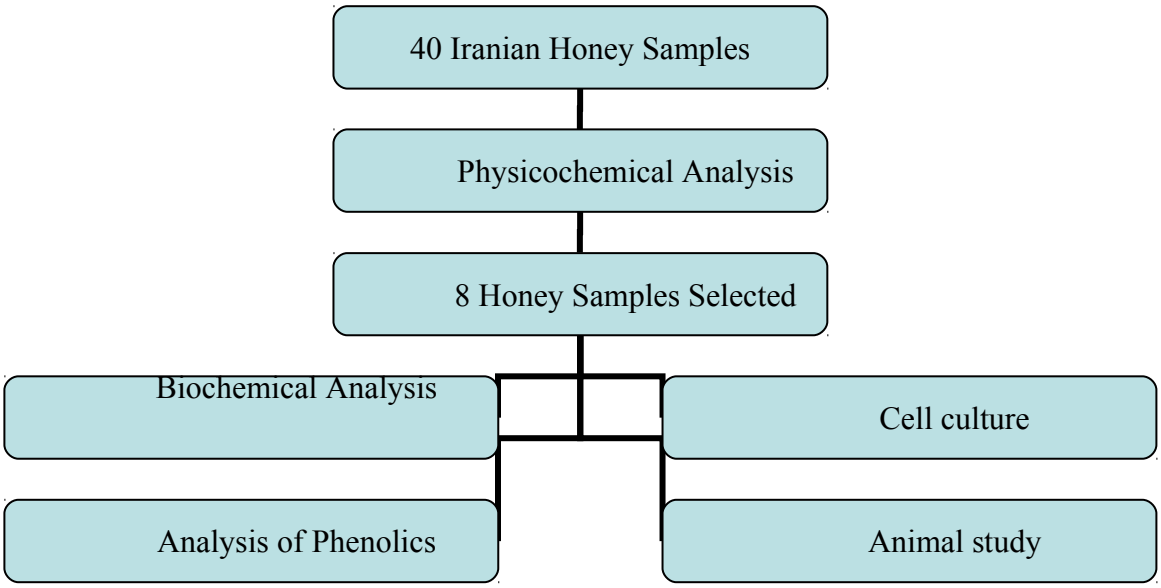

Figure 2.2: Overall work flow

2.2.1 Sampling

In this study, 40 honey samples were initially harvested from Khorasan province in Iran. Subsequently, eight samples were selected based on Malaysian honey standards as well as pH values and moisture content. The physico-chemical properties of these eight Iranian honeys were studied.

2.2.1.1 Liquid Honey

The samples of honey were processed as described. Uncapping the hives is the first step in processing of the honey. The top of the waxes was then cut with a sharp thin knife and this was followed by the removal of impurities such as particles of wax and other impurities that were present during extraction. Finally pure honey is obtained and poured into bottles and vacuum package (Figure 2.3).

If any foreign matter, such as wax, sticks, bees and particles of comb were present in honeys, they were heated at 40˚C in a water-bath and strained through cheesecloth before sampling. If the samples were free from granulation, they were mixed thoroughly by stirring or shaking; if they were granulated, they were placed in closed containers in a water-bath and heated for 30 min at 60˚C until melted. The temperature might be increased to 65˚C if necessary. Occasional shaking might be essential during the heating process. The incubation conditions were controlled by a Gallenkamp model IH-1 50 incubator. The temperature of the incubator was electronically controlled and verified against a calibrated thermometer. All these processing were done in the honey laboratory in Iran (Saleh honey Institute).

Figure 2.3: Uncapping, centrifugal extraction, filtering and bottling of honey .

2.2.1.2 Analyses of pH values

The procedure described by was used. Honey samples (2 g) were diluted with deionised water (5 mL). The solution was mixed until complete dissolution. The pH of the solution was then directly measured with a pH meter (Accumet® 950 pH/ion Meter) after it reached room temperature.

2.2.1.3 Analyses of moisture content

Data on the moisture content of honey is an important parameter in determining the quality of honey, since it affects storage life and processing characteristics. The protocol for this study was based on the refractometric method of Chataway (1932), revised by Wedmore .

The refractive index of the honey sample was determined using a refractometer at a constant temperature of 20˚C. The refractive index reading was converted to moisture content (percent) using the table given below (Table 2.2).

Table 2.2: Refractive Index for the determination of moisture content of honey.

Refractive Index (20° C)	Moisture Content (%)	Refractive Index (20°)	Moisture Content (%)	Refractive Index (20 °C)	Moisture Content (%)
1.5044	13.0	1.4935	17.2	1.4830	21.4
1.5038	13.2	1.4930	17.4	1.4825	21.6
1.5033	13.4	1.4925	17.6	1.4820	21.8
1.5028	13.6	1.4920	17.8	1.4815	22.0
1.5023	13.8	1.4915	18.0	1.4810	22.2
1.5018	14.0	1.4910	18.2	1.4805	22.4
1.5012	14.2	1.4905	18.4	1.4800	22.6
1.5007	14.4	1.4900	18.6	1.4795	22.8
1.5002	14.6	1.4895	18.8	1.4790	23.0
1.4997	14.8	1.4890	19.0	1.4785	23.2
1.4992	15.0	1.4885	19.2	1.4780	23.4
1.4987	15.2	1.4880	19.4	1.4775	23.6
1.4982	15.4	1.4875	19.6	1.4770	23.8
1.4976	15.6	1.4870	19.8	1.4765	24.0
1.4971	15.8	1.4865	20.0	1.4760	24.2
1.4966	16.0	1.4860	20.2	1.4755	24.4
1.4961	16.2	1.4855	20.4	1.4750	24.6
1.4956	16.4	1.4850	20.6	1.4745	24.8
1.4951	16.6	1.4845	20.8	1.4740	25.0
1.4946	16.8	1.4840	21.0		
1.4940	17.0	1.4835	21.2		

2.2.1.4 Analyses of hydroxymethylfurfural (HMF) content

HMF formation resulted from the acid-catalyzed dehydration of hexose sugars with levulose. Fresh honey normally contained small amounts of HMF . For example, if natural honey is stored for one year at temperature lower than 25°C, the HMF content will increase by 3 mg/100 g honey. Acid-catalyzed invert syrup invariably contains high amounts of HMF. Prolonged storage or hot climate can result in an increase in HMF content of honey. In this study, spectrophotometer was used to measure the HMF content in the honey sample.

The determination of HMF content was based on the UV absorbance of HMF at 284 nm. In order to avoid interference from other components at this wavelength, the difference between the absorbance of a clear aqueous honey solution and the same solution after addition of bisulphate was determined. The HMF content was calculated after subtraction of the background absorbance at 336 nm. This method was based on the original work of White et al. .

The protocol for the HMF analysis is as below:

1. 5.0 g honey was weighed in a beaker and 25.0 ml distilled water was added. The mixture was stirred until honey was completely dissolved.

2. The solution was transferred to a 50 ml volumetric flask.

3. 0.5 ml Carrez I solution was added and was mixed thoroughly.

4. 0.5 ml Carrez II solution was added and was mixed thoroughly.

5. Then distilled water was added up to the 50 ml mark of the volumetric flask. If necessary, a drop of ethanol was added to suppress the formation of foam.

6. The solution was filtered using a filter paper. The first 10 ml solution was discarded. The

rest was collected in a separate vial.

7. 1.0 ml solution was pipetted into two separate test tubes.

8. For the test solution, 1.0 ml distilled water was added to the solution and was mixed well. For the reference solution, 1.0 ml sodium bisulphate (0.2%) was added and was mixed well.

9. Using a Quartz cuvette, the absorbance of the sample solution against reference solution at wavelengths of 284 nm and 336 nm were obtained within an hour. If the absorbance at 284 nm was more than a value of 0.6, appropriate dilution of the sample with distilled water and the reference solution with sodium bisulphate was carried out in order to obtain a sample absorbance low enough for accuracy.

The HMF content, expressed in mg/kg sample was calculated using the following formula:

$(A_{284} - A_{336}) \times 149.7 \times 5 \times D/W$

Where;

A_{284} is the absorbance at 284 nm;

A_{336} is the absorbance at 336 nm;

149.7 is the factor = $126 \times 1000 \times 1000/16830 \times 10 \times 5$

126 is the molecular weight of HMF

16830 is the molar absorption of HMF at 284 nm

1000 is the conversion of g to mg

10 is the conversion 5 into 50 ml

1000 is the conversion g of honey into kg

W is the sample weight

D is the dilution factor

2.2.2 Characterization of sugar profile

2.2.2.1 Determination of sugar content using gas chromatography

Some factors such as the Fructose/Glucose ratio, total reducing sugars and also antioxidant activity must be taken into consideration to establish honey profile.

A gas chromatography (GC) method was adapted from Doner et al with some modification to determine the sugar content of the 8 honey samples .

In this experiment, pyridine was used as solvent. Hexamethyldisilazane (HMDS) was used as methylating agent. Triflouroacetic acid was used to promote the methylation of sugar into methylated hydrocarbons. This method was chosen because of its high sensitivity, reliability and most importantly for its rapidity. This is because after sugar is methylated, it can be injected into the GC without further processing. The sugar content in honey was compared to the standards; levulose, dextrose, maltose and saccharose. Codex alimentarious commission (CAC) suggested a minimum total reducing sugar of 65% for pure honey. This criterion is set to detect sample that is adulterated. The samples were first derivatized before being injected into the column. The different standards will give different peaks and these were compared to the peaks from the honey samples so that each individual standard could be injected and their contents could be determined.

The standard sugars (1.0 mg) was individually placed inside a glass vial and labeled. 5.0 mg honey samples were placed inside glass vials. Then 0.45 ml of pyridine was added to each glass vial. After that, the vials were immersed in a water bath at 80°C for 10 minutes. 0.5 ml of HMDS was then added and mixed well. 0.5 ml of TFA was added drop by drop. The sample was shaken for 30 seconds and allowed to stand for 15 minutes. A homogenous clear solution was obtained. If the sample was cloudy, a new sample would be prepared again. The unclouded samples and standards were left for at least 24 hours before being injected onto the GC column. 1.0 µl of solution was injected into the GC column. The following formula was used to calculate the percentage of sugar in honey:

The weight of sugar in honey (mg) = (area peak in honey samples / area peak in standard) × Weight of standard (mg)

% sugar in honey= [Sugar in honey (mg) / weight of honey samples (mg)] × 100%

2.2.2.2 Determination of sugar content using high performance liquid chromatography (HPLC)

To analyse sugar profiles in the honey samples using HPLC, Bogdanov and Baumann method was followed . Sugar standards were maltose, glucose, fructose and sucrose. For each sugar, a standard 4.0 g/L solution was prepared using deionized water. Honey samples (250 mg) were prepared in 5 ml of deionized water. Three replicates were used for each honey sample. Prior to HPLC analyses, the honey samples were passed through Isolute solid phase extraction (SPE) columns and eluted using VacMaster vacuum manifolds (International Sorbent Technology Ltd Hengoed, Mid Glamorgan.UK) before being filtered using a 0.2 mm filter (Satorius-Minister® NML). The SPE cartridge was made active by eluting with methanol and cleaned with 2 ml water.

HPLC analysis was performed on an LC-10A series liquid chromatography system (Shimadzu, Japan), equipped with a C_{18} column (ZORBAX Eclipse Plus C_{18} 5µm 4.6 x 150 mm), thermostated at 40˚C. A gradient elution was modified to separate the extracted sugars. Solvent (A) was 5.0% acetic acid in ultra-high quality water and solvent (B) was 5.0% acetic acid in methanol. All solvents were degassed prior to use. Elution was performed at a solvent flow rate of 1.0 ml/min.

The gradient profile of the system was 8% solvent B at the initial stage (2 minutes), 20% solvent B at 5 min, 35% solvent B at 10 min, 55% solvent B at 10 min, 90% solvent B at 12 min, 100% solvent B at 12 min and 8% solvent B at 15 min. The sugar standard was injected into the HPLC prior to analyses of the honey samples (20 µl injections).

Table 2.3: HPLC gradient profile

Time (min)	Solvent B (%)
2	8
5	20

10	35
10	55
12	90
12	100
15	8

A syringe and pre-mounted membrane filter were used to transfer the solution to sample vials. The standard solutions were stable for four weeks in the refrigerator at 4˚C and for six months at -18˚C. After filtration of the solution, the sugar content was determined by HPLC with Refractive index (RI) detection. Peaks were identified on the basis of their retention times. Quantification was performed using external standards according to peak area or peak height.

2.2.3 Analyses for total phenolic content and antioxidant activities

2.2.3.1 Determination of total phenolic content

The total phenolic content of the honey samples were estimated by a colorimetric method based on the Folin-Ciocalteau assay. Dilutions of samples (0.25 – 4.0 mg/ml) were prepared in deionised water; 0.5 ml of each was placed in test tubes, and 9.5 ml of deionized water was added. Three ml of 20% NaCO solution (w/v) was added to each tube, followed by 1 ml Folin-Ciocalteau reagent. The solution was mixed well, and allowed to stand for 1 hour at room temperature; after which the absorbance was read at 750 nm in a spectrophotometer against deionized water as blank. The total phenolic content was calculated from the calibration curve prepared from gallic acid in the concentration range of 10-250 µg.

2.2.3.2 Liquid-liquid extraction of polyphenols from honey

10 g of honey sample was made up to 50 ml using water , after which 50 ml of 3N NaOH was added and the solution kept for 4 hours at room temperature under nitrogen pressure. The pH of the hydrolysate was adjusted to pH 3.5 using 4N HCl. Then, 100 ml ethyl acetate was added, followed by 2 g sodium metabisulphate. The solution was poured into a separating funnel, shook for 5 minutes and allowed to separate. The ethyl acetate layer was recovered and this procedure was repeated six times. Finally, the recovered ethyl acetate solution was pooled and concentrated under vacuum at 35˚C and was dried under nitrogen pressure. These samples were used for analyses of antioxidant activities and identification of antioxidant compounds.

2.2.3.3 Determination of the free radical-scavenging activity of honey (DPPH Assay)

The spectrophotometric DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging assay that was used by Chen et al (2000) and Almahdi and Kamaruddin (2004) for the

measurement of the antioxidant activity of honey was followed in this study. This technique was first described by Glavind .

In order to evaluate the radical scavenging activity of compounds or extracts, samples are allowed to react with stable free radicals such as DPPH in the presence of an antioxidant which can donate an electron (hydrogen atom) to DPPH. The purple colour, which is typical to DPPH radical decays, and the change in absorbance can be followed spectrophotometrically at 517 nm . This test can provide information on the ability of a compound to donate a hydrogen atom, or the number of electrons a given molecule can donate. In cases where the structure of the electron donor is unknown, this method can provide data on the reduction potential of the sample, and hence can be useful in comparing the reduction potential of unknown materials .

Honey was dissolved in warm deionized water (0.1 g/ml), and then 0.75 ml of this solution was mixed with 1.5 ml of DPPH solution (0.09 mg/ml in methanol) and allowed to incubate at room temperature for 5 minutes. Each honey sample was then completely reduced, and the absorbance was measured at 517 nm against a test blank consisting of honey solution with 1.5 ml of distilled water: methanol. The two readings were subtracted from each other to eliminate the absorbance value due to chemicals other than antioxidants present in honey. The antiradical activity (ARA) of honey was expressed as percentage inhibition of DPPH radicals by honey and was calculated as follow:

ARA (%) = [(AB-AT)/AB] x 100

AB = Absorbance of DPPH radical blank

AT = Absorbance of test sample with DPPH radical

2.2.3.4 Determination of the total antioxidant power of honey (FRAP Assay)

The ferric reducing/antioxidant power (FRAP) assay was adopted in this study to measure the total antioxidant power of honey. It is a simple and accurate test developed by Benzie and Strain (1996) and modified by Almahdi and Kamaruddin (2004) as a direct method for measuring the total antioxidant power of honey.

At low pH, reduction of ferric tripyridyltriazine (Fe^{3+}-TPTZ) complex to the ferrous form, which has an intense blue colour, can be monitored by measuring the change in absorbance at 593 nm. The reaction is nonspecific, in that any half-reaction that has a lower redox potential, under reaction conditions, than that of the ferric/ferrous half-reaction will drive the ferric (Fe^{3+}) to ferrous (Fe^{2+}) reaction. The change in absorbance, therefore, is directly related to the combined or "total" reducing power of the antioxidants present in the reaction mixture .

Reagents included 300 mM acetate buffer, pH 3.6 [3.1 g sodium acetate trihydrate, plus 16 ml glacial acetic acid in 1 liter of deionized water]; 10 mM 2,4,6- tripyridyl-s-triazine (TPTZ) in 40 mM HCl and 20 mM $FeCl_3.6H_2O$. Working FRAP reagent was prepared fresh as required by mixing 10% acetate buffer, 1% TPTZ solution and 1% $FeCl_3.6H_2O$ solution.

Freshly prepared working FRAP reagent was prepared by mixing 10 volumes of 300 mmol/L acetate buffer, pH 3.6 with a volume of 10 mmol of TPTZ in 40 mmol/L HCl and a volume of 20 mmol/L of $FeCl_3.6H_2O$ and was warmed to 37˚C. Two hundred microlitres of honey solution (0.1 g/ml) was added to 1.5 ml of freshly prepared FRAP reagent. The mixture was then incubated at 37°C for 4 minutes, then the absorbance at 593

nm was measured against a blank (200 µl of distilled water). The difference between the absorbance of honey sample and the blank was calculated. Aqueous solutions of Fe^{2+} ($FeSO_4.7H_2O$) in the range of 100-1000 µmol/L were used for calibration. The reducing power of honey was expressed as µM Fe^{2+} equivalents /L.

2.2.4 Analyses on the effect of honey on performance and immune response using an animal model

In order to investigate the effect of honey on performance and immune response of chicks, healthy inbred male broilers (Lohman), weighing 25-30 g were used for the study. They were individually housed and maintained on normal food and water ad lib. Animals were periodically weighed before and after the experiment. Animals were closely observed for any infection and if they showed signs of infection, they were separated, excluded from the study and replaced.

Commercial male broilers (Lohman) were obtained from a local hatchery. One-day old chicks were weighed and randomly allocated to six dietary treatment groups. The basal corn-soybean meal diet was prepared according to NRC and specific treatment was added to the diet (3% of the diet) . Twenty-four pens of chicks (10 chicks per pen) were assigned to each of six treatment groups. The experimental design was a completely randomized design with six treatments. The amount of sugars added to the diet was not more than 3 g/kg as it could harm the broilers. The treatment groups consisted of: Normal diet as control (T1), diet + 3 g/kg dextrose (T2), diet + 3 g/kg saccharose (T3), diet + 3 g/kg polyfloral honey (T4), diet + 3 g/kg levulose (T5), and diet + 3 g/kg maltose (T6).

The chicks' body weight, daily weight gain, food consumption and food conversion

ratio were recorded at the ages of 21, 42 and 62 days, in all the treatment groups.

At 56 days of age, one bird from each pen was killed to measure the weight of gastrointestinal tract, liver, pancreas, spleen and abdominal fat. All groups were fed a practical corn soy-based diet. Serum samples were collected from 240 broilers. Haemagglutination inhibition (HI) test was done on the serum samples by a commercial lab using routine standard procedure. The HI test is a neutralisation test with red blood cell agglutination as the indicator. The principle of the test is to determine, by dilution, the level of (agglutination) inhibiting antibodies in the chick's serum that will prevent the agglutination of the indicator system (susceptible red blood cells).

2.2.5 Cytotoxicity of honey on breast cancer cell lines

To study the cytotoxicity of the honey sample on human breast cancer, cell lines (MCF-7 & MDA-MB-231) were obtained from American Type Cell Culture (ATCC).

2.2.5.1 Cell Culture and Treatment

The cells were cultured in 1640 media, which was prepared as described by Freshney (1994). MCF-7 and MDA-MB-231 cells were propagated in RPMI media, supplemented with 10% fetal bovine serum and 1 unit/ml penicillin/streptomycin. The cell lines were maintained at 37˚C in 5% CO_2 humidified atmosphere.

2.2.5.2 MTT Assay

The cytotoxicity of the honey sample was studied in cell culture media and compared with their antioxidant activities. Saffron and sunflower honeys were chosen for the cytotoxicity study. Various concentrations of the honey extracts were studied and cytotoxicity was determined using the 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. The human breast cancer cell lines (MCF 7 and MDA-MBA 231) were cultured in RPMI 1640 with 10% FBS and incubated in 37^0C with 5% CO_2. After an overnight incubation, cells were seeded at a density of 7000 cells per well of 96-well plates (with 100 µl of medium) and incubated at 37^0C with 5% CO_2 for 24 hours. Cells were treated with various concentrations of honey extracts for 24 and 48 hours. After the incubation period, media was removed and 10 µl MTT reagent was added to each well, followed by another 4 hour incubation period. MTT reagent was then removed and 100 µl DMSO added to each well. The absorbance of the 96-well plates was measured with an ELISA reader at λ=595nm. Each test was repeated at least three times. The concentration of the samples, which shows 50% growth inhibition value, corresponds to the IC_{50}.

2.2.6 Identification of antioxidant compounds

2.2.6.1 Solid phase extraction (SPE) of phenolics

All the 8 selected honeys were analyses for antioxidant compounds. The honey samples were initially subjected to SPE to remove impurities and to obtain clean samples with a high recovery. The SPE cartridge was subjected to a conditioning process before sample was loaded. For this, 5 mL methanol was added to the cartridge. The use of a C_{18} SPE cartridge allowed for a fast sample treatment with low solvent consumption . Vacuum was applied and the eluent was discarded. The conditioning process was repeated with 5 mL H_2O. The cartridge was not allowed to go dry at any point during this step .

One mL of sample which was prepared by liquid-liquid extraction (section 2.2.3.2) was added to the cartridge. Vacuum was applied and the eluent was discarded. Then, 5 mL H_2O was added to the cartridge and vacuum was applied and the eluent was discarded.

A collection tube was then placed beneath the cartridge and 4 mL of methanol was added to the cartridge. Vacuum was applied and the eluent was collected. 2.5 mL of isopropanol was then added to the cartridge. Vacuum was applied and the eluent was collected in the same collection tube and was concentrated to dryness under a gentle stream of nitrogen. The residue was dissolved in 1 mL of mobile phase (methanol). Table 2.3 summarises the SPE process.

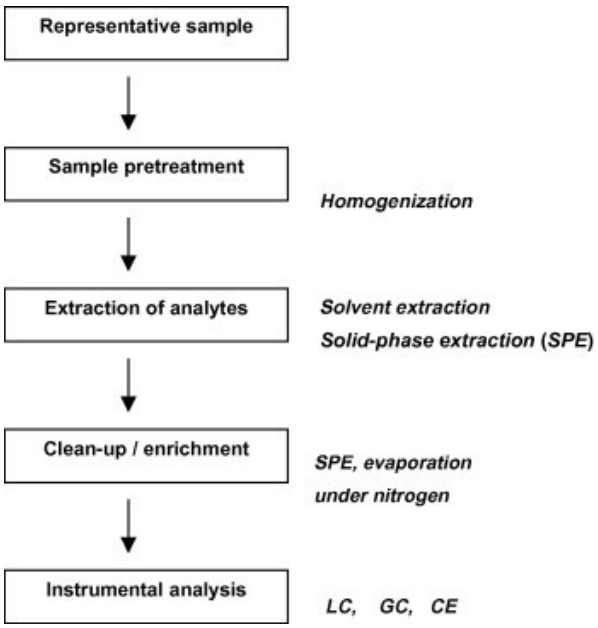

Table 2.4: SPE methods

2.2.6.2 Identification of polyphenolics in Iranian honey using High Performance Liquid Chromatography (HPLC)

Prior to HPLC analysis, the dried phenolic extracts obtained from SPE were redissolved in methanol and filtered through a 0.45 mm Millipore filter (Millipore Corp., Bedford, MA). HPLC analysis was performed on an LC-10A series liquid chromatography (Shimadzu, Japan), equipped with a C_{18} column (ZORBAX Eclipse Plus C_{18} 5µm 4.6 x 150 mm), thermostated at 40˚C. A gradient elution was modified to separate the extracted flavonols. Solvent (A) was 5.0% acetic acid in water and solvent (B) was 5.0% acetic acid in methanol. Elution was performed at a solvent flow rate of 1.0 ml/min. The gradient profile of the system was 15% solvent B at the initial stage, 35% solvent B at 10 min, 40% solvent B at 20 min, 97% solvent B at 30 min, 40% solvent B at 50 min, 35% solvent B at 75 min, and again 15% solvent B at 90 min. The eluted flavonoids were monitored at 280

nm, and identified by comparing the chromatography retention times with those of authentic standards. Quantitative levels were determined from the UV absorption during HPLC, and from the extinction coefficient which was obtained from the external standards. Recovery was measured by adding pure standards to the honey samples prior to analysis; the percentage of recovery was estimated by comparing the individual HPLC peak areas measured for the samples before and after the addition of the standards.

2.2.6.3 Identification of polyphenolics in Iranian honey using gas chromatography-mass spectrometry (GCMS)

The analysis of the extracts for phenolic acids was performed using an Agilent 5890 II GC, equipped with an Agilent 5972 MS detector. The column used was an HP-5MS (Crosslinked 5% phenylmethylsiloxane) capillary column (30 m X 0.25 mm i.d., 0.25 mm film thickness) and the gas carrier was helium, at 1 mL/min rate. The injector and MS transfer line temperatures were maintained at 220˚C and 290˚C respectively. For the analysis of the honey, the oven temperature was held at 40˚C for 3 min, raised to 180˚C at 4˚C/min and then to 250˚C at 10˚C/min (5 min hold). As honey is a complex sample, slower temperature increase is required to achieve a better separation of the isolated compounds. 1 mL of the extract was injected in a split less mode. The identification of the isolated compounds was achieved by comparing retention times and mass spectra of the honey samples with authentic standards from the database.

2.2.6.4 Identification of polyphenolics in Iranian honey using liquid chromatography-mass spectrometry (LCMS)

The identification of phenolic compounds in the Iranian honey samples was done using liquid chromatography-mass spectrometry (LC–MS) at a Shimadzu Laboratory in Singapore.

The commercial phenolics standards used in this study were catechin, epicatechin, chlorogenic acid, gallic acid, coumaric acid, ellagic acid, ferulic acid, morin hydrate, eriodictyol, quercetin, naringenin, apigenin, kaempferol, hesperetin, chrysin, galangin and rutin. Stock solutions of phenolic acid standards were prepared with water and stock solutions of flavonoids standards were prepared with methanol. Diluted standard mix was prepared with water. All solutions were filtered through 0.45 m membranes (Millipore) and degassed prior to use.

2.2.6.5 Identification of polyphenolics in Iranian honey using atmospheric pressure chemical ionization liquid chromatography-mass spectrometry (LCMS-APCI)

Polyphenolic analyses by atmospheric pressure chemical ionization liquid chromatography-mass spectrometry LC-APCI-MS were carried out using an LC and LC/MS Trap (VL) mass spectrometer (Agilent with atmospheric pressure chemical ionization). The LC system includes a G1379A on line degasser, a G1311A quaternary pump, a 1313A auto sampler, a G1316A thermostatic column control, and a G1315A diode array detection (DAD), all of which were controlled by the Lab Solution software. The LC separation was performed on a reversed-phase Agilent C_{18} column at 40^0C. The mobile phase consisted of 1% formic acid in water (solvent A) and 1% formic acid in methanol (Solvent B) with the following gradient; 10% methanol (B) flowed through the column with 20% solvent A for 0.50 min, then was increased to 87% methanol (B) by 5.50 min, to

87% methanol (B) by 6.50 min, followed by binary gradient system. Finally, the gradient was changed to 20% methanol by 7.00 min and this composition was held until 8 min. The flow rate was 1.0 ml min. The injection volume was 20 µl with the UV detector set to an absorbance wavelength of 210-260 nm. The following APCI parameters were used (optimized depending on compounds); dry gas (N_2) flow, 2.50 L/min and dry gas temperature 400^0 C; the ion trap mass spectrometer was operated in negative and positive ion mode with a scanning range from m/z 1000. In addition, the activation energy for the MS experiment was set to 1V.

2.2.6.6 Identification of polyphenolics in Iranian honey using electrospray ionization-liquid chromatography-mass spectrometry (LCMS-ESI)

The identification of phenolic compounds of Iranian honeys was done using liquid chromatography-mass spectrometry (LC–MS-ESI) at a Shimadzu Laboratory in Singapore.

The honey samples were analyzed using a Shimadzu liquid chromatography system consisting of binary pumps LC20-AD, degasser DGU-20A5, column oven CTO-20AC, autosampler SIL-20AC, detector UV SPD 20A connected to 3200 QTRAP Mass spectrometer (Applied Biosystem/MDS SCIEX). Compounds were separated on an Agilent C_{18} column. The mobile phase consisted of eluent A (1% formic acid in water) and eluent B (1% formic acid in methanol). The mobile phase was delivered at 0.2 ml/min in linear gradient mode: 0–3 min 22% B, 10 min 100% B, 12 min 100% B, 13 min 22% B, and 16 min 22% B. An MS system equipped with ESI operated in negative and positive ion modes.

2.2.6.7 Identification of polyphenolics in Iranian honey using liquid chromatography-mass spectrometry-mass spectrometry-ion trap-time-of-flight (LCMSMS-IT-TOF)

The Shimadzu LCMS-IT-TOF system comprised of a liquid chromatography mass spectrometer which is connected to an auto sampler (SIL-20AC HT), a solvent delivery pump (LC-20AD), and a column oven (CTO-20AC) with a photodiode array detector (SPD-M20A). A C_{18} (ZORBAX Eclipse Plus C_{18} 5 µm, 4.6 x 150 mm) column was used with a flow rate of 1.0000 mL/min at 40°C. Reversed-phase HPLC separations were carried out using a Phenomenex (Torrance, CA, USA), ZORBAX Eclipse Plus C_{18} 5µm 4.6 x 150 mm column, protected by a Gemini C_{18} guard cartridge. A Micromass Quattro Micro triple quadrupole mass spectrometer equipped with an electrospray ionisation (ESI) source was operated in negative ion mode (Waters, Milford, MA). The mobile phase was acetonitrile (B) and water with 0.1 % formic acid (A). The gradient was programmed at 8% B (1 min), 15% B (3 min), 25% B (5 min), 40% B (10 min), 50% B (35 min), 100% B (50 min), 100% B (55 min), 8% B (60 min), and stopped at 65 min. The column was maintained at a flow rate of 1.0 mL/min at 40 °C. The injection volume was 15 µL. UV–Vis absorption spectra were recorded on-line during each HPLC analysis. The mass spectra were obtained by electrospray ionisation in negative ion mode, with the following operating parameters: capillary voltage, 3 kV; cone voltage, 25 V; extractor, 5 V. The source temperature was 100˚C, and the desolvation temperature was 350˚C. The cone gas flow was set to 70 L/h and the desolvation gas flow was set to 500 L/h.

Flavonoids were identified by comparisons of their retention times and spectral characteristics with the reference compounds, using the MS solution software. The tentative identification of compounds for which standard compounds were available was obtained by comparing the elution order, UV spectra, m/z values and MS characteristics with data from the literature.

2.2.7 Statistical analysis

Data were analyzed using SPSS and the SAS software computer package . A complete random design was used. Arcsine transformation of all percentage and ratio data before ANOVA analysis was done in order to normalize data.

CHAPTER THREE

RESULTS AND DISCUSION

3.1 PHYSICO-CHEMICAL PROPERTIES OF HONEY

3.1.1 Honey color

In Iran the wide assortment of honeys available is a result of production in different regions with specific climatic conditions and a wide range of floral sources. The colour of honey is one of the characteristics that serve to indicate the plant source. It ranges from yellow through orange, red and red-brown to nearly brown (Table 3.1).

There is a wide variety of honeys with different tastes and colours, depending on their botanical origin . Many researchers found that honeys with dark colour have a higher total phenolic content and consequently a higher antioxidant capacity . The dark colour is related to the content of minerals, pollen and phenolics, and is characteristic of floral origin . Darkening of honey during storage may occur because of Maillard reactions, fructose caramelization and reactions of polyphenols. The degree of darkening depends on the temperature and/or time of storage .

Table 3.1: 40 Colour of Iranian honey samples.

Sample no.	Honey type	Colour	Sample no.	Honey type	Colour
1	Crocus sativus	red-brown	21	Mentha piperita	brown
2	Berberis	brown	22	Polyfloral	orange
3	Foeniculum vulgare	brown	23	Malus domestica	brown
4	Citrus	yellow	24	Polyfloral	brown
5	Thyme (Astragallus,Thistle)	brown	25	Vitex agnus castus	brown
6	Tamarix(Rosmarinus officinalis)	brown	26	cotton	yellow
7	Polyfloral	brown	27	helianthus	brown
8	Sunflower (alfalfa, Lotus)	yellow	28	Polyfloral	orange
9	polyfloral	brown	29	Polyfloral	brown
10	polyfloral	orange	30	eucalypyus	yellow
11	Lavandula angustifolia	brown	31	Ferula gumosa	brown
12	polyfloral	brown	32	Centaurea cyanus	brown
13	polyfloral	orange	32	Polyfloral	red
14	Prunus	brown	34	Robinia pseudoacacia	brown
15	polyfloral	brown	35	Polyfloral	brown
16	polyfloral	orange	36	Prunus domestica	brown
17	Rosmarinus officinalis	brown	37	Prunus persica	brown
18	polyfloral	brown	38	polyfloral	orange
19	Foeniculum vulgare	brown	39	Pyrus communis	brown
20	polyfloral	orange	40	Citrus limon	yellow

3.1.2 Analyses of pH values

Previously, the pH of honey was evaluated by measuring the percent acidity of formic acid. However, with the discovery of other acids in honey, it is now known that formic acid is one of the least important acids in honey. In terms of sugar acids, gluconic acid was found to be the main acid in honey and results are normally expressed as "percentage of gluconic acid" by multiplying the numbers by 0.0196. Nevertheless, the term "milli equivalents per kilo" is used to avoid suggesting that honey contains a single

acid .

All the honey samples in this study have weak acidic properties with the pH ranging from 3.14 to 4.95 (Table 3.2). In general, honey is acidic in nature, regardless of their geographical origin.

Honey generally tastes like weak acid. Acids in honey represent less than 0.5 percent of its total composition. The level of acidity not only contributes to the taste, but is also responsible for the stability of honey against various microorganisms. Various acids have been characterized in honey. Gluconic acid is the most important. Gluconic acid is produced by glucose oxidase from the stomach of bees. Other acids present in honey are formic, acetic, butyric, lactic, oxalic, tartaric, maleic, succinic, pyruvic, pyroglutamic, α-ketoglutaric glycolic, citric and malic acids .

Table 3.2: pH values of Iranian honey samples.

Sample no.	pH	Sample no.	pH
1	3.91±11	21	3.50±22
2	4.85±18	22	3.14±18
3	4.73±14	23	3.81±13
4	3.77±17	24	3.79±15
5	4.87±22	25	3.82±14
6	4.95±07	26	3.74±19
7	4.68±15	27	3.79±22

8	4.82±09	28	3.19±15
9	3.92±14	29	3.23±24
10	3.54±22	30	3.98±18
11	3.22±72	31	3.20±14
12	4.11±38	32	3.18±11
13	3.81±41	32	3.14±10
14	3.62±10	34	3.88±12
15	3.42±22	35	3.47±15
16	3.59±15	36	4.12±19
17	3.14±14	37	3.71±13
18	3.77±19	38	3.96±19
19	3.49±12	39	3.84±25
20	3.40±17	40	3.59±17

n=3

3.1.3 Analyses of moisture content

Water content is a good criterion to establish honey quality; high moisture content can produce honey fermentation during storage, resulting in the formation of ethyl alcohol and carbon dioxide. The alcohol can be further oxidized into acetic acid and water with the ensuing sour taste . Honey moisture content depends on various factors such as harvesting season; degree of maturity reached in the hive and climatic factors . Values between 17.1 g/100 g (honey number 1 which is saffron honey) and 19.8 g/100 g (honey number 4 which

is citrus) were obtained (Table 3.3). In addition to honey number 4, the other top five honey samples with high moisture content were honey numbers 5, 2, 3, 6 and 8, with moisture content of 18.1%, 18.3%, 18.7%, 19.2%, 19.5% and 19.6%, respectively. All the tested honeys had moisture content below 20%, which is the maximum prescribed limit as per the Codex standard for honey .

Table 3.3: Iranian Honey Moisture content.

Sample no.	Moisture content (%)	Sample no.	Moisture content (%)
1	19.1	21	17.1
2	18.3	22	19.2
3	18.7	23	18.2
4	19.8	24	18.5
5	18.1	25	18.0
6	19.2	26	17.6
7	19.6	27	17.8
8	19.5	28	17.4

9	18.2	29	17.3
10	18.4	30	18.7
11	18.7	31	18.3
12	17.9	32	18.1
13	19.2	32	18.5
14	17.5	34	17.3
15	18.0	35	17.4
16	18.7	36	17.1
17	17.1	37	17.3
18	18.4	38	17.7
19	17.7	39	17.9
20	17.4	40	19.1

3.1.4 Analyses of hydroxymethylfurfural (HMF) content

Subsequent analyses were only performed on 8 honey samples that were selected based on their moisture content and pH as well as their medicinal uses. Table 3.4 shows that the HMF content is highest in berberis honey with a value of 0.78 ± 3.7 mg/kg and is the lowest in citrus honey which contained 0.011± 3.6 mg/kg HMF. Analyses for the HMF content of the selected 8 honeys were carried out based on the Institute of Standards and Industrial Research of IRAN (ISIRI, 1998). The standardization of honey is possible if the analytical analysis is controlled. ISIRI is responsible for setting the standard of Persian honeys. In this research, the Iranian honeys were selected based on ISIRI (1998).

Table 3.4: HMF content of 8 selected Iranian honeys analysed by spectrophotometry.

Honey Type	Saffron	Berberis	Vulgare	Citrus	Thyme	Tamarix	Polyfloral	Sunflower
HMF content (mg/kg)	0.18 ± 2.1	0.78 ± 3.7	0.34 ± 0.9	0.01 ± 3.6	0.45 ± 1.5	0.29 ± 3.2	0.70 ± 2.7	0.69 ± 1.5

3.1.5 Characterization of sugar profiles in honey using gas chromatography (GC) and high performance liquid chromatography (HPLC)

Levulose, dextrose, saccharose and maltose were analyzed using GC and HPLC. The average content of sugar, sugar composition and Fructose/Glucose (F/G) ratio are presented in Table 3.5. Levulose was the main sugar found in all 8 honey samples followed by dextrose, maltose and saccharose. Generally, honey with a high fructose content are sweeter compared to those with high glucose content. Fructose can be produced from the action of glucose oxidase. The higher fructose content of honey will increase the Fructose/Glucose ratio.

Based on Table 3.5, there are differences between the results of the sugar content measured using HPLC and GC. In this study, it is assumed that sugars could ionize better in GC than HPLC. Therefore, the differences in sugar content between GC and HPLC could be due to differences in the polarity of the sugars. Generally, GC is more reliable for analysis of polar compounds like sugars whereas HPLC is better for non-polar compounds.

Table 3.5: The amount and composition of sugars and fructose/glucose (F/G) ratio in 8 selected honey samples measured using gas chromatography and high performance liquid chromatography

Honey type	Levulose (%)		Dextrose (%)		Saccharose (%)		Maltose (%)		F/G ratio	
	GC	HPLC	GC	HPLC	GC	HPLC	GC	HPLC	GC	HPLC
Saffron	35.47± 2.17	37.77 ±1.81	31.66±3 .8	29.47± 1.93	1.24± 0.61	0.02± 0.03	5.28± 0.16	4.07± 0.77	1.08	1.28
Berberis	38.31± 1.72	32.38± 3.19	22.09±2 .32	29.0± 3.0	0.55± 0.41	N.D.	7.13± 0.29	3.0± 0.17	1.73	1.10
Vulgare	39.21± 2.48	34.44± 4.57	28.34±1 .79	27.80± 2.66	0.26± 0.11	0.07± 0.05	9.29± 0.18	7.19± 1.10	1.38	1.24
Citrus	45.52± 2.53	32.76± 2.61	25.81±1 .43	31.38± 2.40	0.50± 0.51	0.50± 0.11	5.19± 0.49	4.61± 0.05	1.76	1.04
Thyme	34.25± 1.52	33.46± 3.17	28.22±2 .37	29.49± 2.85	0.23± 0.79	0.13± 0.13	11.65± 0.72	4.20± 0.61	1.21	1.13
Tamarix	37.73± 3.71	38.37± 8.56	19.11±3 .9	31.0± 6.0	0.62± 0.15	N.D.	15.21± 0.66	4.42± 1.04	1.97	1.21
Polyfloral	42.28± 3.12	32.76± 1.56	27.68±5 .29	26.0± 1.0	0.20± 0.2	N.D.	6.59± 1.03	4.93± 0.64	1.52	1.25
Sunflower	39.44± 1.56	32.83± 2.13	28.88±2 .03	30.0± 1.0	1.75± 0.29	N.D.	6.9± 0.81	4.86± 0.21	1.36	1.06

(All results are expressed as mean ± std dev).

3.2 Analyses for total phenolic content and antioxidant activities

3.2.1 Determination of total phenolic content

Eight of the selected Iranian honeys were used in this study. Varying antioxidant activities and total phenolic content were expected for each honey sample because of different floral sources of the honeys . Honey phenolics were extracted and recovered by base hydrolysis and ethyl acetate liquid-liquid extraction (LLE). For all the eight honeys, the recovered polyphenolic fractions from each 10 g of honey yielded approximately 124.5±1.85 mg extract (results are the mean of 20 observations). Appropriate dilutions were prepared with a final concentration of 4, 2, 1, 0.5 and 0.25 mg/ml; and then tested for total phenolic content and antioxidant activity.

Figure 3.1 shows the gallic acid standard curve for measuring the phenolic content of the honeys. The total phenolic content of saffron honey was found to be highest with a value of 2.09 ±1.25 μg/mg which differs significantly in comparison with other types of Iranian honeys ($P < 0.001$) (Table 3.6). However, sunflower honey (0.29±1.48 μg/mg) showed the lowest total phenolic content. As shown in Figure 3.2, a linear relationship

existed between extract concentrations and their total phenolic content.

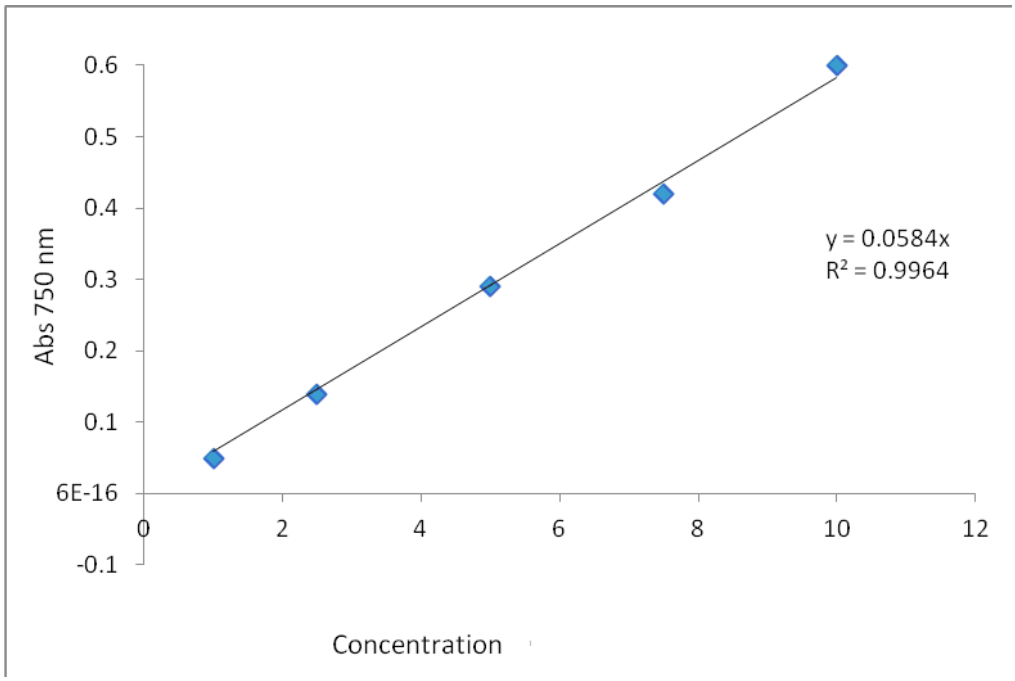

Figure 3.1: Total phenolic content calibration curve prepared from 1, 2.5, 5, 7.5 and 10 µg/mg gallic acid. Determination was carried out using Folin-Ciocalteau reagent procedure.

Table 3.6: Determination of total phenolic content of honey.

Type of Honey	Total phenolic content (µg/mg)
Saffron	2.09 ±1.25

Berberis	1.95±1.82
Vulgare	1.30±2.74
Citrus	1.22±1.57
Thyme	1.15±2.39
Tamarix	1.09±1.05
Polyfloral	0.71±2.56
Sunflower	0.29±1.48

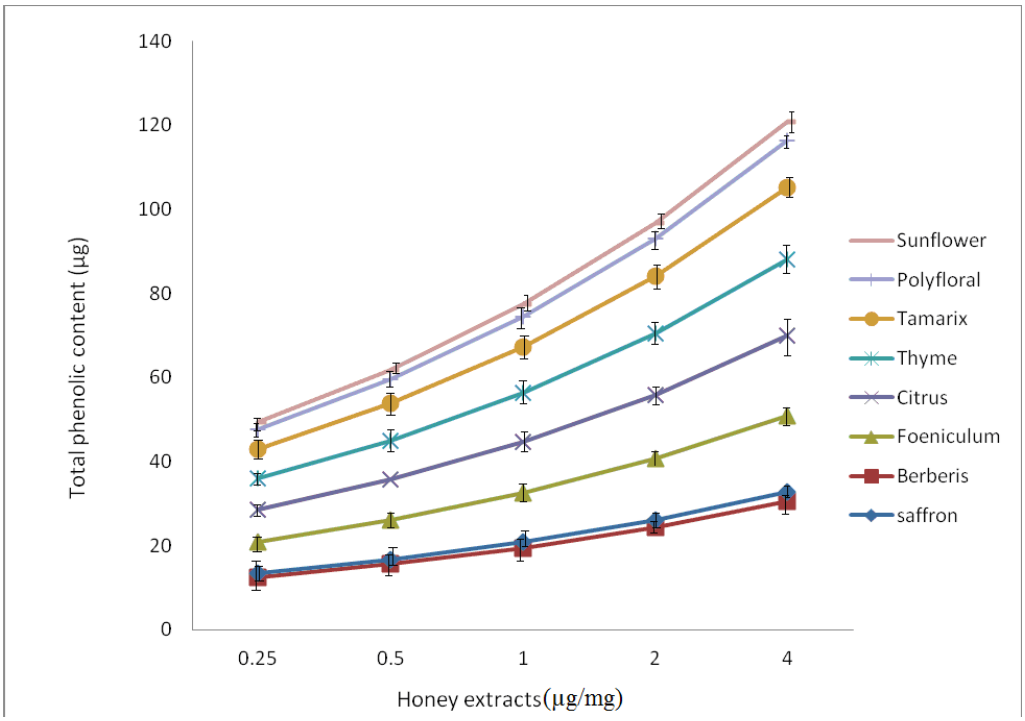

Figure 3.2: Total phenolic content of the ethyl acetate extracts of honey. Determination was carried out using Folin-Ciocalteau reagent procedure, and calculated from standard gallic acid graph.

3.2.2 Determination of the DPPH radical-scavenging activity of honey (DPPH Assay)

The highest DPPH radical scavenging activity was in saffron honey with a percentage inhibition of 82.4 ± 7.4% whereas the lowest was in polyfloral honey (36.29 ± 31.9%) (Table3.7). However, following the liquid-liquid extraction, there was a significant loss of DPPH radical scavenging activities. In this instance, thyme honey extract showed the highest DPPH radical scavenging activity (35.21 ± 0.04%), while the lowest was seen in the berberis honey extract (13.44 ± 0.04%). This implies that antioxidants responsible for the DPPH radical scavenging activities of honey are not only present in the ethyl acetate extract.

Each crude honey and honey extract have specific DPPH radical scavenging capacity. The dose–response curves of the DPPH radical scavenging activities of honey extracts are shown in Figure 3.4. It was found that through all concentrations that were tested, the DPPH radical scavenging ability of thyme and sunflower honey extracts were significantly higher ($P < 0.001$) than the other 6 honey extracts. The DPPH radical scavenging activities of the honey extracts increased in response to increasing extract concentration up to 2 mg/ml, at which it reached a steady state and the reaction curve showed a plateau; indicating that addition of 2 mg/ml of citrus, polyfloral, tamarix, berberis, vulgare and saffron and honey extracts resulted in a complete reduction of DPPH

used in the test. Therefore, any increase of the extract concentration would not show any increase in the activity. On the other hand, the DPPH radical scavenging activity of thyme and sunflower honey extracts also increased in response to the increase in extract concentration but without reaching an endpoint even by using a concentration of 4 mg/ml.

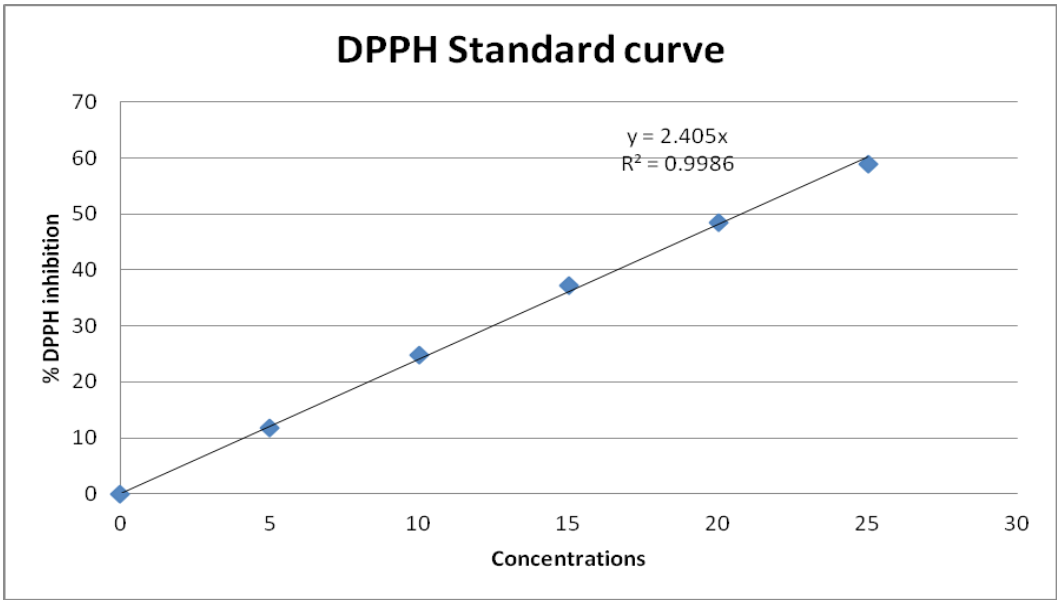

Figure 3.3: Dose-dependent activity of vitamin C on scavenging the DPPH radicals.

Table 3.7: The DPPH radical scavenging activities of crude honey and ethyl acetate extract of honey samples.

Type of Honey	DPPH radical scavenging activity (% inhibition)	
	Crude honey	Ethyl acetate extract of honey
Saffron	82.4 ± 7.4	17.93 ± 0.04
Berberis	80.23 ± 38.2	13.44 ± 0.04
Vulgare	51.38 ± 16.5	15.32 ± 0.03
Citrus	66 ± 46.1	25.13 ± 0.13
Thyme	76.72 ± 30.8	35.21 ± 0.04

Tamarix	43.91 ± 24.7	24.39 ± 0.07
Polyfloral	36.29 ± 31.9	18.12 ± 0.01
Sunflower	45.77 ± 21.6	32.41 ± 0.02

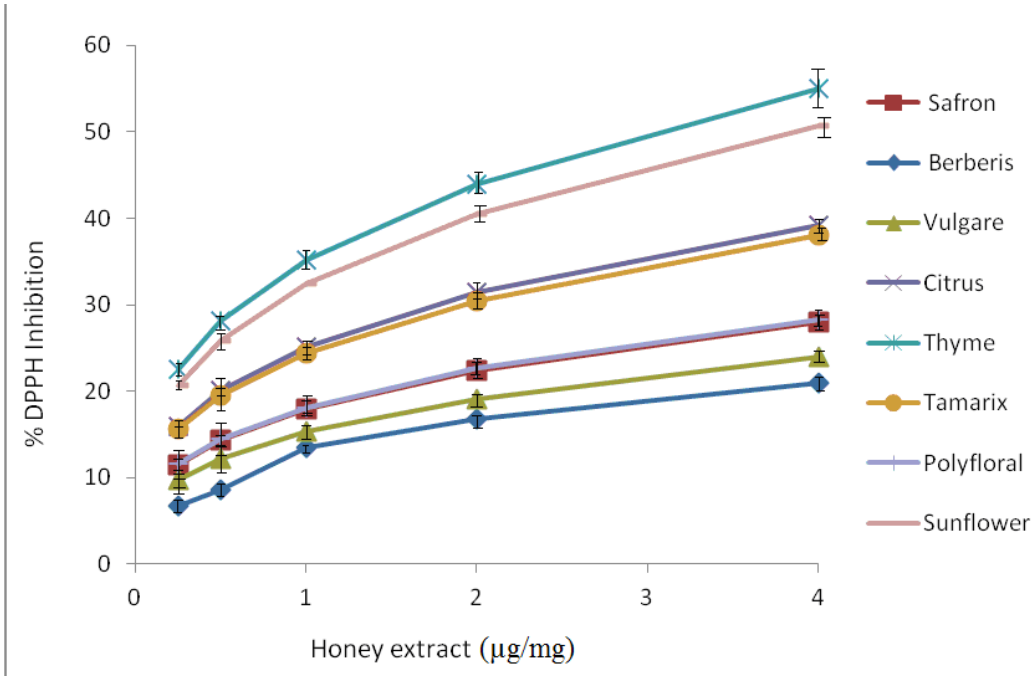

Figure 3.4: Dose–response curves for the DPPH radical scavenging activity of the ethyl acetate extract of honey.

3.2.3 Determination of the total antioxidant power of honey (FRAP Assay)

Different honeys show different antioxidant activities in this study (Figure 3.7), depending on their botanical sources . The average FRAP values of the crude honey extracts range from 309.7 ± 24.1 µM, in sunflower honey, to 1247.5 ± 13.5 µM, in saffron honey (Figure 3.8). Following extraction with ethyl acetate, there was significant loss of ferric reducing activities (from 31.67 ± 14.2 µM in sunflower honey to 82.22 ± 9.7 µM in saffron honey) (Figure 3.9). This again implies that antioxidants responsible for the

antioxidant activities of honey are not only present in the ethyl acetate extract. Figure 3.10 shows the dose-response curve for the FRAP analyses of the honey samples. Activities were more rapid at lower concentrations and gradually slowed down at higher concentrations of honeys.

The Iranian honeys with high content of antioxidant activities are potentially useful as a source of nutraceuticals. All the extracts of selected Iranian honeys showed the presence of antioxidant activities. Honey phenolics exhibit different antioxidant activities. Generally, the darker the honey, the higher is its phenolic content and antioxidative power. This result shows similar pattern to the study by Almahdi and Kamaruddin .

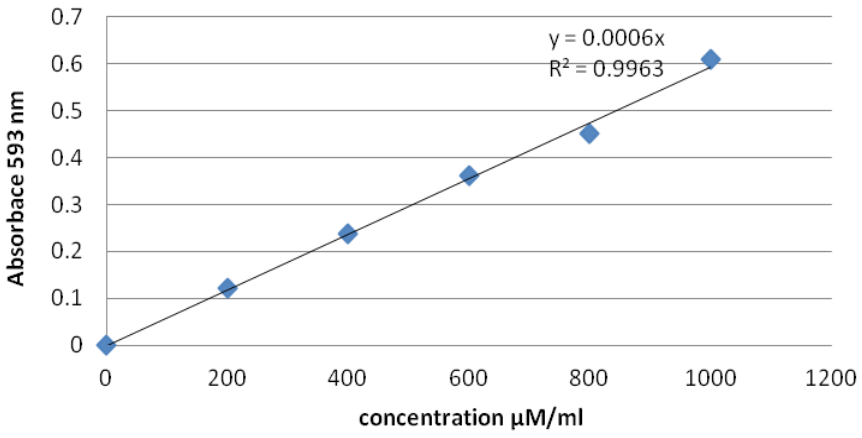

Figure 3.5: $FeSO_4$ standard curve for analysis of ferric reducing antioxidant potential (FRAP) of honey. Absorbance change due to Fe (III) - TPTZ reduction by $FeSO_4$ was monitored at 593 nm.

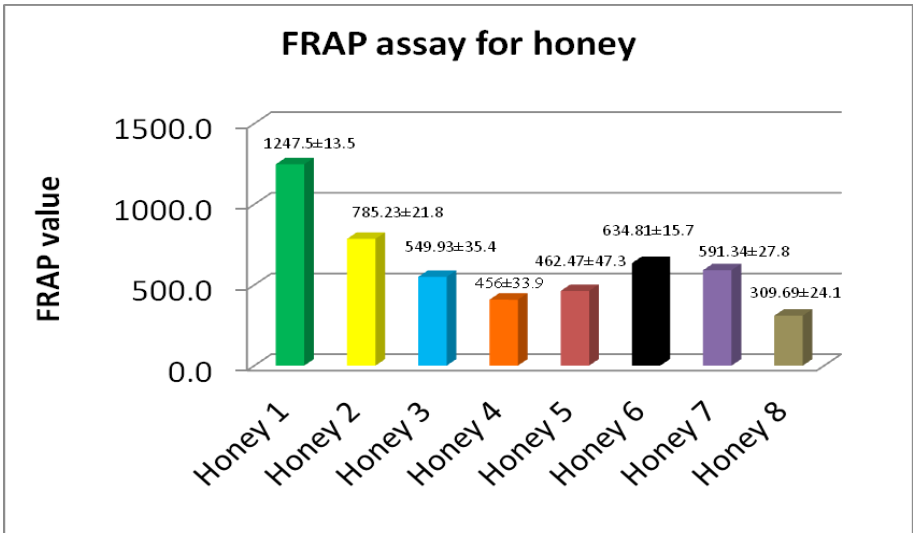

Figure 3.6: Ferric reducing antioxidant power (FRAP) of crude honey extracts (µM). Absorbance change due to Fe (III) - TPTZ reduction by antioxidants in honey extract was monitored at 593nm. Values were then related to that given by a standard of Fe (II). Results represent mean of five determinations (± Std. Dev.).

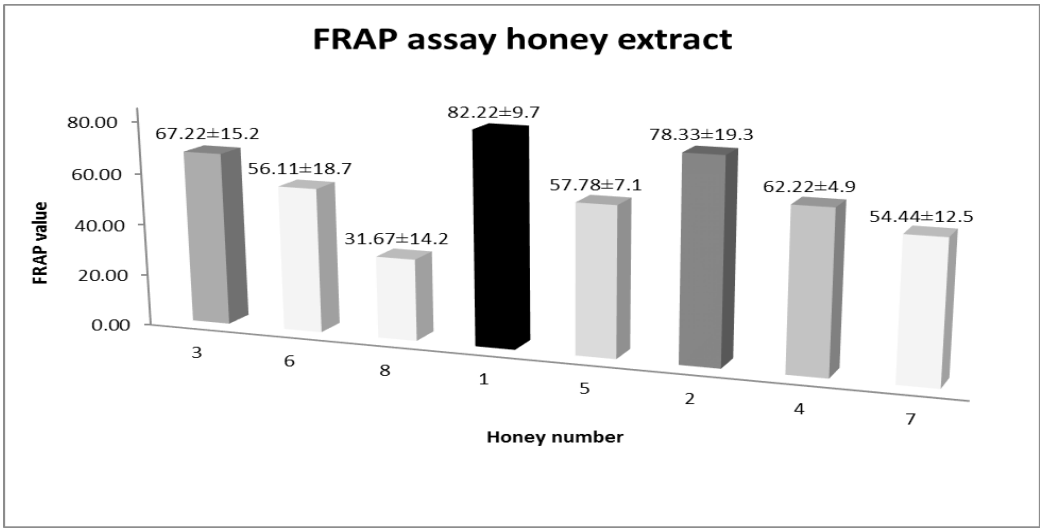

Figure 3.7: Ferric reducing antioxidant power (FRAP) of the ethyl acetate extracts of honey (µM). Absorbance change due to Fe (III) - TPTZ reduction by antioxidants in honey extract was monitored at 593nm; Values were then related to that given by a standard of Fe (II). Results represent mean of five determinations (± Std. Dev.).

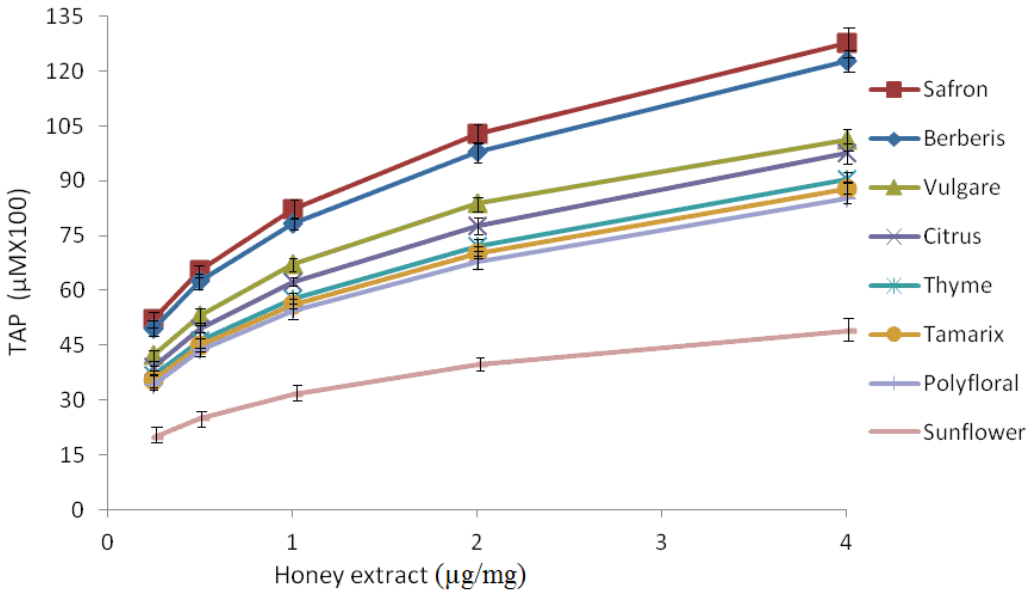

Figure 3.8: Dose–response curves for the TAP of the crude honey extracts.

3.2.4 Relationship between the antioxidant activities of honey and their phenolic content

A high positive correlation was found between the two parameters which were between the antioxidant activities of honeys, and their phenolic content, indicating the role of phenolics in the antioxidant activity of honey. In addition to phenolics, there may be other minor organic compounds contributing to the antioxidant activities of honey.

As a result, a strong positive correlation was seen between DPPH radical scavenging activities and total phenolic content of the honey samples (Figure 3.10). A moderately strong positive correlation was observed between FRAP and total phenolic content (Figure 3.9), further strengthening the contribution that polyphenols play in providing antioxidant activities.

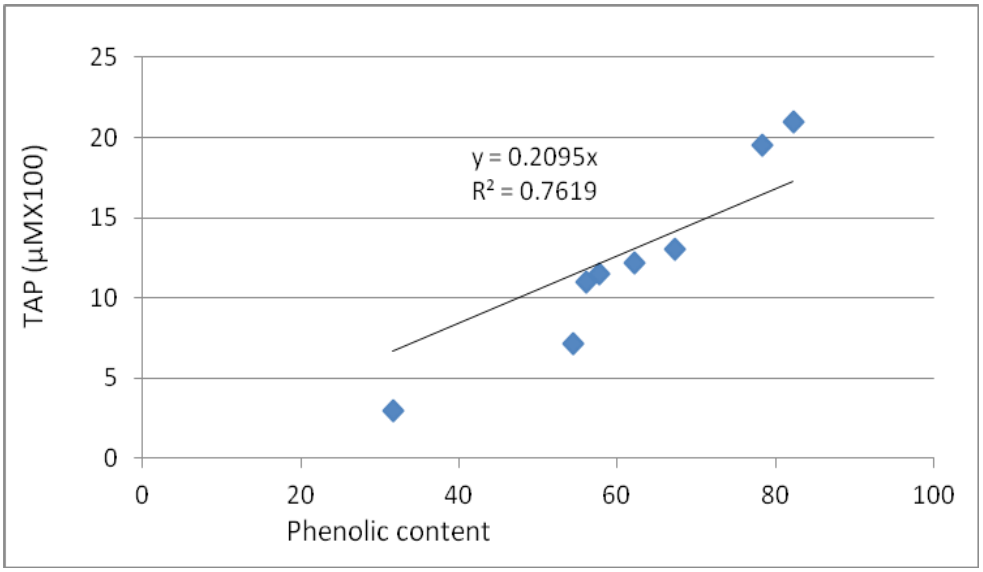

Figure 3.9: Correlation between the ferric reducing antioxidant power of honey extracts and their total phenolic content.

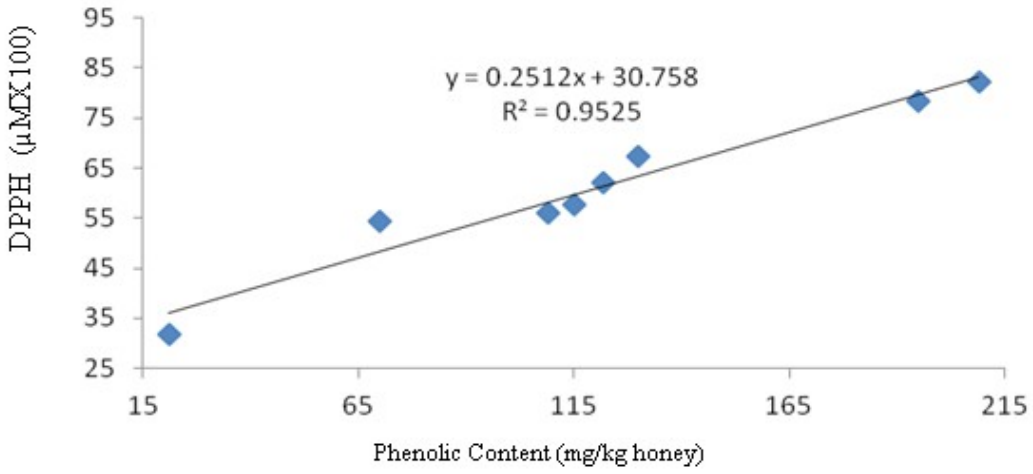

Figure 3.10: Correlation between the DPPH radical scavenging activity of honey and their total phenolic content.

3.3 Analyses on the effect of honey on performance and immune response using an animal model

Honey can play an important role in providing feed antioxidants in a highly palatable form , and can be used as a natural antioxidant to reduce the negative effects of lipid oxidation in chicks and during meat processing, as recommended by Antony et al. . A previous study by Raji (2006) showed that the honey supplement had metabolizable energy content increased by about 10%. This content might be derived from the breakdown of non-starch polysaccharides. Chicken fed honey in water showed better performance characteristics and feed conversion ratio .

Depending on the botanical sources, honeys showed various antioxidant activities and carbohydrate profiles. In this study, the polyfloral honey that was used was harvested from north east of Iran. Honey with high antioxidant activity is potentially a good source of nutraceuticals. The antioxidant properties of honey were measured successfully and generally, TPC, DPPH and FRAP showed a variety of activities.

Animal studies were designed to directly examine daily feed or dry matter intake of broilers. Since the chicks were fed ad libitum, the primary research have been to examine the response to different diets at some fixed point associated with final weight, rather than to characterize feed intake. Nevertheless, exposure of chicks to infectious agents induces immune responses that result in reductions in food consumption and weight gain. The effect of honey on performance and immune response remains unclear. Six treatments were conducted to investigate their effects on the chicks' immune system and performance. In this study, the chicks were feed polyfloral honey and different types of carbohydrates (the carbohydrate selected was based on the main carbohydrates found in honey) for three days. The treatment groups consisted of: Normal diet as control (T1), diet + 3 g/kg dextrose

(T2), diet + 3 g/kg saccharose (T3), diet + 3 g/kg polyfloral honey (T4), diet + 3 g/kg levulose (T5), and diet + 3 g/kg maltose (T6).

As a result, feed administration of honey, or other treatments, did not change the chicks' body weight (Table 3.8), chicks' daily weight gain (Table 3.9), food consumption (Table 3.10), food conversion ratio (Table 3.11) and the organ weight of the broilers (Table 3.12), of performance utilization. The food conversion ratio (FCR is a measure of a broiler efficiency in converting feed mass into increased body mass) accretion was not influenced by the honey supplements or carbohydrate intake. It was seen that polyfloral honey (T4) was the most effective treatment on daily weight gain (Table 3.9) among the different groups, and could be effective on the food conversion ratio (FCR) of the chicks below 21 days old (Table 3.11). In this regard, the dietary honey required to maximize chicks' food efficiency maybe for longer period (more than 3 initial days).

Results from this study show that the different dietary treatments did not change weight gain, food intake, and efficiency of food utilization; nevertheless, the immune response in avian influenza anti body titer (Table 3.8) and newcastle anti body titer (Table 3.9) of the animals was not affected significantly. As a result, feeding of honey administration did not affect performance, nor did it affect the diet required to maximize immune response.

As a result, the addition of polyfloral honey treatment (T4), especially during the early ages of the chickens may improve performance (Table 3.8), and may decrease the need for other supplements such as carbohydrates. Moreover, for instance, honey contains more than 80% different sugars, mostly fructose and glucose (Cherbuliez, 2001). This was possibly due to the antioxidant content of polyfloral honey, which might affect the chickens' performance and which was mixed to the oligosaccharides and sugars inside

honey. Morover, carbohydrate may affect chickens as prebiotic. Research has shown that prebiotics have the ability to enhance immune responses and anecdotal evidence suggests that honey can stimulate immune function. In addition, honey has been demonstrated to stimulate antibody production during primary and secondary immune responses against thymus-dependent and thymus- independent antigens . Long-term feeding of honey has been shown in the laboratory to have a number of health benefits, including improved weight regulation, decreased oxidative damage and improved cognitive function compared with animals fed sucrose or a sugar-free diet and it appears that honey may also stimulate immune function in animals. It is also possible that the non-sugar components of the honey may be responsible for the enhanced immune-modulatory effects. It is also possible that the antioxidant content of the honey may have contributed to its immune-modulatory effects. Although there are no studies directly investigating the effects of honey antioxidants on immune function, other antioxidant compounds have been shown to stimulate immune function *in vitro* and *in vivo* .

The increase of antibody titer for avian influenza (Table 3.13), only in honey supplemented diet, may have been due to the antioxidant properties of honey. It seems honey antioxidant was able to protect immature lymphocytes from damage by free radicals due to oxidation, thus enhancing the immune system of the broilers. It assumed antioxidants in honey may improve chicks' performance and immune response.

Table 3.8: Effect of different treatment on chick body weight

Treatments	CHICK BODY WEIGHT (gram)		
	21 DAY	42 DAY	56 DAY
T1	555.5±8.09b	1867.5±15.7	2374.75±12.5
T2	591.25±7.03ab	1858.25±13.4	2398.75±13.08
T3	598.25±9.93ab	1836.75±16.47	2381.25±11.61
T4	646.25±5.64a	1857.25±14.1	2422.0±10.03
T5	555.5±10.27b	1833.0±14.51	2385.25±13.11
T6	604.75±8.24ab	1869.25±16.9	2399.75±16.41

Table 3.9: Effect of different treatment on the chicks' daily weight gain.

Treatments	DAILY WEIGHT GAIN (g/chicks/day)			
	0-21DAY	21-42 DAY	42-56 DAY	TOTAL
T1	521.25±14.4b	1302±12.22	507.25±15.21	2330.5±11.07
T2	548±15.01b	1267±8.09	540.5±13.03	2355.5±14.32
T3	554.5±13.6ab	1238.5±14.42	544.5±11.73	2337.5±13.74
T4	602.5±10.07a	1225.5±11.04	564.75± 16.2	2378.25±12.07
T5	512.25±8.9b	1291.25±15.55	552.25±8.51	2342.0±10.44
T6	561.5±11.0ab	1289.75±13.06	530.5±15.85	2356.5±13.02

Table 3.10: Effect of different treatment on food consumption.

Treatments	FOOD CONSUMPTION (g/chicks/day)			
	0-21 DAY	21-42 DAY	42-56 DAY	TOTAL
T1	911±15.05	2594.25±18.66	1762.75±18.9	5268.0±10.01
T2	909.25±19.8	2536±10.31	1793±11.42	5238.25±13.41
T3	924.75±16.17	2558±13.33	1776.5±14.07	5259.25±11.28
T4	917.75±10.51	2537.75±11.19	1866.25±15.99	5321.75±12.39
T5	881.0±18.2	2653.5±19.2	1751.5±11.78	5259.75±14.37
T6	898.75±11.09	2617.5±17.46	1744±20.41	5260.25±17.3

Table 3.11: Effect of different treatment on food conversion ratio (FCR: for every one broiler, the certain amount of feed was consumed).

Treatments	**Food Conversion Ratio**			
	0-21DAY	21-42 DAY	42-56 DAY	TOTAL
T1	1.75±0.04a	1.99±0.02	3.48±0.01	2.26±0.01
T2	1.67±0.06ab	2.01±0.04	3.32±0.03	2.22±0.02
T3	1.67±0.01ab	2.07±0.09	3.27±0.06	2.25±0.03
T4	1.52±0.05b	2.07±0.07	3.31±0.05	2.24±0.04
T5	1.72±0.02a	2.06±0.06	3.19±0.09	2.25±0.0
T6	1.61±0.09ab	2.03±0.01	3.29±0.04	2.23±0.06

Table 3.12: Effect of different treatments on the organ weight of the broilers.

Treatments	Organ weight of broilers				
	Liver	Pancreas	Abdominal Fat	Spleen	Ventriculus Weight
T1	55.75±3.5	6.75±0.35	31.5±6.29	4±0.33	274.25±8.21
T2	57±7.27	6.25±0.28	57.25±5.52	4.75±0.39	309.75±4.35
T3	49±6.54	5±0.33	40.5±3.48	3±0.29	243±5.8
T4	49.25±5.71	6.25±0.31	46.25±4.95	3.25±0.35	287±7.19
T5	56.25±4.39	4.5±0.35	31.25±8.17	3.25±0.31	278.25±6.5
T6	48.75±8.4	5±0.3	38.75±7.32	3.5±0.37	268.75±3.9

Table 3.13: HI Test for Avian Influenza

Treatment	Titer (%)
1	3.75
2	3.5
3	5
4	5.5
5	5.25
6	4.5

Table 3.14: HI Test for New

Treatment	Titer (%)
1	5.5
2	5.75
3	5.5
4	5.25
5	5.25
6	5

In this experiment, chicks given polyfloral honey (T4) showed significantly higher weight gain (Table 3.8). Moreover, feed conversion ratio (Table 3.11) decreased meaningfully (0-21 days). There were no significant differences in food consumption

between the chicks treated with the different carbohydrate treatments and those treated with poly floral honey treatment (Table 3.10).

3.4 Cytotoxicity of honey on breast cancer cell lines

The present study was undertaken with the goal to determine the anti-cancer properties of two Iranian honey extracts, sunflower (which has the lowest antioxidant activities) and saffron (which has the highest antioxidant activities) by measuring their effects on cell viability/proliferation of breast cancer cells.

The dose response curve related to the effect of honey on the viability of MCF-7 cells is illustrated in Figure 3.11. Sunflower honey extract showed minimal inhibition of MCF-7 cells while Saffron honey extract could inhibit MCF-7 cell viability. Some of the polyphenols of honey extract like caffeic acid (CA), caffeic acid phenyl ester (CAPE), chrysin (CR), galangin (GA), quercetin (QU), acacetin (AC), kaempferol (KF),Zinocembrin (PC), pinobanksin (PB) and apigenin (AP) as effective pharmacological agents play pivotal roles in curing different cancers. Polyphenols and phenolic acids (vanillic acid and protocatechuic acid and p-hydroxybenzoic acid) are found to be able to constrain cancer-related pathways and processes ; ; . Figure 3.12 shows the dose response curve related to the honey effect on the viability of MDA MB 231 breast cancer cells. Both honey samples could inhibit the viability of MDA MB 231 cells although Saffron honey was more potent. The higher content of phenolic compounds in Saffron honey could have contributed to the higher cytotoxic effect of this honey on the breast cancer cells.

The MTT assay assesses indirectly the effect of honey on the survival and proliferation of cells. Honey extracts from saffron honey reduced significantly the MCF7

and MBA 231 cell viability.

A main characteristic of most breast cancers is an uncontrolled cellular growth whilst substances that monitor cell proliferation may be useful in cancer prevention. Estrogens are known stimulants of breast cancer whereas the viability/proliferation of MCF-7 cells is a well-established biological test for screening extracts that may function as oestrogen agonists/antagonists.

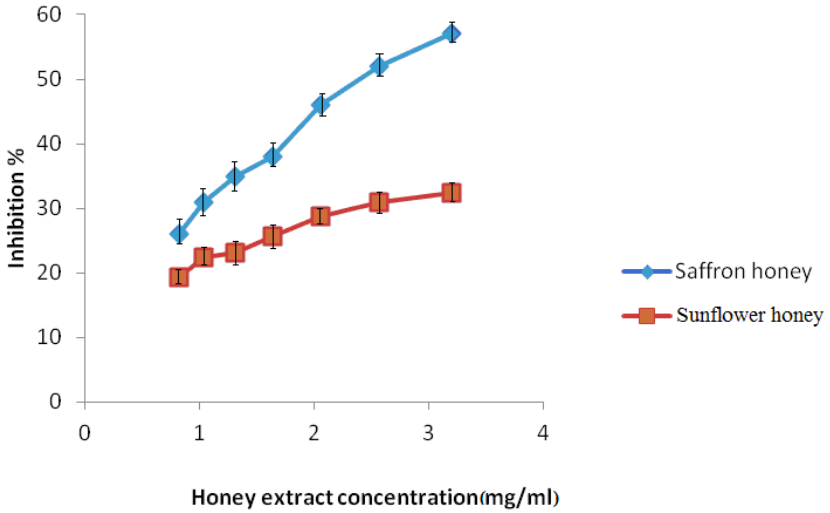

Figure 3.11: Anti-proliferative effect of honey on MCF-7 breast cancer cells

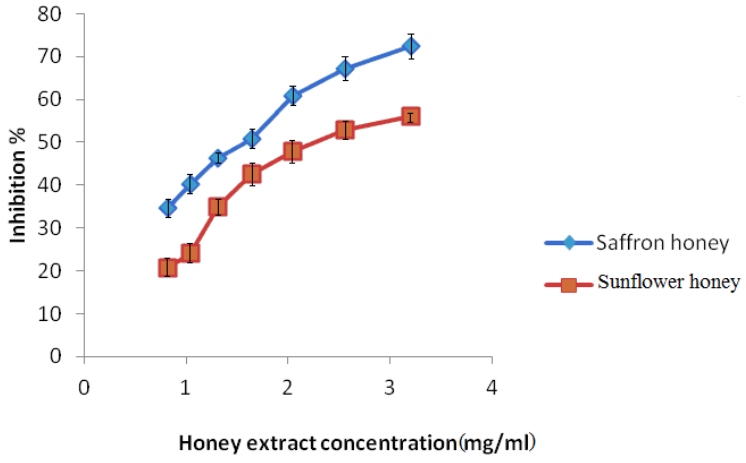

Figure 3.12: Anti-proliferative effect of honey on MDA-MB-231 breast cancer cells

3.5 IDENTIFICATION OF ANTIOXIDANT COMPOUNDS

3.5.1 Isolation, identification and quantification of phenolic compounds using high performance liquid chromatography (HPLC)

3.5.1.1 Solid phase extraction

The recovery of phenolic compounds in honey extracts ranges from 25% to 85%. Nevertheless, some compounds did not have peak area to measure recoveries (like isorhamnetin). Apigenin had percentage recoveries of 62%. The other flavonoids chrysin, galangin, hesperetin, isorhamnetin, kaempferol, pinocemberin, quercetin and rutin had percentage recoveries of 85%, 37%, 71%, 0, 25%, 43%, 62% and 79%, respectively. The use of a C_{18} cartridge allowed for a fast sample treatment with low solvent consumption , good clean up and recovery values were 25% to 85% for phenolic compounds in honey samples. The SPE method showed good sensitivity and linearity. This SPE-LC method was found to be suitable for quality control and routine analysis of honey .

3.5.1.2 High performance liquid chromatography (HPLC)

A calibration curve of quercetin at 280 nm was used to calculate polyphenol concentration. This is because the different polyphenols show better absorbance at this wavelength. Phenolics in the honey samples were quantified using a calibration curve based on concentrations of 25, 50, and 100 μg/mL . In the HPLC condition, most polyphenols were detected better at 280 nm, however, some flavonoids were detected clearly at 360 nm and some phenolic acids were detected clearly at 290 nm . Although ellagic acid is a phenolic acid, it is better detected at 345 nm due to its stronger absorbance at this

wavelength .

Figure 3.13 shows HPLC chromatogram of all the standard compounds: apigenin, chrysin, galangin, hesperetin, isorhamnetin, kaempferol, pinocemberin, quercetin and rutin which were detected at 280 nm over 40 minutes. Table 3.15 shows the flavonoid content (ng/100µl) detected in the methanolic extract of all honey samples. Figures 3.14 to 3.21 show the HPLC chromatograms of the 8 honey samples. Quercetin and apigenin were detected at varying levels in all the 8 honey samples. In addition to quercetin and apigenin, kaempferol was also detected but at low levels in thyme honey (Figure 3.18). Several unknown flavonoid peaks were also detected in the samples, which did not correspond with the retention times of the standards used in these analyses.

HPLC is the method of choice for food phenolic investigation and was used in this study for the identification of honey polyphenols . Phenolic compounds were detected at 280 nm and most of the compounds showed convincingly high absorbance at this value . Although there is a similarity between the HPLC chromatograms of all honeys tested, quantitative and qualitative differences were also shown. As a result, the levels of most identified and unidentified compounds were determined from the peak areas .

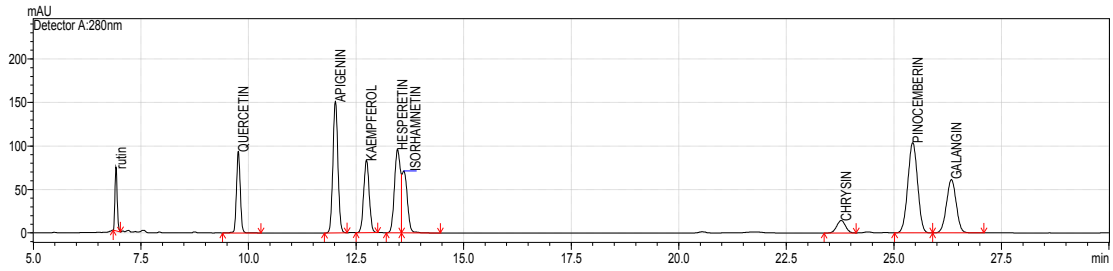

Figure 3.13: UV chromatogram of phenolic standards at 280 nm.

Table 3.15: Quantification of flavonoids in the methanolic extract of honeys

No.	Honey types	Apigenin	Quercetin	Kaempferol
1	Crocus sativus	41.74±0.03	3.38±0.01	ND
2	Berberis	19.64±0.02	0.64±0.03	ND
3	Foeniculum vulgare	8.15±0.05	0.64±0.02	ND
4	Citrus	13.54±0.01	0.73±0.04	ND
5	Thyme (Astragallus,Thistle)	29.36 ± 0.04	1.78±0.05	15.23 ± 0.02
6	Tamarix (Rosmarinusofficinalis)	26.82±0.03	13.88±0.02	ND
7	Polyfloral	60.11±0.02	3.39±0.04	ND
8	Sunflower (alfalfa, Lotus)	52.29±0.05	5.53±0.01	ND

The concentration of flavonoids was expressed as ng/100 µl ± std. dev.
ND: not detected.

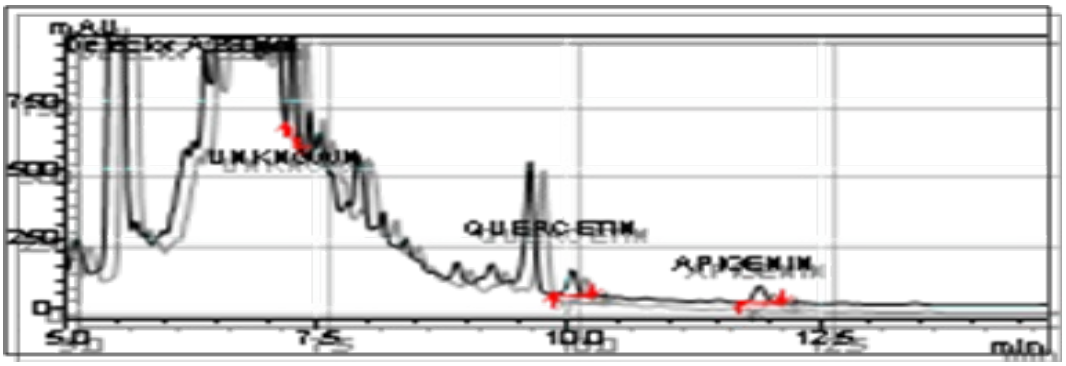

Figure 3.14: Chromatogram of saffron honey detected by UV absorption at 280 nm.

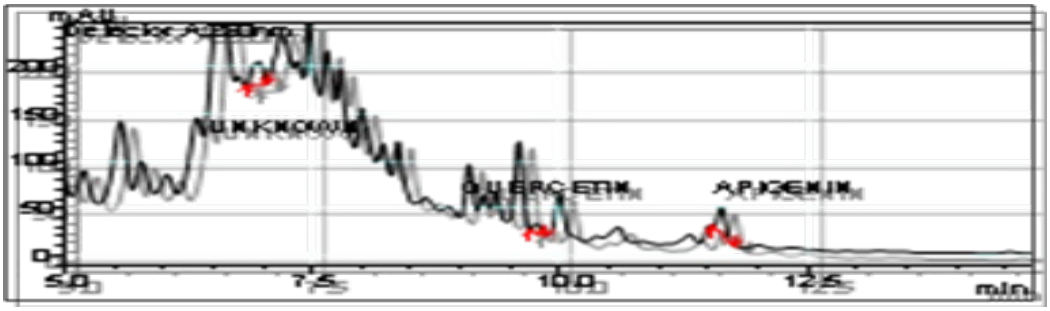

Figure 3.15: Chromatogram of berberis honey detected by UV absorption at 280 nm.

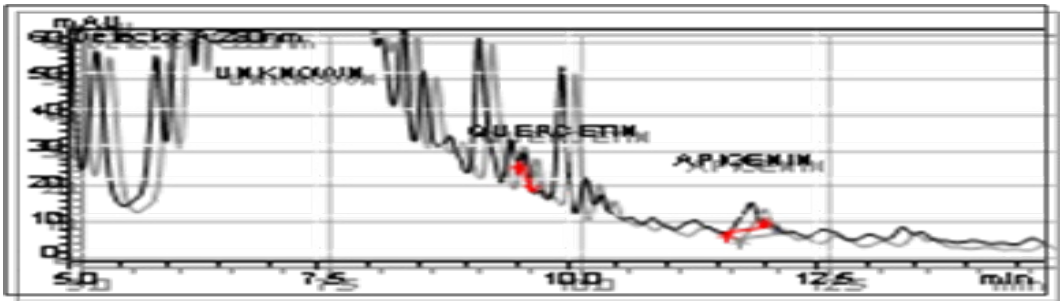

Figure 3.16: Chromatogram of vulgare honey detected by UV absorption at 280 nm.

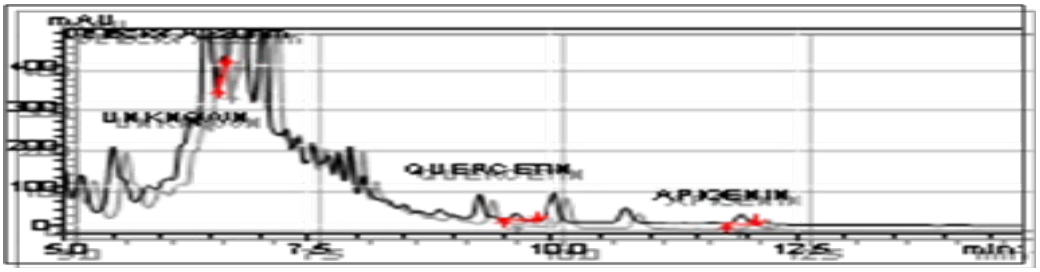

Figure 3.17: Chromatogram of citrus honey detected by UV absorption at 280 nm.

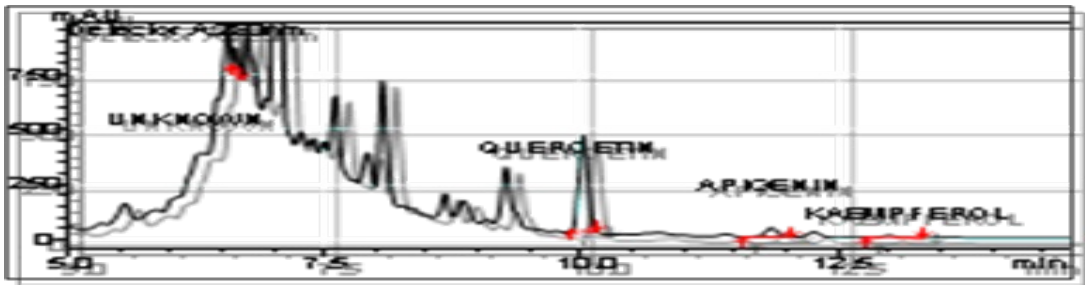

Figure 3.18: Chromatogram of thyme honey detected by UV absorption at 280 nm.

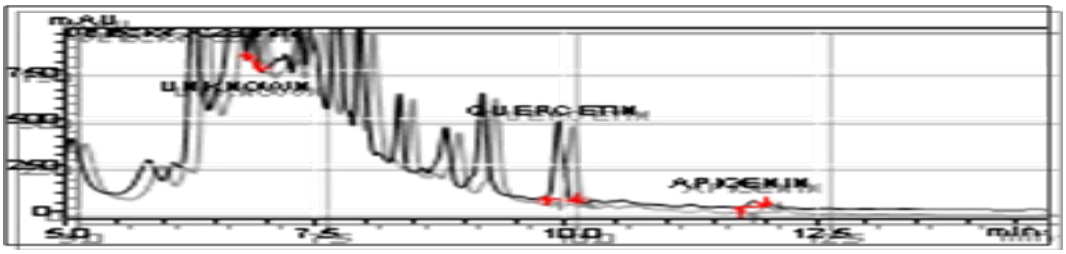

Figure 3.19: Chromatogram of tamarix honey detected by UV absorption at 280 nm.

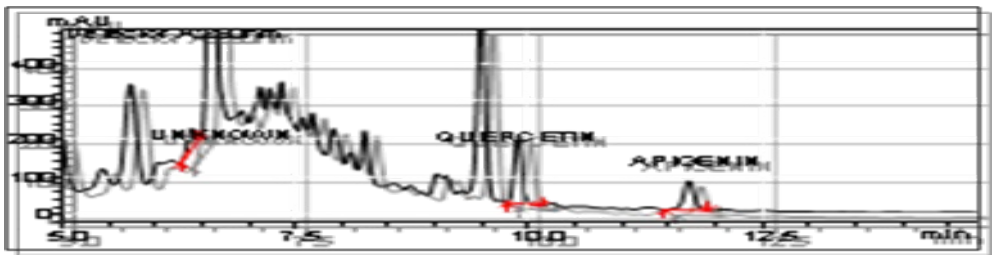

Figure 3.20: Chromatogram of polyfloral honey detected by UV absorption at 280 nm.

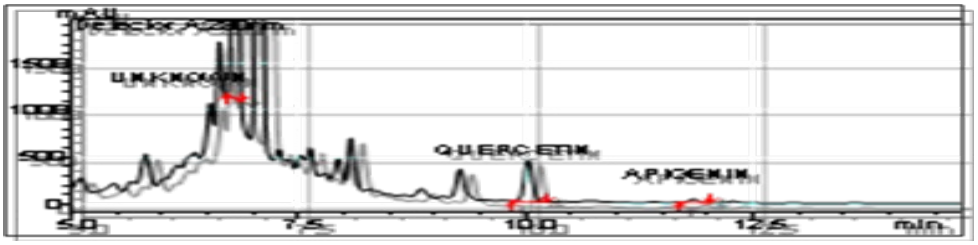

Figure 3.21: Chromatogram of sunflower honey detected by UV absorption at 280 nm.

3.5.2 Identification of phenolic acids in honey using GCMS

In this study, the phenolic acids (Table 3.16) were analysed using GCMS as they were less polar and have high basicity in the gas phase . Flavonoids were not analysed by GCMS as they do not hold nitrogen atoms and have low basicity in the liquid phase . The assignment of the hydroxyl-group at p-position for the honey extracts was based on relative abundance of fragments in comparison with MS spectra and retention time of standards . After methylation, the honey phenolic extracts were subjected to GCMS spectrometry . They were analysed under the conditions as described in materials and methods.

No.	Phenolic acids	Formula	Exact mass	Melting point (˚C)	Molecular Weight	RT (min)
1	Benzoic acid	$C_7H_6O_2$	122.12	122.38 °C	122.12	28.9
2	Caffeic acid	$C_9H_8O_4$	180.042259	223-225 °C	180.16	22.4
3	Chlorogenic acid	$C_{16}H_{18}O_9$	354.095082	207 - 209 °C	516.45	11.9
4	Gallic acid	$C_7H_6O_5$	170.021523	250˚C	170.12	5.7
5	p-Coumaric acid	$C_9H_8O_3$	164.047344	210–213 °C	164.16	13.9
6	Ellagic acid	$C_{14}H_6O_8$	302.006267	360˚C	302.20	29.3
7	Ferulic acid	$C_{10}H_{10}O_4$	194.057909	168-172 °C	194.18	28.1
8	Fumaric acid	$C_4H_4O_4$	116.07	287 °C	116.07	7.2

Table 3.16: GCMS profiles of phenolic acid standards used in this study.

Some researchers like D'Arcy (2002) and Yao (2004) used LC for identification of phenolic acids ; however, HPLC is a better instrument for detection of flavonoids in honey while GCMS is better for identification of phenolic acids . Gas chromatography with flame ionization detector (GC-FID) analysis can confirm the results obtained from HPLC analysis: combined GCMS analysis also provides the mass spectra for polyphenols.

A variety of phenolic acids were identified in the Iranian honey samples. The phenolic acids that were detected in Iranian honeys included gallic, ellagic, ferrulic, and chlorogenic acids. Figures 3.22-3.29 show the phenolic mass spectra of the honey extracts. Their identities were confirmed by direct comparison with authentic standards and retention times, which were remarkably stable and reproducible , although there were also several unknown peaks which could not be identified.

To our knowledge, this current work is the first report highlighting the use and probable combination of MS spectra in GCMS fingerprinting studies. The results indicate the applicability of this instrumentation in targeted chemometrics studies of phenolic acid

compounds of honey.

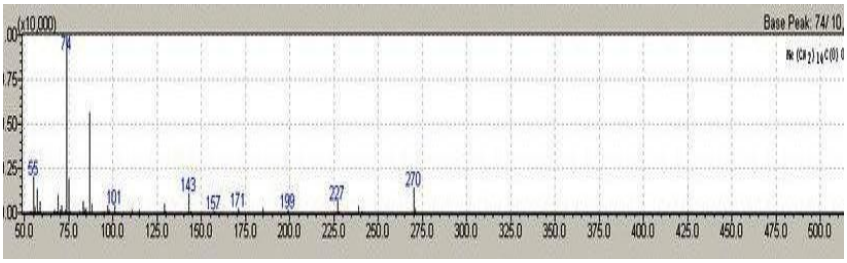

Figure 3.22: Phenolic acid mass in the methanolic extract of saffron honey.

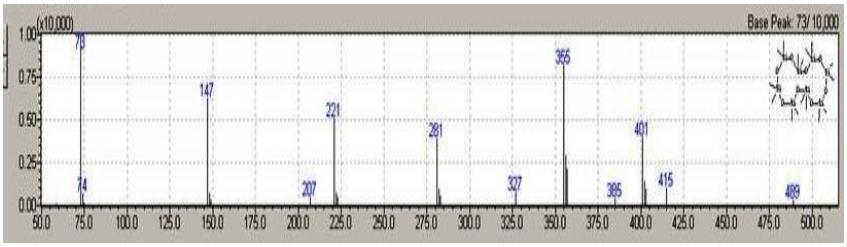

Figure 3.23: Phenolic acid mass in the methanolic extract of berberis honey .

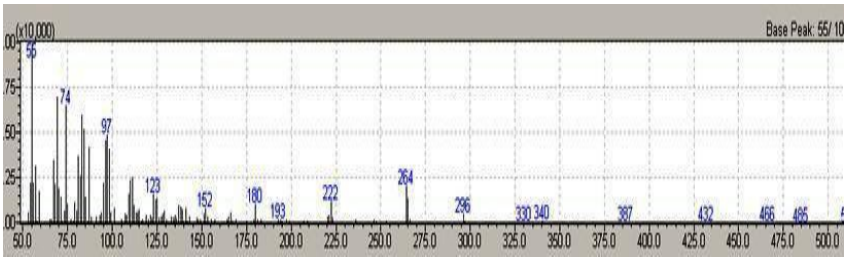

Figure 3.24: Phenolic acid mass in the methanolic extract of vulgare honey.

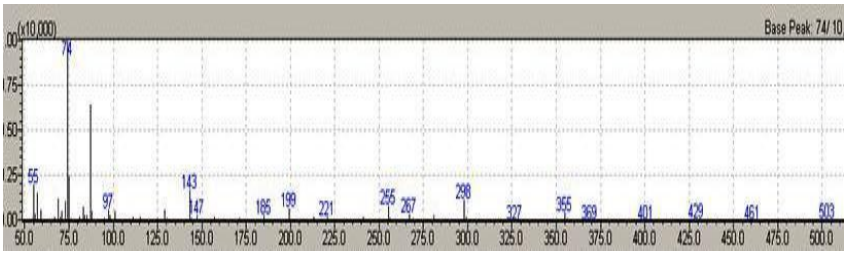

Figure 3.25: Phenolic acid mass in the methanolic extract of citrus honey.

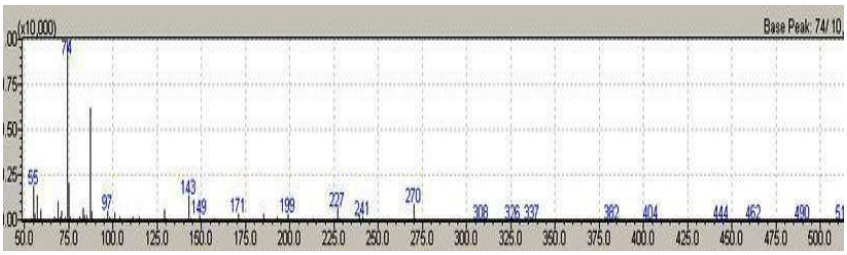

Figure 3.26: Phenolic acid mass in the methanolic extract of thyme honey.

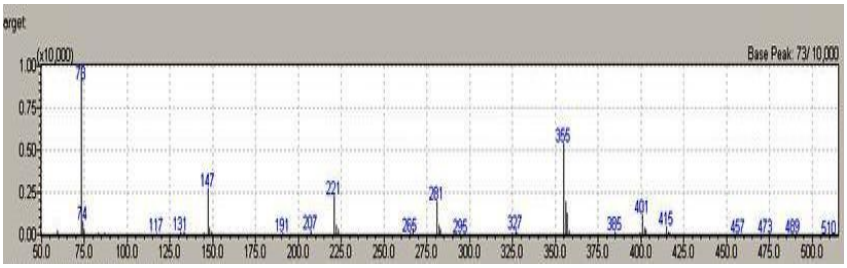

Figure 3.27: Phenolic acid mass in the methanolic extract of tamarix honey.

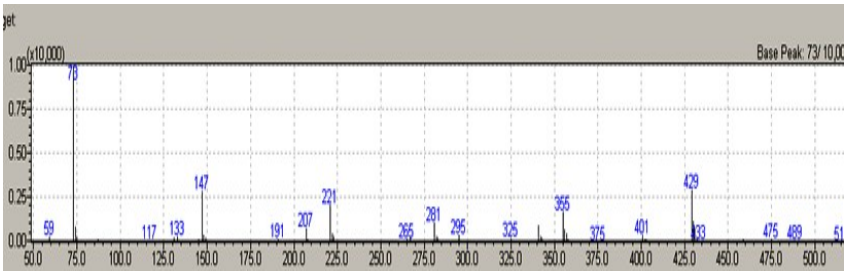

Figure 3.28: Phenolic acid mass in the methanolic extract of polyfloral honey.

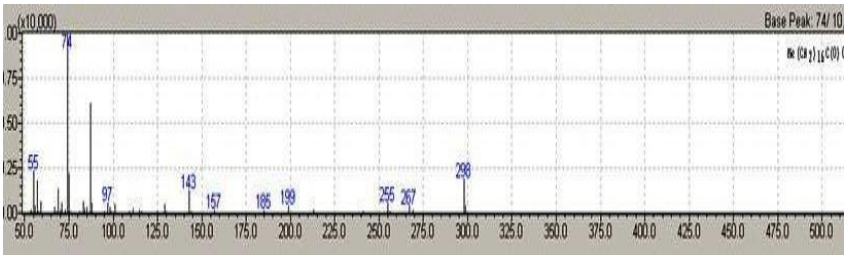

Figure 3.29: Phenolic acid mass in the methanolic extract of honey sunflower.

3.5.3 Identification of polyphenols in honey using liquid chromatograpy-mass spectrometry-atmospheric pressure chemical ionization (LCMS-APCI)

LCMS atmospheric pressure chemical ionization (APCI) was investigated in negative and positive modes. The negative mode results in limited fragmentation but provides the highest sensitivity for phenolics . The mass spectra of gallic acid, coumaric acid and ferrulic acid ions occur in negative ionization mode while ellagic acid ions occur in positive ionization mode. As expected, the high level of noise in ionization and the formation of protonated molecules [M+H] + were lower in positive mode than in negative mode . The limit of detection for polyphenols for APCI in the positive and negative mode was 10 mg/L, which could not detect low weight polyphenols . However, the eluent composition could have a significant influence on the (APCI) analysis ionization. The total ion current (TIC) chromatogram represents the summed intensity across the entire range of masses being detected.

Table 3.17 shows the APCI LCMS profiles of phenolic standards used in this analysis. Figures 3.30 and 3.31 show the UV spectra of the phenolic standards measured at 210 nm and 260 nm, respectively. In the 8 honey samples, the TIC chromatograms show 5 peaks as multiple analytes that elute simultaneously, obscuring individual species. Phenolic

acids were not detected in all the 8 honey samples but three flavonoids, quercetin, apigenin and hesperetin were detected. Apigenin was detected in all the 8 honey samples. Quercetin was also found in the honey samples except in citrus honey. Hesperetin was detected in citrus honey. The quercetin (MW 302.236) peak being RT = 6.715 and peaks in negative ionization and positive ionization were 208.05 and 209.55, respectively which was identified in all the honey samples by the LC-MS of total scan PDA. The same peak with a retention time of 3.3 in all the eight honeys were identified as apigenin (MW 270.24) whereby negative ionization and positive ionization were 268.95 and 270.65, respectively. In honey number 4 (citrus honey), only hesperetin was detected in negative ionization and positive ionization which were 301 and 303.08, respectively, showing at RT = 5.578. Generally, the linear range of (APCI) LCMS is usually very wide, hence is useful in analyzing large numbers of phenolics. The advantage of (APCI) LCMS is the fast ionization method for analyzing phenolics with high efficiency . APCI have emerged as a highly useful LCMS method.

Figure 3.32 shows the peaks when standards were injected and the eluting peaks were showing front tailing besides the overlapping observed. However, the gradient system showed reasonable resolution for some of the standards and some overlap between apigenin and hesperetin. The problem of the co-eluting peaks was not possible to solve, although different gradient systems and wavelenghts were used. However, since hesperetin is found at very low concentration in honey samples, therefore it is expected to have negligible interference in the assay of apigenin at 340 nm.

The analysis did not show any specific compounds that can be used as markers for determination of the botanical origins of these different types of Iranian honey. However, the benefit of APCI-LCMS is the fast ionization method of analyzing phenolics with high

efficiency .

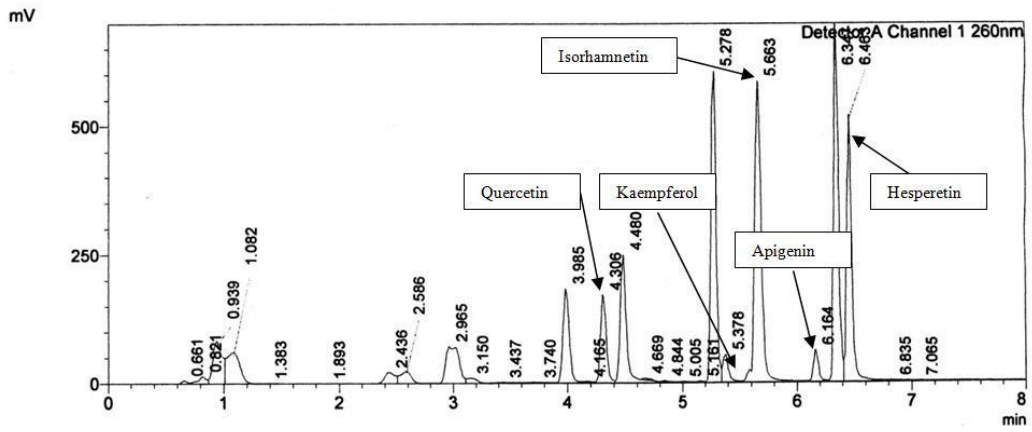

Figure 3.30: Mixed standards chromatogram detected at 210 nm

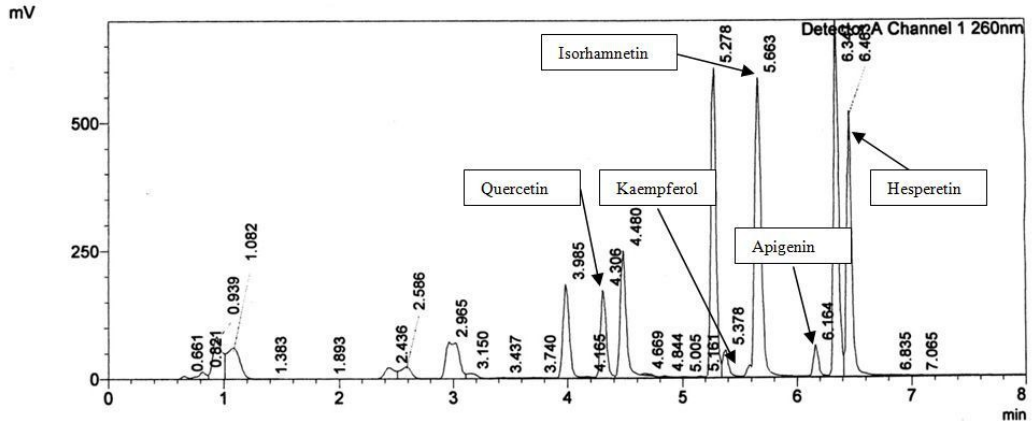

Figure 3.31: Mixed standard chromatogram detected at 260 nm

Table 3.17: APCI LCMS of phenolic standards used in this analysis.

No	Name	Formula	Exact Mass	Positive M/Z	Negative M/Z	RT
1	Catechin	$C_{15}H_{14}O_6$	290.079038	291.0767	289.0615	0.255
2	Epicatechin	$C_{15}H_{14}O_6$	290.079038	291.0767	289.0619	0.300
3	Chlorogenic acid	$C_{16}H_{18}O_9$	354.095082	355.1003	353.0800	0.302
4	Gallic acid	$C_7H_6O_5$	170.021523	ND	169.0062	0.450
5	Coumaric acid	$C_9H_8O_3$	164.047344	ND	163.0366	0.647
6	Ellagic acid	$C_{14}H_6O_8$	302.006267	303.0100	ND	0.683
7	Ferulic acid	$C_{10}H_{10}O_4$	194.057909	ND	193.0467	1.038
8	Morin hydrate	$C_{15}H_{10}O_7$	302.042656	303.0412	301.0244	1.968
9	Eriodictyol	$C_{15}H_{12}O_6$	288.063388	289.0652	287.0510	2.103
10	Quercetin	$C_{15}H_{10}O_7$	302.042653	303.0449	301.0258	2.492
11	Naringenin	$C_{15}H_{12}O_5$	272.2548	273.0670	271.0558	3.095
12	Apigenin	$C_{15}H_{10}O_5$	270.052823	271.0578	269.0446	3.343
13	Kaempferol	$C_{15}H_{10}O_6$	286.0477738	287.0533	285.0420	3.468
14	Hesperetin	$C_{16}H_{14}O_6$	302.079038	303.0810	301.0627	3.632
15	Chrysin	$C_{15}H_{10}O_4$	254.057909	255.0623	253.0476	5.278
16	Galangin	$C_{15}H_{10}O_5$	270.052823	271.0588	269.0442	5.613

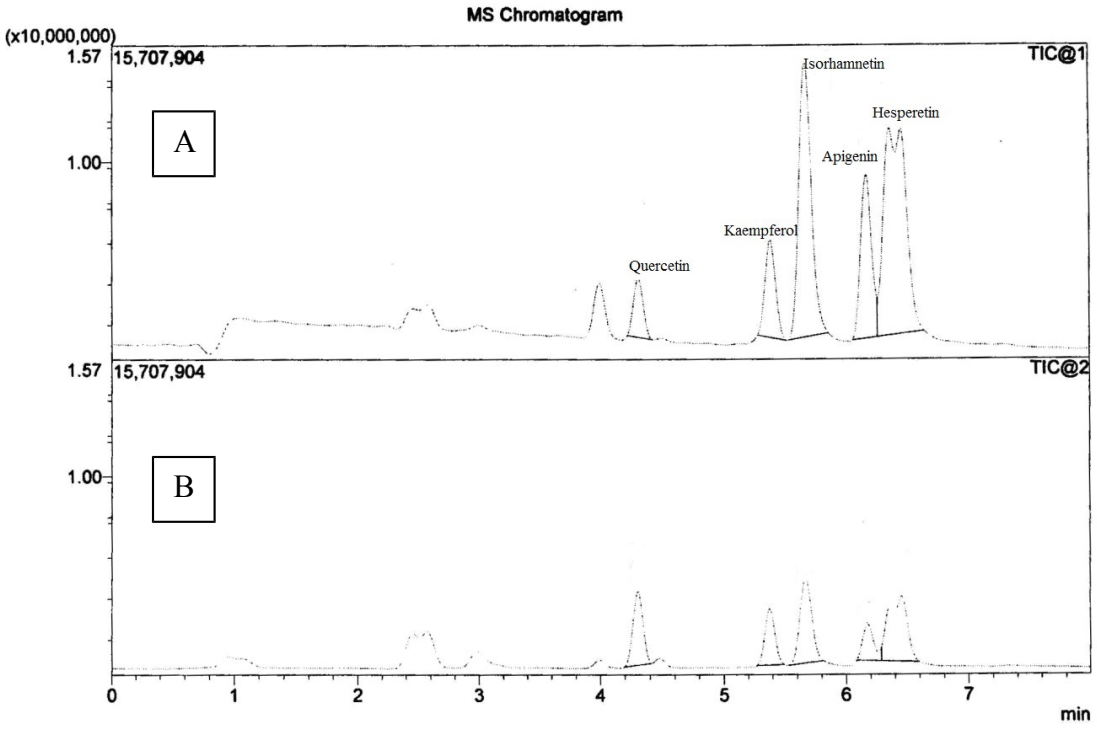

Figure 3.32 : Chromatogram (TIC) of polyphenol standard mix measured at a wavelength of 340 nm (A) and 290 nm (B).

3.5.4 Identification of polyphenols in honey using electrospray ionization LC-MS

(LCMS-ESI)

The 8 honey samples were diluted with acidified water (pH 2), and analyzed using LCMS-ESI in positive and negative ionization under the same chromatographic conditions. Each compound was identified by LC-MS comparisons with the standards and by comparing its mass with those of the authentic standard. These results can provide good references of LCMS data in honey and can be used to analyze phenolics as the new data base library in the LCMS. Mass spectrometry technology is used to determine the identities and abundances of the phenolic compounds in complex sample such as honey. In combination with liquid chromatography, consequently, a deeper knowledge of phenolic profiles can be obtained and this is crucial for the development of new LCMS data base in analyzing honey phenolics. The data sets obtained from an LCMS experiment are large and highly complex. The outcome of such an experiment is called an LCMS profile. The profile is a collection of total scan photodiode array (PDA), Ultraviolet chromatography (UV) spectrum and electrospray ionization-mass spectra (ESI-MS). Standard components that were identified by UV spectrum and their positive and negative ionization are listed in Table 3.18. As it can be seen in Table 3.19 describes the conditions of electrospray ionization-mass spectrometry (ESI-MS).

Three phenolic acids were identified in saffron (Figure 3.33), polyfloral (Figure 3.39) and vulgare honey (Figure 3.35); they were gallic acid, chlorogenic acid and ferrulic acid. Two phenolic acids were identified in berberis (Figure 3.34) and citrus honey (Figure 3.36); they were gallic acid and chlorogenic acid. Two phenolic acids were identified in thyme (Figure 3.37), sunflower (Figure 3.40) and tamarix honey (Figure 3.38); they were gallic acid, chlorogenic acid and ellagic acid.

Three flavonoids were identified in saffron honey; they were rutin, kaempferol and

hesperetin. Two flavonoids were identified in berberis and polyfloral honey; they were rutin and apigenin. Two flavonoids were identified in vulgare honey; they were quercetin and apigenin. Three flavonoids were identified in citrus honey; they were rutin, apigenin and quercetin. Four flavonoids were identified in thyme honey; they were rutin, apigenin, kaempferol and quercetin. One flavonoid was identified in tamarix honey; it was apigenin.

Table 3.18: LCMS-ESI profiles of phenolic standards used in this study.

No	Name	Formula	Exact Mass	Positive M/Z	Negative M/Z	RT
1	Catechin	$C_{15}H_{14}O_6$	290.079038	291.0767	289.0615	7.5
2	Epicatechin	$C_{15}H_{14}O_6$	290.079038	291.0767	289.0619	6.5
3	Chlorogenic acid	$C_{16}H_{18}O_9$	354.095082	355.1003	353.0800	6.2
4	Gallic acid	$C_7H_6O_5$	170.021523	ND	169.0062	4.9
5	Coumaric acid	$C_9H_8O_3$	164.047344	ND	163.0366	12.2
6	Ellagic acid	$C_{14}H_6O_8$	302.006267	303.0100	ND	20.8
7	Ferulic acid	$C_{10}H_{10}O_4$	194.057909	ND	193.0467	13.4
8	Morin hydrate	$C_{15}H_{10}O_7$	302.042656	303.0412	301.0244	15.3
9	Eriodictyol	$C_{15}H_{12}O_6$	288.063388	289.0652	287.0510	25.8
10	Quercetin	$C_{15}H_{10}O_7$	302.042653	303.0449	301.0258	32.2
11	Naringenin	$C_{15}H_{12}O_5$	272.2548	273.0670	271.0558	31.1
12	Apigenin	$C_{15}H_{10}O_5$	270.052823	271.0578	269.0446	33.4
13	Kaempferol	$C_{15}H_{10}O_6$	286.0477738	287.0533	285.0420	31.4
14	Hesperetin	$C_{16}H_{14}O_6$	302.079038	303.0810	301.0627	27.4
15	Chrysin	$C_{15}H_{10}O_4$	254.057909	255.0623	253.0476	32.3
16	Galangin	$C_{15}H_{10}O_5$	270.052823	271.0588	269.0442	31.8

Table 3.19: Electrospray ionisation (ESI-MS) parameters used in this study.

Parameter	measure
Capillary	5 V
Cone	35 V
Extractor	2.5 V
temperature	350 °C

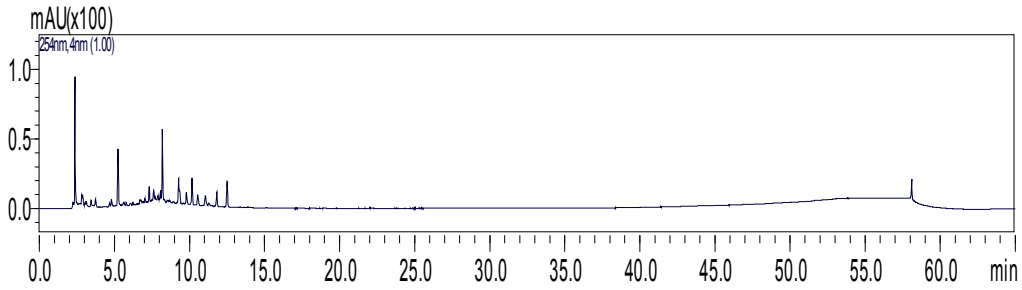

Figure 3.33: UV Chromatography of saffron Honey

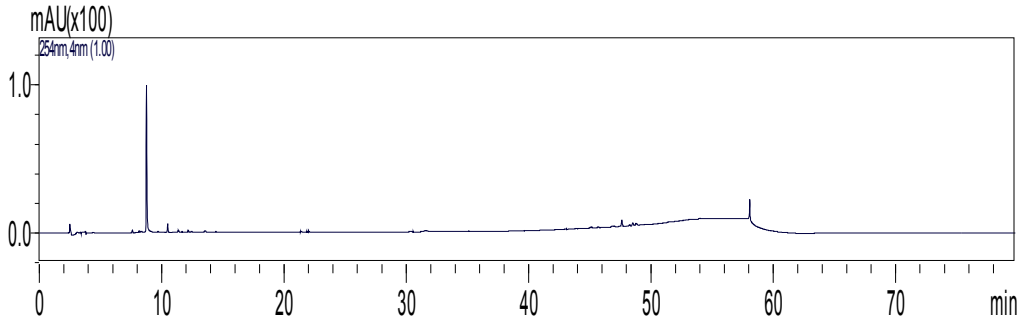

Figure 3.34: UV Chromatography of berberis Honey

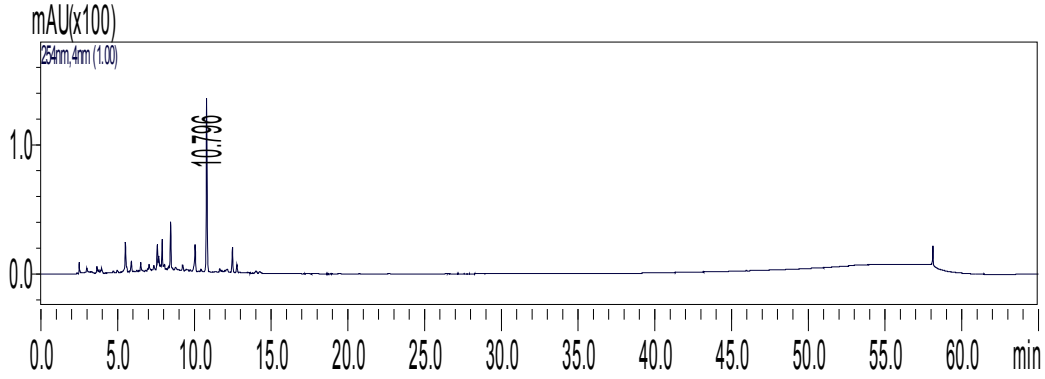

Figure 3.35: UV Chromatography of vulgare Honey

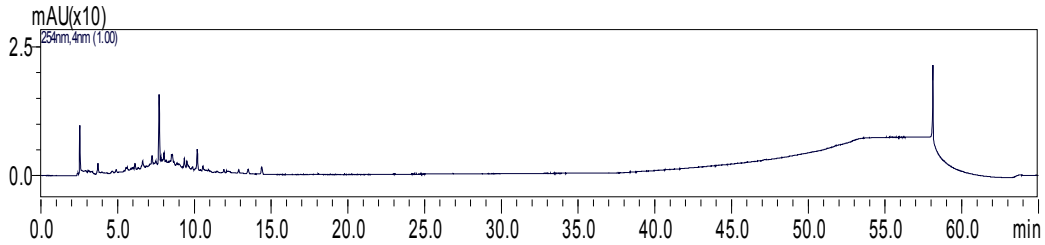

Figure 3.36: UV Chromatography of citrus Honey

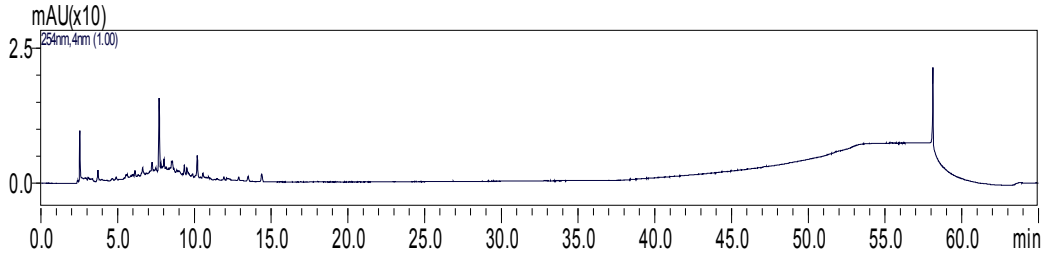

Figure 3.37: UV Chromatography of thyme Honey

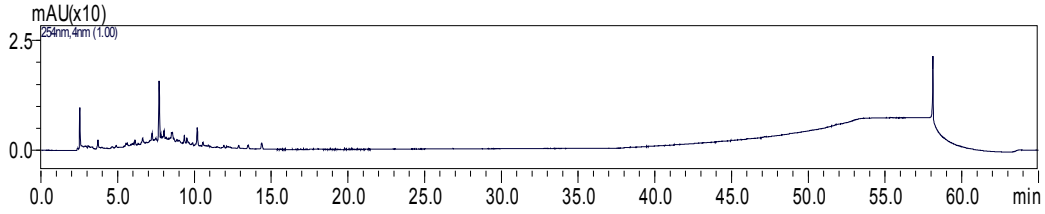

Figure 3.38: UV Chromatography of tamarix Honey

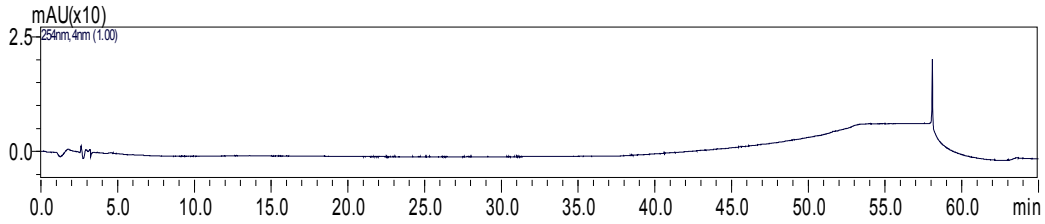

Figure 3.39: UV Chromatography of polyfloral Honey

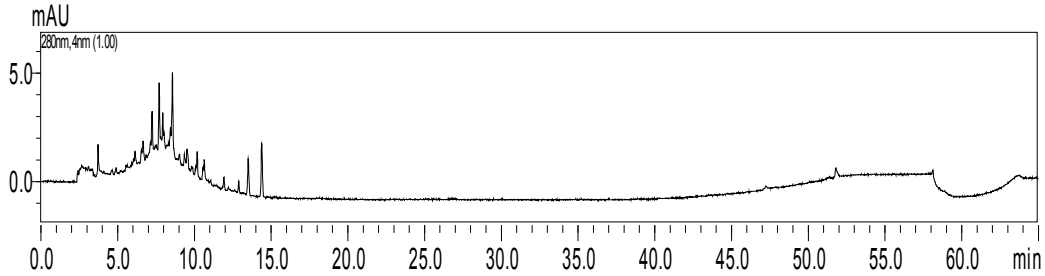

Figure 3.40: UV Chromatography of sunflower Honey

The present study has established that LCMS-DAD-ESI is an influential tool for the classification and quantification of flavonoids in a complex medium like honey . Several flavonoids were detected in different types of Iranian honeys. Flavonoids do not have nitrogen atoms and have low basicity in the liquid phase. For this reason the configuration of protonated molecules in positive-ion mode is lower than in negative ESI. ESI appear to be favored more than APCI, and with methanol and acetonitrile as the mobile phases, it

provides the best sensitivity. The mass spectra in negative ESI were conquered by deprotonated molecules in negative-ion mode and these ions were selected for collision-activated dissociation studies of the precursor ions. The declustering potential (DP) and collision energy (CE), measured as fragmentor voltage value, were optimized in infusion mode for each compound in the range from 400 to 10V and 130 to 5V, respectively. The voltage required for significant fragmentation was compound dependent parameter (Table 3.19).

3.5.5 Identification of polyphenols in honey using time of flight mass spectrometer (TOF–MS)

The exact mass and retention time of 18 phenolic standards were investigated in this study (Table 3.20). Figure 3.41 shows total ion chromatograms (TIC) for 18 phenolic standards as listed in Table 3.15. Study of ion suppression was used as a method of detection; it was known that this ionization source (ESI) may undergo loss of sensitivity owing to the presence of other compounds that co-elute with the analytes of interest, a phenomenon known as ion suppression . Moreover, this effect might be more pronounced when large sample volumes were injected. Here, we studied the possible effect of ion suppression on the signals of the analytes when 500 μL of honey sample was injected into the LC-MS/MS system. To accomplish this, the TIC were recorded (Figure 3.41) for a standard aqueous sample at a concentration of 20 ng g−1 in methanol and for a honey sample at a concentration of 20 ng g−1 in UHQ water using the proposed LC-MS/MS positive and negative methodology. This honey sample was also directly injected into the LC-MSMS/MS positive and negative system. It may be observed that the total signal of the analytes in the standard in UHQ water and in the honey injected was similar both in shape

and in intensity . However, when fortified honey was injected directly into the LC-MS/MS system, the signal of the analytes underwent a strong decrease owing to ion suppression. Tandem MS analysis of phenolics needs to be further studied, in order to improve its suitability, while it fits very well with flavonoid detection .The results obtained should be further confirmed through the analysis of more extended samplings.

Table 3.20: ITTOF of phenolics standards used in this study.

NO	NAME	FORMULA	EXACT MASS	POSITIVE m/z	NEGATIVE m/z	RT
1	Catechin	$C_{15}H_{14}O_6$	290.079038	291.0767	289.0615	25.2
2	Epicatechin	$C_{15}H_{14}O_6$	290.079038	291.0767	289.0619	22.6
3	Chlorogenic acid	$C_{16}H_{18}O_9$	354.095082	355.1003	353.0800	7.3
4	Gallic acid	$C_7H_6O_5$	170.021523	172.0158	169.0062	4.1
5	Coumaric acid	$C_9H_8O_3$	164.047344	166.0247	163.0366	10.4
6	Ellagic acid	$C_{14}H_6O_8$	302.006267	303.0100	301.0317	9.2
7	Ferulic acid	$C_{10}H_{10}O_4$	194.057909	196.0317	193.0467	14.5
8	Morin hydrate	$C_{15}H_{10}O_7$	302.042656	303.0412	301.0244	14.6
9	Eriodictyol	$C_{15}H_{12}O_6$	288.063388	289.0652	287.0510	19.2
10	Quercetin	$C_{15}H_{10}O_7$	302.042653	303.0449	301.0258	9.7
11	Naringenin	$C_{15}H_{12}O_5$	272.2548	273.0670	271.0558	17.3
12	Apigenin	$C_{15}H_{10}O_5$	270.052823	271.0578	269.0446	12.1
13	Kaempferol	$C_{15}H_{10}O_6$	286.0477738	287.0533	285.0420	12.7
14	Hesperetin	$C_{16}H_{14}O_6$	302.079038	303.0810	301.0627	13.5
15	Chrysin	$C_{15}H_{10}O_4$	254.057909	255.0623	253.0476	23.8
16	Galangin	$C_{15}H_{10}O_5$	270.052823	271.0588	269.0442	26.4
17	Rutin	$C_{27}H_{30}O_{16}$	610	612.0380	609.0264	6.99
18	Pinocembrin	$C_{15}H_{12}O_4$	256.25	258.0341	255.0716	26

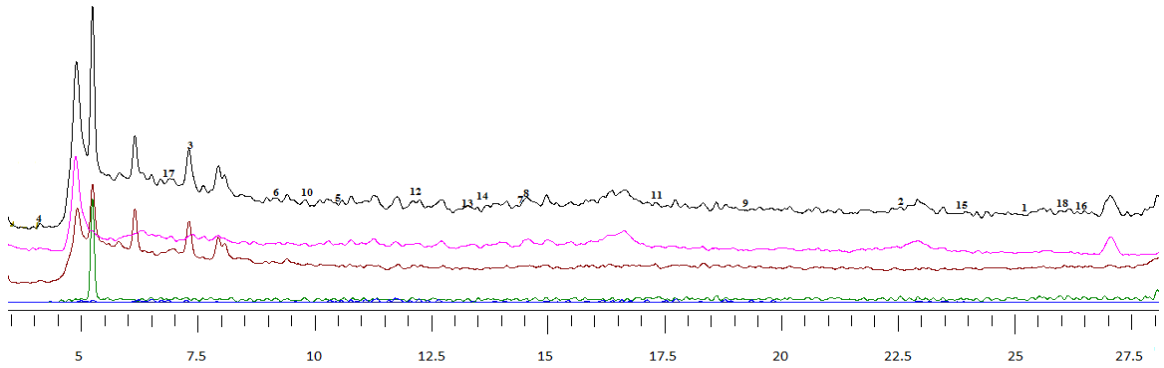

Figure 3.41: Chromatograms of the phenolic standard solution detected by mass spectral total ion current (1= Catechin, 2= Epicatechin, 3= Chlorogenic acid, 4= Coumaric acid, 5= Coumaric acid, 6= Ellagic acid, 7= Ferulic acid, 8= Morin hydrate, 9= Eriodictyol, 10= Quercetin, 11= Naringenin, 12= Apigenin, 13= Kaempferol, 14= Hesperetin, 15= Chrysin, 16= Galangin, 17= Rutin and 18= Pinocembrin)

The flavonoids galangin, naringenin and quercetin TIC, MIC, MSMS positive and MSMS negative in all honey samples analyzed. Identification of galangin, naringenin and quercetin were obtained by comparing total scan photodiode array (PDA), Ultraviolet chromatography spectra (UV) and electrospray ionization-mass spectrometry (ESI-MS) with TIC chromatogram and tandem MS (MS/MS) spectrum .

Figures 3.42-3.49 show the chromatograms of the 8 honey samples. The chromatograms were obtained using two mass TIC spectrums. It was indicated that most of the honeys had similar, but quantitatively different, phenolic profiles. The Iranian honeys were investigated in different wavelengths which were UV spectrum in 340 nm and UV spectrum 290 nm . Differences in the flavonoid contents were seen among different types of honeys. The main flavonoids detected in all of the honey samples analysed was apigenin, on the basis of its retention time, m/z, UV spectrum and the new profile data which was

made as new data base. Additional MS/MS positive and negative analysis produces the typical ion honey sample of honeys.

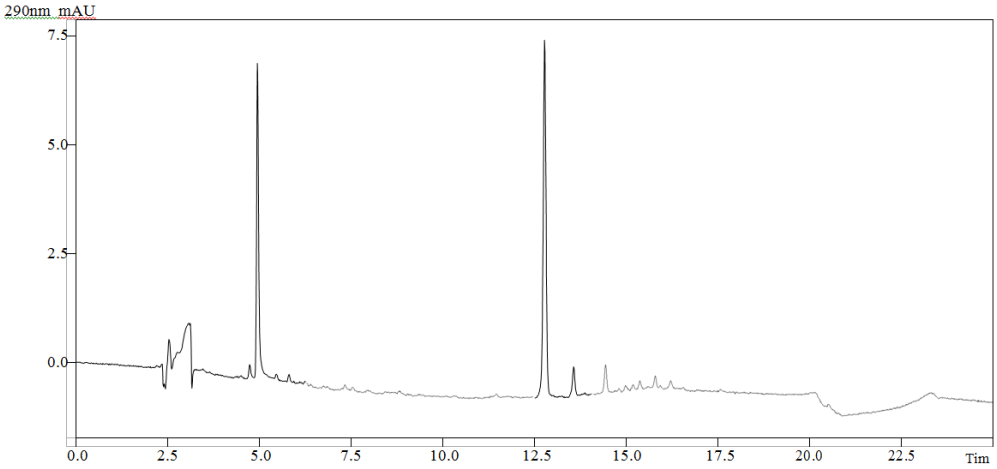

Figure 3.42: UV chromatography of saffron honey using TOF

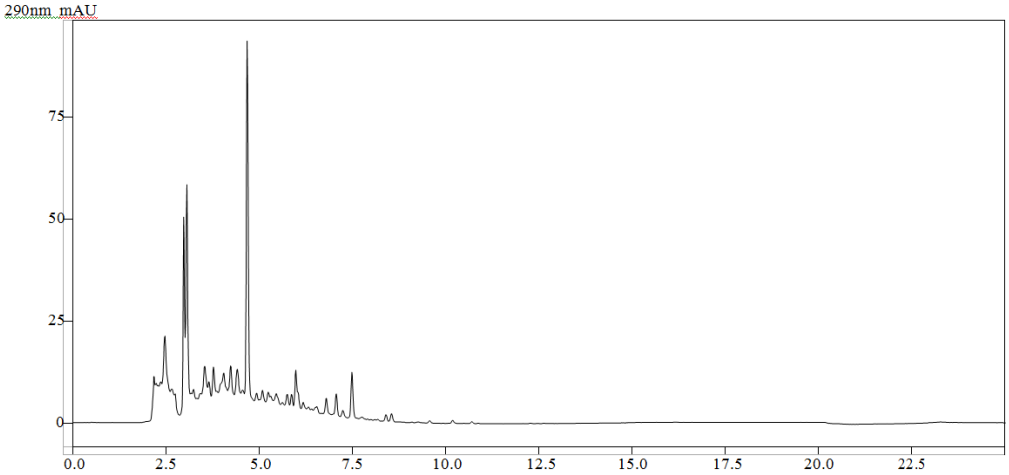

Figure 3.43: UV chromatography of berberis honey using TOF

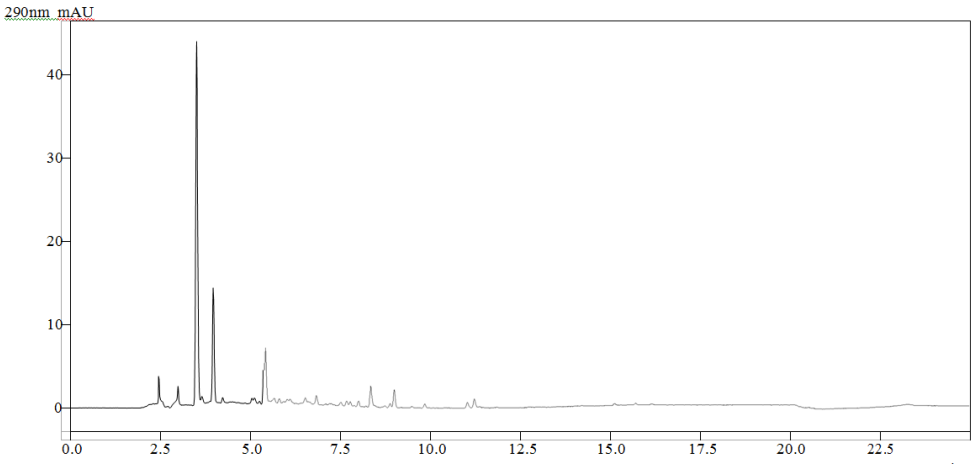

Figure 3.44: UV chromatography of vulgare honey using TOF

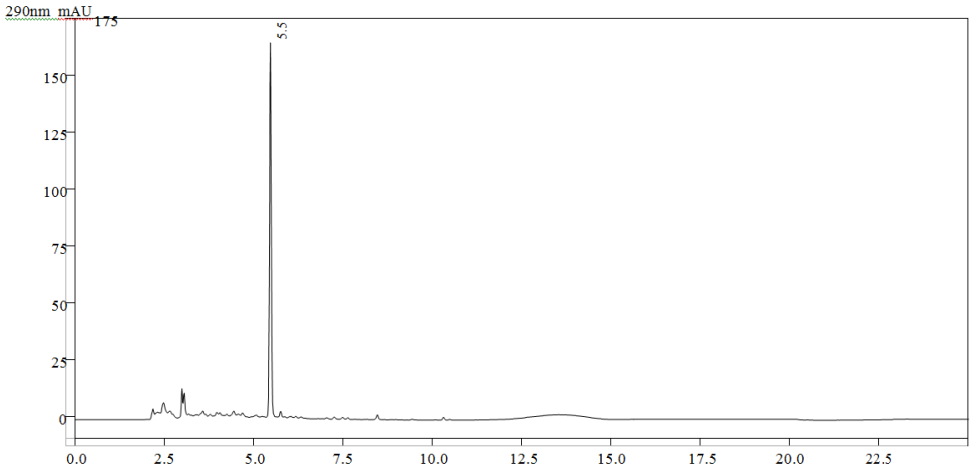

Figure 3.45: UV chromatography of citrus honey using TOF

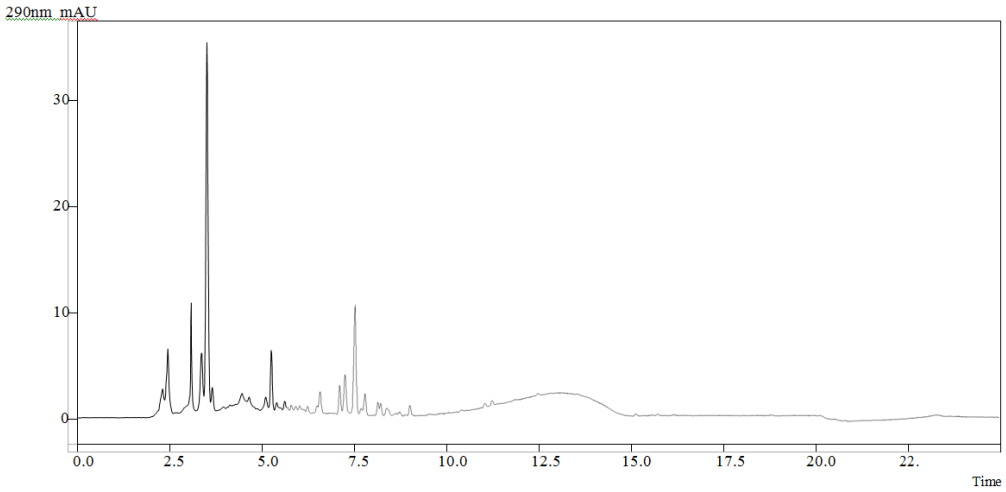

Figure 3.46: UV chromatography of thyme honey using TOF

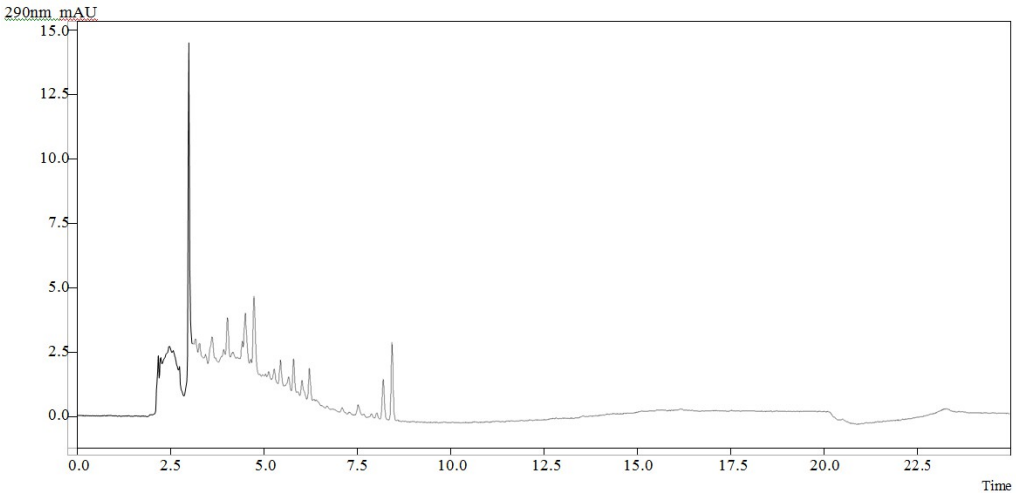

Figure 3.47: UV chromatography of tamarix honey using TOF

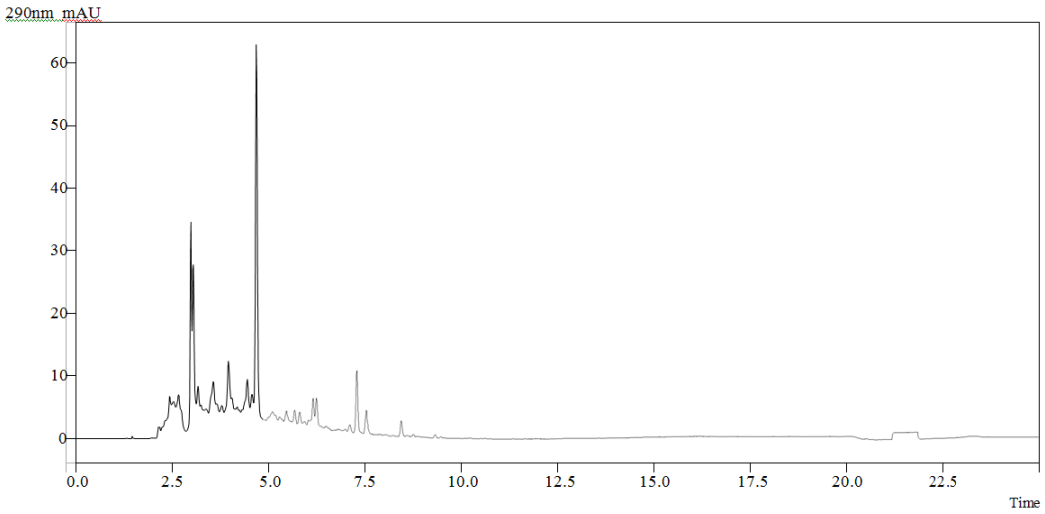

Figure 3.48: UV chromatography of polyfloral honey using TOF

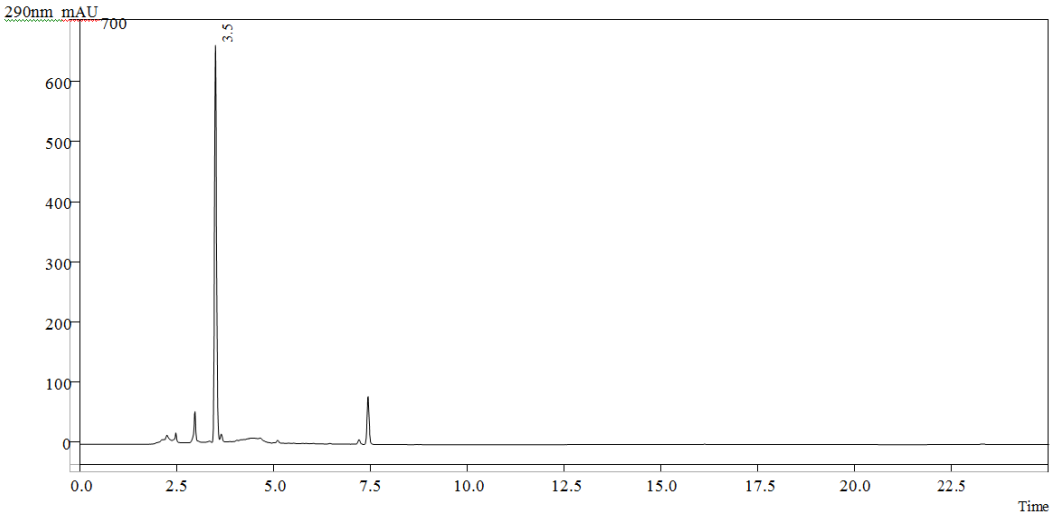

Figure 3.49: UV chromatography of sunflower honey using TOF

This present work provides a preliminary report highlighting the potential use of combined MS spectra in LCMS-TOF fingerprinting of Iranian honeys. The use of tandem MSMS spectra provides an important tool in the identification of the isolated compounds. The results revealed the applicability of this method in chemometrics studies of phenolics of honey. The flavonoids detected in the honey samples were quercetin, apigenin and kaempferol. The flavonoid quercetin, which originates from nectar, and apigenin were seen in all of the honey samples while kaempferol was identified in only one honey sample.

This study indicated that ITTOF is the best instrument for analysis of phenolics in honey. LC/MS ESI is a better method than LC/MS APCI to identify flavonoids in complex matrix such as honey. Several flavonoids and five phenolic acids were found in different types of honey. However, the results obtained do not support possible markers to confirm the type of honey and further analyses and optimization are needed. It would be necessary to analyse a bigger number of honey samples, with the inclusion of more phenolic standards, in order to establish biomarkers for confirmation of honey types . Therefore, to determine the phenolic profile of honey, it would be necessary to study other phytochemicals such as phenolic acids and flavonoids, followed by the same statistical approach to multivariate analysis . Study of honey extract ion mass spectra of MS and fragment ions made in the source was simplified by the use of elevated cone voltages. The test of product ion mass spectra at high mass resolution allowed definite determination of the important composition of fragment ions. Classification of the fragmentation patterns for positive [M+H] and negative [M−H] ions of phenolic compounds are currently utilised to facilitate investigation of honey flavonoids.

3.5.5.1 Combination studies on the botanical origin of honey and TOF–MS

In this study, the phenolic content of Iranian honeys was investigated using SPE, HPLC, GCMS, LCMS (APCI and ESI) and IT-TOF analysis. Table 3.21 summarises the phenolic compounds detected in the Iranian honey samples using the different instrumentation.

Flavonoid markers i.e. quercetin and apigenin were found in sunflower honey. Apigenin and hesperetin found in the citrus honey can be markers of the floral origin.

This is the first time that saffron honey is characterized as a new type of honey. Flavonoid markers rutin and phenolic acid marker gallic acid were found in saffron honey after liquid-liquid extraction with ethyl acetate and solid phase extraction. The gallic acid and rutin as bioactive markers present in saffron honey are found in high quantities in comparison to other phenolics.

Table 3.21: Comparisons among the different methods for analyses of phenolics in the Iranian honeys.

Honey	**HPLC**	**GCMS**	**LCMS-APCI**	**LCMS-ESI**		**LCMS-TOF**
	Flavonoid	**Phenolic acid**	**Flavonoid**	**Phenolic acid**	**Flavonoid**	
Saffron	apigenin, quercetin	gallic acid, ferrulic acid, chlorogenic acid,	apigenin, quercetin	gallic acid, chlorogenic acid, ferrulic acid	rutin, kaempferol, hesperetin	ND
Berberis	apigenin, quercetin	gallic acid, chlorogenic acid	apigenin, quercetin	gallic acid, chlorogenic acid	rutin, apigenin	ND
Vulgare	apigenin, quercetin	gallic acid, chlorogenic acid, ellagic acid	apigenin, quercetin	gallic acid, chlorogenic acid, ferrulic acid	quercetin, apigenin	ND
Citrus	apigenin, quercetin	gallic acid, chlorogenic acid, ellagic acid	apigenin, hesperetin	gallic acid, chlorogenic acid	rutin, apigenin, quercetin	ND
Thyme	apigenin, quercetin, kaempferol	gallic acid, chlorogenic acid, ellagic acid	apigenin, quercetin	gallic acid, chlorogenic acid, ellagic acid	rutin, apigenin, kaempferol, quercetin	ND
Tamarix	apigenin, quercetin	gallic acid, chlorogenic acid, ellagic acid	apigenin, quercetin	gallic acid, chlorogenic acid, ellagic acid	apigenin	ND
Polyfloral	apigenin, quercetin	gallic acid, chlorogenic acid,	apigenin, quercetin	gallic acid, chlorogenic acid, ferrulic acid	rutin, apigenin	ND
Sunflower	apigenin, quercetin	gallic acid, chlorogenic acid, ellagic acid	apigenin, quercetin	gallic acid, chlorogenic acid, ellagic acid	-	ND

CHAPTER 4

CONCLUSION

Medicinal herbs and organic products instead of chemical substances have been studied for their potential in curing different diseases and for preventing the formation of oxidants in food preservation process. Honey as one of these organic and herbal products has received much attention and has variety of uses in medicinal food industry.

Honey, a complex natural tonic, can be used as natural food with antioxidant contents which improves and maintains human and animal health. The biochemical analysis of honey shows that it contains sugars, proteins, moisture, vitamins, minerals, hydroxymethylfurfural, enzymes, phenolics and volatile compounds. Honey composition varies from one country to another. Geographically, Iran is located in a part of the world where multi climates exist in a season which provides bees with various flowers belonging to different seasons. Moreover, mountainous areas rich in medicinal herbs provides ideal environment for producing honey of high quality. Most phenolic compounds discovered in honey possess antioxidant activities . This in turn has led to the exploration of the use of honey in diseases known to involve free radicals such as cancer . There is growing support that food phenolic compounds play a role in preventing cancer .

The study investigates the physicochemical parameters of eight Iranian honey samples with different botanical origins. Different values of pH, moisture and sugar content were observed in these eight types of Iranian honeys. The Iranian honeys contain different carbohydrate's structures. They contain more maltose and less sucrose in comparison to other type of honeys.

The total phenolic content of eight types of Iranian honeys was estimated by a colorimetric method based on the Folin-Ciocalteau reagent. The ferric reducing/antioxidant power (FRAP) assay and the spectrophotometric DPPH (2, 2-Diphenyl-1-Picrylhydrazyl) radical scavenging assay were used for the measurement of the antioxidant activity of the samples. The antioxidant property of the honey samples is mainly due to their phenolic constituents. Moreover, the antioxidant activity

varies, mainly depending on its floral source, phenolic contents, harvest climatic area and environmental conditions. Among these factors, honey phenolics are found to be responsible for most of the antioxidant activities even though their amount is very low in certain honeys. The antioxidant activities of phenolic compounds are mostly attributed to their redox properties. These redox properties can play an important role in quenching and neutralizing free radicals or decomposing peroxides and thus providing protection against various diseases .

A mongst the eight honeys analyzed in this study; the phenolic content, total antioxidant power and radical scavenging activity of saffron honey was found to be highest and showed a dose-response relationship. To analyze the correlation between the antioxidant activity of honeys and their phenolic content, the values for the total antioxidant power of honeys were plotted against the values of their total phenolic content. A significant correlation was found between the total antioxidant power of honeys and their total phenolic content, indicating the role of the phenolic compounds on the antioxidant activity of the honeys. Since different plants contain different phenolic compounds and show variation in their total phenolic content, the significant variation between honeys in their total phenolic contents is due to the variation in their floral sources.

Honey phenolics were extracted and recovered base on hydrolysis and ethyl acetate liquid-liquid extraction, together with solid phase extractiony using isolute C_{18} columns, which was used for the first time to recover Iranian honey phenolics. The recovery of phenolic compounds in honey extracts ranges over 25% to 85%.

The separation and quantification of phenolics were carried out. The characterization of phenolics depends on the method of identification. Chromatographic methods were developed for characterization of phenolics in the honey samples. The most important classes of antioxidant polyphenols are flavanone, flavone, flavan,flavonols and phenolic acids. The phenolic acids that were detected in the Iranian honeys included gallic, ellagic, ferulic, and chlorogenic acids. HPLC

was used in this study for the identification of honey phenolics and characterization of flavonoids. Although there is a similarity between the HPLC chromatograms of all honeys tested, quantitative and qualitative differences were also shown. The identification and quantification of quercetin, kaempferol and apigenin were carried out successfully using HPLC. Ten flavonoids were selected in this study. The initial separation of the flavonoids were performed by HPLC. The eluted phenolic compounds were detected at 280 nm because most of the phenolic compounds showed reasonably high absorbance at this value among different wavelengths. HPLC is an efficient instrument for detecting flavonoids in honey whereas GCMS is more suitable for identifying phenolic acids. Phenolic acids including gallic acid, ferulic acid, chlorogenic acid and ellagic acid were identified in the honey samples using GCMS. The results revealed the applicability of this approach in targeted chemometrics studies of phenolics of honey samples.

Mass spectrometric methods illustrate an enormous promise for the characterization of the honey phenolic compounds. LC-MS/MS techniques have been employed for the identification and quantification of phenolic compounds. The linear range for APCI- LCMS is very wide, and is useful in analyzing large numbers of phenolics. The present study has established that ESI-MS was an influential tool for the classification and quantification of flavonoids in honey. This study showed that ITTOF is the best instrument for analysis of phenolics in honey.

This is the first time that saffron honey marker is known. Gallic acid and rutin as bioactive markers present in saffron honey are found in high quantities in comparison to other phenolics. Available literature indicates that no previous studies on antioxidant property have been done on Iranian honeys. This is the first study to evaluate the selected Iranian honeys and may provide useful data for their potential medicinal uses.

Addition of honey to animal feed may provide beneficial effects on weight gain of animals and food conversion efficiency. There was no significant difference in feed intake, weight gain, feed to gain ratio, gastrointestinal tract, abdominal fat, liver, spleen and pancreas in the animal model.

Future work related to this research project should focus on the isolation of new phenolic compounds. Confirmation of the fragmentation pattern with high resolution GCTOFMS should also be done. The purified phenolics should be tested for potential antioxidant properties.

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