Comparative Assessment of Selected Metals, Polyphenols and Antioxidant Potential of Various Types of Honey

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2017

Comparative Assessment of Selected Metals, Polyphenols and Antioxidant Potential of Various Types of *Honey*

A dissertation submitted in partial fulfilment of the requirements for the degree of

Master of Philosophy

In

Analytical/Inorganic Chemistry

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In the name of Allah,

The most Beneficent,

The most Merciful

Dedicated to

My Mother, Father and Siblings

The most strengthening part of my life, whom sincere devotion stood aside me in my whole life.

DECLARATION

This is to certify that this dissertation entitled "**Comparative Assessment of Selected Metals, Polyphenols and Antioxidant Potential of Various Types of Honey"** *by* **Adnan Khan** *is accepted in its present form by the Department of Chemistry, Quaid-i-Azam University, Islamabad, Pakistan as satisfying the dissertation requirements for the degree of* **Master of Philosophy** *in* **Analytical/ Inorganic Chemistry**.

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ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to my supervisor *Dr. Munir Hussain Shah* for his invaluable direction, time, guidelines, encouragement and provision of scholarly articles in making this research work possible and also helping to boost my morale in developing positive attitude towards research. I am indebted to all respected teachers at Department of Chemistry for their guidance and encouragements. I also am thankful to lab attendant and support staff. Profound thanks are due to my entire course mates especially Saba Ghaffar and Kalsoom Bibi for helping in Lab work. Finally, my deepest appreciation and gratitude is reserved for Yasir Karim Khan for his hard working during metal analysis.

Adnan Khan

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ABSTRACT

Branded honey samples from local markets and unbranded honey samples from local vendors and beekeepers were collected. The honey samples were processed for metal analysis using flame atomic absorption spectrometer. Digestion of honey samples was done according to the reported method for complete digestion. Honey samples were processed to find out the essential and toxic metals along with their phytochemical contents and the antioxidant properties. For phytochemical contents and the antioxidant properties, honey samples were extracted using methanol. Total polyphenols and flavonoids were determined along with the DPPH radical scavenging activity was also evaluated. Among major essential metals, K showed highest concentration in all the honey samples, followed by Na and Ca whereas in case of minor essential metals Fe showed highest concentration on average basis. Most of the trace metals showed random distribution in honey samples. Very strong and significant correlations were observed in most of the cases and quartile distribution pattern showed that metals were more dispersed and asymmetrically distributed in honeys. Among phytochemicals, methanol extracts of most of the honey samples showed exceedingly high polyphenols and flavonoid contents and random pattern in both the cases was observed. DPPH scavenging activity also showed very high values for honey samples; most of the varieties showed values higher than 70%. Health risk assessment for selected metals in honey showed that hazard quotient and cancer risk indicated values much below the limit values (unity and 1×10^{-4}, respectively) indicating no long term non-carcinogenic as well as carcinogenic health risks are associated with the consumption of these honeys.

Chapter 1

INTRODUCTION

Honey is the sweet food produced by honey bees from the sugary secretion of plants or insects living on plants. The nectar which bees collect is subjected to further process of dehydration and enzymatic activity in honey comb, until fully ripen and mature (Codex Standard for Honey, 2001). Honey contain more than 200 substances (Escuredo *et al*., 2013) and composed of largely carbohydrates, water, and other materials such as proteins, organic acids, vitamins, various minerals, pigments, phenolic compounds, volatile compounds and solid suspensions (Alqarni *et al*., 2012; Ciulu *et al*., 2011; Pontes *et al*., 2007). Honey is mixture of different carbohydrates, such as fructose (27.3–44.3%), glucose (22.0– 40.8%), maltose (2.7–16.0%), sucrose (1.5–3.0%) and high sugars (0.1–8.5%) (Poul, 2009). Sugar composition of honey varies mostly with plant nectar and geographical origin, and also with environment, processing and storage (Escuredo *et al*., 2014; Tornuk *et al*., 2013).

Honey is one of the most essential human foods. It has therapeutic, nutritive and disease protective potential due to its chemical composition (Kohler and Mwangi, 2012). In order to have a useful effect, honey must be free of any contaminants (Ruschioni *et al*., 2013). Honeybees are exposed to the pollutants during their foraging activities; they can be contaminated via food resources when gathering pollen and nectar from flowers, or through water (Ruschioni *et al*., 2013). The metals are collected through the roots system and distributed in the whole plant including the nectar and pollen. The amount of metal in the plant increases with the amount of metal in the soil. Also, in regions where air contain large amount of Pb, it may fall on the plant and stick to the surfaces of pollen. Bees can collect the polluted pollen and nectar. It is, therefore, necessary to analyse honey to make sure that it is free of pollutants such as heavy metals (Kohler and Mwangi, 2012)**.**

1.1 Physicochemical Properties of Honey

1.1.1 Sugars

The carbohydrates are main components of honey, which are about 95% based on dry weight. The glucose and fructose comprise of about 65–85% of the total soluble solids (De La Fuente *et al*., 2011). According to the standards of the Codex Alimentarius

Committee on Sugars, the minimum amount of reducing sugars is 60 g/100 g for floral honey (Codex Standard for Honey, 2001). In addition to reducing sugars, sucrose amount is a very important parameter in evaluating the honeys' maturity and identifying any improper treatment of honey. High quantity of sucrose may show a variety of adulterations, such as adding sweeteners like cane sugar or refined beet sugar. In case of early harvest, the sucrose is not completely converted into glucose and fructose (Escuredo *et al*., 2014; Tornuk *et al*., 2013). Due to these factors the Codex Alimentarius Committee on Sugars specifies a maximum value of 5 g of total sugar in 100 g of floral honey (Codex Standard for Honey, 2001).

1.1.2 Free Acidity and pH

Free acidity is an important parameter regarding the quality of honey. It is due to the presence of organic acids and some ions such as phosphates, sulphates and chlorides. Similarly, pH is an important parameter because it affects quality, taste, stability and shelf life of honey (Terrab *et al*., 2003). Acidity of honey is due to the presence of naturally occurring organic acids, but higher acidity may propose the fermentation of honey, and the resulting alcohols can be transformed to organic acid. Other factors such as floral source and season also affect the acidity (Ojeda De Rodriguez *et al*., 2004). Codex Standard (2001) stipulates that acidity should be 50 mEq/kg or less. Acidity, increases the antioxidant activity of honey and decreases microorganism's growth (Cavia *et al*., 2007).

1.1.3 Colour

Colour is the first eye-catching attribute of honey from commercialization point of view. It is an important parameter regarding the honey quality, acceptance and preference of consumers (Boussaid *et al*., 2014). Honeys range from pale yellow through dark red to almost black (Aubert and Gonnet, 1983), depending on mineral content and pollen colour (González-Miret *et al*., 2005), as well as temperature and storage conditions. Colour intensity is related to pigments (e.g., carotenoids, flavonoids), which show antioxidant properties (Frankel *et al*., 1998) and health benefits.

1.1.4 Electrical Conductivity

Electrical conductivity of honey is due to the presence of mineral salts, organic acids, and proteins. It shows the variation according to floral origin, allowing differentiation between blossom honey and honeydews (Terrab *et al*., 2002; Bogdanov,

2009). The electrical conductivity for nectar honey should be less than 0.08 S/cm, with few exceptions (Codex Standard, 2001). Electrical conductivity is also an important parameter for determining the origins of honeys and has replaced the ash content in international standards (Mateo and Bosch-Reig, 1998; Codex Standard, 2001).

1.1.5 Moisture and Water Activity

The moisture content is one of the important characteristics of honey. It affects the physical properties such as viscosity and crystallization, as well as colour, flavour, taste, specific gravity, solubility and preservation (Escuredo *et al*., 2013). Water content of honey vary depending on factors such as season, maturity, climate (Conti, 2000; Finola *et al*., 2007) and conditions during collection and processing (Acquarone *et al*., 2007). However, it affects the shelf life of honey because high moisture can cause objectionable fermentation during storage (Saxena *et al*., 2010; Al *et al*., 2009). Honeys with 18–20% moisture content are considered mature and stable (Codex Standard, 2001; EU, 2001).

1.1.6 5-Hydroxymethylfurfural

5-Hydroxymethylfurfural (5-HMF) content is used as good indicator of honey deterioration (Tornuk *et al*., 2013). Normally 5-HMF is formed as a result of monosaccharide decomposition or the Maillard reaction, when honey is heated or stored for a long time. As the heat treatment and the storage time increase, the concentration of 5-HMF increases extensively. However, along with excess heat treatment, other factors can affect the levels of 5-HMF, such as the sugar profile, presence of organic acids, pH, moisture content, water activity and the floral source. Therefore, the 5-HMF content is sign of overheating or poor storage conditions. In addition, 5-HMF can also be formed at low temperatures, even under acidic conditions, from subsequent dehydration reactions of sugars (Barra *et al*., 2010; Castro-Vázquez*et al*., 2007; Tornuk *et al*., 2013; Wang *et al*., 2009). The standards established by the Codex Alimentarius Committee on Sugars recommend a maximum value of 40.0 mg/kg for the processed honey and a maximum value of 80.0 mg/kg for the topical honey (Codex Standard, 2001).

1.1.7 Diastase Activity

Diastases are the enzymes naturally present in honey. Diastase content depends on both floral and geographical origins of the honey. They are heat sensitive and show overheating of the product and the degree of preservation (Ahmed *et al*., 2013). The

diastatic activity can be used as an indicator of honey maturity and excessive heating because the diastatic activity may decrease during storage or when the product is heated above 60°C (Yücel and Sultanoglu, 2013). The diastatic activity is related to the activity of the enzyme present in 1 g of honey, which can hydrolyse 0.01 g of starch in 1 h at 40°C, expressed as the diastase number in Göthe units (Ahmed *et al*., 2013). The current law requires a minimum value of 8.0 Göthe units. However, honeys with naturally lower diastase activity tolerate a minimum of 3 Göthe units if honeys have up to 15 mg/kg of 5-HMF (Codex Standard for Honey, 2001). The content of diastase activity in honey may vary depending on the age of the bees, the nectar collection period, and the physiological period of the colony. The large quantity of nectar flow and its sugar content will lower the enzyme content and pollen utilization (Oddo *et al*., 1999).

1.2 Health Benefits of Honey

The harmful effects of engineered medications and chemicals on human health in the age of technology have supported the utilization of more conventional and natural methods (Can *et al*., 2015). Research suggests that honey has useful properties in human health development that depend on a great extent on the flower source. These properties may be due to high osmolality, antibacterial properties and antioxidant capacity of honey (Alvarez-Suarez *et al*., 2010). Some of the well-established health benefits of honey are discussed below.

1.2.1 Anti-microbial Activity

Generally, honey reduces the growth of micro-organisms and fungi. It has well known antibacterial effect mostly against gram-positive bacteria. The antimicrobial effect of honey is due to different materials and depends on the botanical origin of honey. The low water contents of honey slow down bacterial growth. Honey glucose oxidase produces the antibacterial agent hydrogen peroxide. There are many non-peroxide antibacterial substances, e.g. aromatic acids, phenolics and flavonoids. The low pH can also be factor responsible for the antibacterial activity of honey. The peroxide activity in comparison to non-peroxide one can be destroyed by heat, light and storage. These factors affect the antibacterial activity of blossom honey more than honeydew honey. Thus, for optimum antibacterial activity, honey should be kept in a cool, dark place (Bogdanov *et al*., 2008).

1.2.2 Anti-inflammatory Effects

Honey has been used mostly for topical treatment of diseases as a medicine to cure infected wounds and inflammations. The primary focus of wound treatment therapy is to kill the contagious microbes present in the wound and to remove any dead tissue that may provide an environment which can favour the growth of microorganisms. Inflammation not only makes the wound painful and difficult to control, but also inhibits repairing of wound through the healing processes. Honey rapidly cleans the wound and infected area by removing the dead tissue and destroying the bacteria, thus reducing the inflammation, in addition to stimulating cells and tissues involved in the production of new tissue to repair the wound and infected tissue (Hadagali and Chua, 2014).

1.2.3 Anti-tumour Activity

Multifactorial processes are involved in honeys' antitumor activity such as, cytotoxicity of H_2O_2 (Bang *et al*., 2003) inhibiting directly the activities of COX-1 and COX-2 by some specific constituent caffeic acid penyl ethyl ester (CAPE) a phenolic antioxidant (Michaluart *et al*., 1999) and scavenging action against different reactive oxygen species (ROS) which stimulate of the inflammatory burst which is ultimate cause of cancer (Greten *et al*., 2004).

1.2.4 Wound Healing

Honey has properties that are supposed to help the healing process. It is acidic with a pH ranging from 3.2 to 4.5, which serves to stall growth of microbes as the majority thrives at a pH between 7.2 and 7.4. Higher sugar contents extract water from the wound, decreasing the water accessibility to microorganisms, which further inhibits microbial growth. Honey contains glucose-oxidase enzyme that stimulates the production of hydrogen peroxide in body tissue, which has a sterilizing impact. Honey encourages an increase in lymphocytes and phagocytes and helps monocytes to discharge cytokines and interleukins, consequently invigorating the healing process. In addition to encouraging faster healing, honey has additionally been appeared to avert the need for suturing, remove the malodour of wounds, diminish irritation and reduce scarring (Bardy *et al*., 2008).

1.2.5 Source of Important Minerals

Honey contains different amount of minerals which vary from 0.02 g/ 100 g to 1.03 g/100 g, with potassium being the most abundant element approximately one-third of

the total mineral content. Macro-elements, such as potassium, calcium, sodium, and trace metals, such as, iron, copper, zinc, and manganese, play a significant role in biological systems. These elements are involved in physiological reactions, general metabolism, germination, circulatory systems and control reproduction as catalysts of various biochemical reactions (Alqarni *et al*., 2014).

1.3 Phytochemicals in Honey

Phytochemicals are the plant nutrients protecting it against microbial infections or other pathogens. The mostly they are not required by the human body for sustaining life (Rao, 2012). Photochemical utilization decreases the risk of several types of chronic diseases owing to their antioxidant and free radical scavenging activity (Sung and Lee, 2010; Soobrattee *et al*., 2005). Polyphenols are of plant source and major constituent of our food. The key nutritional sources of polyphenols are the fruits including apple, grape, pear, cherry, and various berries containing up to 200–300 mg polyphenols per 100 g fresh weight. The total dietary intake is about 1 g/day. Polyphenols are classified into flavonoids and phenolic acids. Flavonoids are further divided into several classes including flavones, flavonols, flavanones, isoflavones, proanthocyanidins, and anthocyanins (Scalbert *et al*., 2005). Natural polyphenols are the most abundant antioxidants, and their antiradical activities are due to replacement of hydroxyl groups in the aromatic rings of phenolics (Zhang *et al*., 2015). Honey contains more than 150 polyphenolic compounds, with flavonoids, flavonols, phenolic acids, catechins, and cinnamic acid derivatives are of prime importance (Ferreira *et al*., 2009). The natural antioxidants, particularly flavonoids, show a broad array of biological properties, such as antibacterial, anti-inflammatory, anti-allergic, anti-thrombotic, and vasodilatory actions (Al-Mamarya *et al*., 2002).

1.4 Antioxidant Potential of Honey

An antioxidant is ‘any substance that, when present at low concentrations compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate’. The term ‘oxidizable substrate’ including proteins, lipids, carbohydrates and DNA found in living cell (Barry Halliwell, 1995). Various studies have reported that oxidative damage is responsible for diseases such as cancer, coronary, and neurological deterioration. Therapeutic action of honey is related with antioxidant capacity against reactive oxygen species (ROS). Therefore, recent studies have mostly focused on the

composition of honeys and their biological and therapeutic properties (Chua *et al*., 2013). It has been established that the components present in honey having antioxidant properties include phenolic acids, flavonoids, some enzymes (glucose oxidase and catalase), ascorbic acid, protein and carotenoid (Alvarez-Suarez *et al*., 2010). Gheldof *et al*., (2002) showed a significant correlation between total phenolic content and oxygen radical absorbance capacity (ORAC) of the honeys. ORAC values of honey were in the same range as ORAC values of many fruits and vegetables that are (3-17 µmol TE/g) for honey and (0.5-19 µmol TE/g fresh weight) for fruit and vegetable. These results indicate that antioxidant potential of honey is comparable to the fruits and vegetables on a fresh weight basis. Honeys from various floral sources exhibit a wide range of antioxidant activities, and a linear correlation with honey colour has been observed (Frankel *et al*., 1998). Frankel *et al*., (1998) established an important correlation between colour and antioxidant capacity of honey and showed that darker coloured honeys have higher antioxidant content. Extensive data are available for honeys' antioxidant properties which support that the bioactivities of honeys depend on botanical and geographical origin and varies in honey from different botanical sources (Al *et al*., 2009; Al-Waili *et al*., 2013). For example, radical scavenging activity of natural Pakistani honeys measured by DPPH assay was in the range of 30.50 to 77.43% (Noor *et al*., 2014). Radical scavenging activity of Romanian honey samples was from 35.80 to 64.83% (Al *et al*., 2009). Similarly, the DPPH radical scavenging activity of rhododendron honeys from Turkey varies from 36.11 to 90.73% (Silici *et al*., 2010). Al-Waili *et al*., (2003) studied the effect of honey consumption on antioxidative capacity of blood and reported that honey ingestion has increased the antioxidant agents of blood vitamin-C by 47%, b-carotene by 3%, uric acid by 12% and glutathione reductase by 7%.

1.5 Metal Constituents in Honey and their Health Impacts

The mineral content in honey varies from 0.04% in pale to 0.2% in dark honeys (Anklam, 1998). The most abundant metal in honey is K, which accounts for one third of the total mineral content (Alqarni, 2013). Honey contains macro and micro elements vital for health. These elements are derived from soil and become part of nectar through plant's root system and then honey. As a result, the concentration of elements in honey will be different depending on the composition of soil (Stankovska *et al*., 2008). Honey may contain conceivably harmful species, such as toxic metals (Cd, Pb). Such defilements may be due to external sources such as industrial pollution, discharge from factories, leaded

petrol or by faulty measures during the honey processing and storing. The acidic nature of honey may also cause the release of metals such as Cr, Pb and Zn from metallic tools or containers (Pisani *et al*., 2008).

1.5.1 Potassium

Potassium (K) is an essential macro nutrient and it is the abundant cation in intracellular fluid and takes part in acid-base balance, control of osmotic pressure, transmission of nerve impulses, muscle contraction, cell membrane function (Soetan *et al*., 2013). The significance of potassium to human health has been well documented and new studies continue to highlight its positive effects and potential in public health. High potassium intake decreases the blood pressure. Hypertension is considered leading cause of cardiovascular diseases (Houston and Harper 2008). Increased dietary potassium ingestion, may decrease other cardiovascular risks such as risk of stroke and coronary artery disease. Recent study suggests that consumption of 1,640 mg of potassium every day reduce risk of stroke by 21% (He and MacGregor, 2001). A positive relationship has been observed between high intake of potassium and bone mineral density (BMD) and bone mass (Macdonald *et al*., 2005). There is high risk of developing kidney stones during hypercalciuria. High dietary potassium reduces urinary calcium excretion, and thus reduces the risk of kidney stone formation (Lanham-New *et al*., 2012). Hypokalaemia refers to low level of potassium in blood. Normal blood potassium level is 3.6 to 5.2 mmol/L which is maintained by average food ingestion of 80 to 200 mEq potassium per day in adult. Symptoms associated with hypokalaemia are weakness, paralysis, tetany, gastrointestinal disorders although it is less common (Pohl, 2013).

1.5.2 Sodium

Sodium is essential macro mineral for human health. Normal plasma levels for sodium in adults vary from 136 to 146 mEq/L and an average dietary intake of 90 to 250 mEq per day is needed to keep the balance. Sodium is the major cation within extracellular fluid compartment. The water, sodium, and potassium are in constant movement between intracellular and extracellular space and such movement keeps balance of internal fluid and electrolyte. Potassium and sodium ions are important in the renal regulation of acid-base balance and the transmission of nerve impulses. Hyponatremia is low serum sodium level below normal range (136 mEq/L). It is an electrolytic disorder which can cause, liver failure, heart failure, myocardial infarction, and endocrine changes found mostly in older

patients. Hyponatremia may be either due to dilutional disorders or depletional disorders. Hypernatremia represents high serum sodium level exceeding 146 mEq/L. Various risk factors for hypernatremia include chronic renal failure, recovery phase of acute renal failure, hypocalcaemia, hypokalaemia, and sickle cell anaemia. It is due to abnormal renal excretion of water with insufficient water intake disorders (Pohl, 2013).

1.5.3 Calcium

Calcium is the most abundant element in the body and it accounts for about 2% of bodyweight in adults, most of which is found in the skeleton and teeth while the remaining in soft tissues and body fluids. Calcium is an important constituent for bones and teeth and regulates intracellular functions in most body tissues. Calcium plays an important function in muscle contraction and nerve transmission (Theobald, 2005). Hypercalcaemia is the abnormal increase in the concentration of serum ionized calcium. The most common symptoms due to hypercalcaemia are gastrointestinal disorders, such as nausea, vomiting, constipation and abdominal pain. It can cause nephrogenic diabetes insipidus which lead to acute renal failure (Maziarka and Pasternak, 2013). Hypocalcaemia is low ionized calcium concentration in serum. Mild hypocalcaemia is asymptomatic. The periodontal numbness and carpopedal contraction are due to abrupt changes in ionized calcium. Hypocalcaemia is primary cause of tetany (Maziarka and Pasternak, 2013).

1.5.4 Magnesium

Magnesium is important for variety of physiological functions. The adult human body contains 21–28 g of magnesium, 65% of which is present in the skeleton and 34% in intracellular fluids. A relatively low percentage (1%) is found in the extracellular fluids/blood. Magnesium plays vital role as cofactor in more than 300 metabolic reactions. Deficiency of magnesium may cause many diseases such as heart disease, hypertension, heart failure, sickle cell anaemia, metabolic syndrome, diabetes mellitus type II, insulin resistance, polycystic ovary syndrome, tuberculosis pleurisy, growth retardation, arthritis, Parkinson's, anxiety, epilepsy, multiple sclerosis, obsessive–compulsive disorder, Alzheimer's, depression, preeclampsia and eclampsia, chronic kidney disease, and prostate cancer (Glasdam *et al*., 2016). Mild plasma Mg concentration 3.5–5 mmol/ L has side-effects like diarrhoea, abdominal cramps, vomiting, nausea, and other cardiac arrhythmias, double vision, slurred speech and weakness. At extremely high plasma Mg concentrations muscular paralysis, respiratory and cardiac arrest develop (Saris *et al*., 2000).

1.5.5 Strontium

Strontium is an alkaline earth metals and its important minerals are celestite ($SrSO_4$) and strontianite ($SrCO_3$). The alkaline earth metals form divalent cations in biological fluids, and have different protein binding affinity in biological fluids like serum or plasma. The protein binding capacity of Sr in serum or plasma is similar to Ca (Nielsen, 2004). Strontium is not considered to be an essential element although its metabolism is almost similar to that of calcium. Strontium is an osteotrophic or bone-seeking element and strontium ranelate which act as nutritive agent, has reduced the incidence of fractures in osteoporotic patients (Prejac *et al*., 2017). Strontium prevents Caries and its prevalence is inversely related to strontium concentration in water, plaque, and enamel. However, a high quantity of strontium causes hypocalcaemia due to high renal excretion of Ca (Nielsen 2004). ^{90}Sr is radio-toxic and it has severe consequences for the human body in case of exposure after nuclear accidents or use of atomic weapons due to its bone-seeking properties. This radio-isotope is especially toxic for foetus and new-born, as transfer of strontium can occur through the placenta into the foetus mineralized bone tissue during pregnancy and later by means of the maternal milk into the new-born bones (Momčilović *et al*., 1971).

1.5.6 Iron

Iron is an essential trace metal for synthesis of haemoglobin and it is involved in the production of erythrocytes, oxidation–reduction reactions, and cellular proliferation, whereas excess iron is involved in the production of reactive oxygen species (ROS). Human body contains approximately 3–4 g iron of which two-thirds is red blood cell (RBC) iron and recycled iron by RBC destruction; the remaining is stored in ferritin/hemosiderin, while only 1–2 mg of iron is absorbed in the intestinal tract and circulated in the blood. Iron has the ability to accept and donate electrons readily, interconverting between ferric and ferrous forms. It is a useful constituent of cytochromes, oxygen-binding molecules (haemoglobin and myoglobin), and many enzymes. Conversely, iron catalyses the transfer of hydrogen peroxide to free-radical ions that can damage tissues such as attack on cellular membranes, proteins, and DNA (Andrews, 1999). Anaemia is most evident consequence of iron deficiency. However, other the adverse effects of iron deficiency are metabolic processes, including electron transport, catecholamine metabolism, DNA synthesis, and several enzyme systems (Baynes, 1990).

Other signs and symptoms of iron deficiency include pallor, fatigue, poor exercise tolerance, and decreased work performance. However, in severe cases iron deficiencies directly affect the central nervous system (Andrews, 1999).

1.5.7 Zinc

Zinc is an essential and one of the most common trace mineral in human body. It is indispensable for the growth and development of cells. It is vital for RNA transcription, DNA synthesis, cell division and cell activation (Horecka and Pasternak, 2014). Recently, research suggests Zn to be a functionally crucial constituent of more than 200 enzymes (Wu and Wu, 1987). It play an important role in immune system and its deficiency weakens the immune function and resistance to infection (Walker and black, 2004). High concentrations of Zn cause nausea, abdominal cramping, vomiting, tenesmus and diarrhoea (Maret and Sandstead, 2006). Zinc plays an important role in reproduction; it modulates the synthesis and secretion of luteinizing hormone (LH), follicle stimulating hormone (FSH), gonadal differentiation, growth and maturation of spermatozoa and testicular fertilization. Zinc interferes in copper metabolism and is used in treatment of Wilson's disease (Salgueiro *et al*., 2000). Mortality rate reduced 68% among the infants who received zinc supplement (Sazawal *et al*., 2001) while Baqui *et al*., (2002) reported a 51% reduction in mortality among children who received zinc supplementation.

1.5.8 Copper

Copper is an essential trace element required by human body in small amount. It is found in highest concentrations in the liver in comparison to cells and tissues (Turnlund, 1998). it functions as a cofactor and is part of many important enzymes, including Ceruloplasmin for iron metabolism, cytochrome oxidase for electron transport system, and monoamine oxidase which oxidizes amines to aldehydes for cross linking in collagen formation (Fisher, 1975). Exposure to high concentrations of Cu is harmful. Liver is first target of toxicity, because Cu first accumulates in liver after it enters the blood. Overload of this metal cause liver cirrhosis, haemolysis and damage to renal tubules, brain, and other organs (Gaetke and Chow, 2003).

1.5.9 Cobalt

Cobalt (Co) is an essential element. It is part of cyanocobalamin, an essential vitamin (vitamin B12) whose function is to produce red blood cells (RBCs) and to prevent

pernicious anaemia (Barceloux, 1999). Cobalt was used in treatment of certain type of anaemia historically because of its ability to stimulate haemoglobin and synthesis of RBC (Stokinger, 1962). Abnormalities due to cobalt used in treatment of anaemia are thyroid dysfunction in children and, reversible vision and hearing disorder in adults. Certain individuals, particularly children suffering from sickle cell anaemia and adult patients with renal failure, developed these indications at lower Co doses than other patients on similar Co treatment. The use of Co for treating anaemia was abandoned in 1970s, with availability of more effective drugs (Paustenbach *et al*., 2013). The average daily intake of cobalt from the diet is 5–45 mg/day while the concentration of cobalt is relatively high in fish and vegetables. Liver store the highest concentration of cobalt followed by the kidney (Barceloux, 1999). Exposure to large amount of cobalt may cause gastrointestinal irritation, nausea, vomiting and diarrhoea (Jensen and Tuchsen, 1990).

1.5.10 Manganese

Manganese is a trace element and it is crucial for human beings as it is the part of several important enzymes, such as superoxide dismutase, pyruvate carboxylase and glutamine synthetase (Michalke *et al*., 2007). Its role is to activate enzymes that are needed for food metabolism. It is also required for reproduction and bone growth. Manganese superoxide dismutase, act as antioxidant that protects cells by scavenging the free radical (Leach and Harris, 1997). Wide range of problems due to its deficiency have been reported in laboratory animals including impaired growth, skeletal defects, reduced fertility, birth defects, abnormal glucose tolerance and altered lipid and carbohydrate metabolism (Dobson *et al*., 2004). Inhalation of large amount of Mn may result in neurotoxicity. Welders and smelters are highly susceptible to such toxicity (Keen *et al*., 1999). Ingestion of Mn is less problematic than inhalation, because in later case it is transported directly to the brain before it can be metabolized in the liver (Davis, 1998). The indications of Mn toxicity become visible gradually over a period of months to years. Permanent neurological dysfunctions have similar sign and symptoms as Parkinson's disease, including tremble, difficulty in walking, and facial muscle contraction. The syndrome, often called manganism or manganic madness, has severe psychiatric abnormalities, including hyperirritability, violent act, and hallucinations (Pal *et al*., 1999; Aschner and Aschner, 1991). Additionally, environmental or occupational exposure to Mn can result in lungs inflammation (Han *et al*., 2009).

1.5.11 Chromium

Chromium (Cr) is an essential trace mineral required for normal carbohydrate and lipid metabolism. Low intake of Cr may result in sign and symptoms including high blood glucose, insulin, cholesterol, triglycerides, and decreased high density lipoproteins (HDL). More severe signs of Cr deficiency include neurological disorders (Anderson, 1998). It is a naturally occurring element and it exists in Cr (III) to Cr (VI) oxidation states. In humans and animals, Cr(III) is an essential nutrient while hexavalent chromium Cr (VI) is a toxic industrial pollutant classified as carcinogen by International Agency for Research on Cancer. Chromium (VI) compounds cause nose irritation, stomach and small intestine ulcers, anaemia, sperm and male reproductive system damage. A relationship has been observed between increase in stomach tumours of humans and animals and chromium (VI) in drinking water (Tchounwou *et al*., 2012).

1.5.12 Cadmium

Cadmium is an inorganic toxicant. Both occupational and environmental exposure to cadmium has wide variety of hazardous effects. It has a very long biological half-life and accumulates primarily in the liver and kidney where it is bound to metallothionein, low molecular weight proteins which has high affinity for the metal and detoxify it. The toxicity of cadmium is often due to cadmium intrusion with various zinc mediated metabolic processes, and zinc treatments reduce or stop the toxic effects of cadmium. Both natural and anthropogenic sources of cadmium result in contamination of soils and increase cadmium uptake by crops and vegetables from soil which is then consumed by human. Possible sources of human exposure to cadmium are including occupational exposure, eating contaminated food, smoking cigarettes, and working in cadmium-contaminated work places. Various health effects are related to cadmium toxicity such as lung damage, acute gastrointestinal disorders with vomiting and diarrhoea, and kidney injury. Acute ingestion of Cd can also cause gastrointestinal tract erosion, pulmonary, hepatic or renal injury and coma, depending on the route of poisoning. The IARC has classified Cd as carcinogenic (group-I) based on association between its exposure and lung cancer in both humans and experimental animals (Tchounwou *et al*., 2012).

1.5.13 Lead

Lead is a naturally occurring metal present in small amounts in the earth's crust. Although Pb occurs naturally in the environment, anthropogenic activities are major cause

of higher concentrations. Lead has many different industrial, agricultural and domestic applications. It is used in lead-acid batteries, ammunitions, metal products, and in X-rays shields. Research confirms the adverse effects of Pb in children and the adult population. In children, its poisoning can damage brain, cause behavioural and hearing problem, speech and language disability, growth delay, and poor attention. Symptoms of Pb poisoning in adults are reduces sperm count in men and abortions in women. Acute levels may cause brain damage, kidney failure, and gastrointestinal diseases, while chronic Pb toxicity may have harmful effects on the blood, central nervous system, kidneys, and vitamin D metabolism. IARC has classified it as possibly carcinogenic to human (Tchounwou *et al*., 2012).

1.6 Environmental Aspects of Honey

The importance of biological monitoring as a technique for identifying the environmental pollution has been increased. Honeybees (*Apis mellifera*) have potential in such cases, since during foraging they efficiently collect sample constituents from the forage plants and soil of the surrounding area. They easily adjust to different kind of environments and produce a enough material each season for sampling and analysis (Crane, 1975). The bees' foraging area generally expands over a surface of approximately 7 km^2. Such large foraging area makes the honeybees and their products ideal bio-indicators of environmental pollution (Conti and Botre, 2001). Honeybees come in contact not only with plants and air but also with water and soil while searching for nectar, honeydew, and pollen and plant secretions. Thus, surrounding environmental contaminants also become part of the raw materials collected during this foraging. In this way, contaminants reach honey and change its composition and quality. In fact, mining and steelworks, industrial exhaust and urban dense populated areas or highways near the bee's forage area result in increase of concentrations of certain metals in honey. Thus, honey can be considered as good biomarker for environmental pollution and it can indicate the level of air, water, plant and soil contamination of particular area. Study of trace metals in honey can assist to estimate the environmental quality in different regions and countries (Pohl, 2009).

1.7 Aims and Objectives of the Present Study

The present study is based on measurement and monitoring of selected essential and toxic metals in various honey samples available in local markets of Pakistan. Such study is considered imperative to evaluate the health risk and benefits associated with the consumption of honey which is considered as an important and beneficial food product. Therefore, the present study is designed with following broad objectives:

- To chemically characterize the honey samples for trace metals and polyphenolics.
- To compare the antioxidant potential of different honey samples.
- To evaluate the honey quality regarding its essential and toxic metal contents.
- To find out plausible correlation between phytochemicals, antioxidant and mineral contents.
- To compare the metal contents of honey samples collected from different sources.
- To highlight the possible relationships between the chemical composition of honey and its botanical and/or geographical origin.
- To identify the major sources of the metals in honey.
- To assess the health risk associated with the metal contents in honey.
- To compare present metal levels with those reported from other parts of the world.

Chapter 2

METHODOLOGY

2.1 Sample Collection

Two sets of honey samples were collected in the present study; first set consists of the branded samples collected from the local markets, while the second set was collected from the vender and beekeepers. The details of the honey samples collected during the present study are shown in Table 1. The samples were collected in clean dry plastic or glass bottles and were stored at room temperature. A total of 23 branded and unbranded honey samples were collected and analysed for selected essential and toxic metal contents, phytochemicals and antioxidant activity. Before honey collection, the sampling bottles were soaked in detergent and then soaked in 10% HNO_3 for two to three hours. Afterward they were first rinsed with tap water and then with plentiful distilled water and dried.

Table 1. Description of the honey samples included in the present study

Code	Colour	Made	Origin	Nature	Vegetation
M-101	Brown	Langnese	Germany	Multifloral	Mixed
M-102	Dark Brown	Al-shifa	Saudi Arabia	Multifloral	Mixed
M-103	Dark Brown	Dabour	India	Multifloral	Mixed
M-104	Light Brown	Marhaba	Lahore	Multifloral	Mixed
M-105	Amber	Life Style	Lahore	Multifloral	Mixed
M-106	Dark Brown	Langnese	Germany	Multifloral	Mixed
M-107	Light Amber	Langnese	Germany	Multifloral	Mixed
M-108	Brown	Labbaik	Lahore	Multifloral	Mixed
M-109	Amber	Young's	Karachi	Multifloral	Mixed
M-110	Brown	Swat	Karachi	Multifloral	Mixed
V-201	Brown	Vender	Talagang	Unifloral	*Ziziphus spina-christi*
V-202	Brown	Vender	Jhelum	Unfloral	*Acacia modesta*
V-203	Brown	Vender	Karak	Unifloral	*Acacia modesta*
V-204	Brown	Vender	Abbottabad	Multifloral	*Acacia modesta*
V-205	Brown	Vender	Kalarkahar	Unifloral	*Ziziphus spina-christi*
V-206	Amber	Vender	Murree	Multiifloral	Mixed
V-207	Amber	Vender	Gilgit	Unifloral	*Olea europaea*
V-208	Brown	Vender	Wah Garden	Unfloral	*Acacia modesta*
V-209	Brown	Vender	Karak	Unifloral	*Ziziphus spina-christi*
V-210	Brown	Vender	Changa Manga	Multifloral	*Ziziphus spina-christi*
V-211	Brown	Vender	Bahawalpur	Multifloral	Mixed
V-212	Amber	Vender	Kala Bagh	Multifloral	Mixed
V-213	Amber	Vender	Kaghan	Unifloral	*Juglans regia*

2.2 Sample Processing and Preparation for Metal Analysis

Generally honey is subjected to different mineralization procedures before analysis of the metals by atomic absorption or emission spectrometry. Typically, high temperature, dry ashing or wet-acid digestion have been used to decompose the organic matrix and release metals from the complex sample matrix into the solution, which is suitable for introduction into atomizers and excitation sources. There are a number of methods used for this purpose. Some reported methods for honey digestion are given below:

A microwave digestion method was used to minimize the organic matter prior to analysis. Approximately 0.5 g of the honey was placed in a digestion vessel and 2 mL of concentrated HNO_3 and 2 mL of H_2O_2 (30%, v/v) was added. The vessels was capped, tightened and placed in the oven. The digestion was then carried out with the following digestion program: 500 W for 5 min up to 180°C; 0 W for 2 min; then 500 W for 10 min at 180°C. Ventilation was performed for 20 min after the end of the second step. Finally the vessels were cooled, carefully opened and the contents quantitatively transferred to a 50-ml volumetric flask (Stankovska *et al*., 2008).

One gram (1.0 g) of sample was weighed in porcelain crucible. The furnace temperature was gradually increased from room temperature to 450^oC in 1 hour. The sample was ashed for about 8 hours until a white or grey ash residue was obtained. The residue was dissolved in 5 ml of HNO_3 (25%, v/v) and the mixture was heated slowly to dissolve the residue. The solution was transferred to a 10 mL volumetric flask and made up to the volume. A blank digest was treated the same way **(**Tuzen *et al*., 2007).

Even though, direct analysis of honey samples is performed rarely, it certainly reduces the risk of contamination or loss of analysts due to prolonged treatment of the samples or their incomplete digestion. Sample was dissolved and diluted only with water and the result obtain with this method is as reliable as those with microwave assisted, wet-acid digestions. Evidently, the direct analysis decreases the time of analysis. Before measurement, the sample solutions prepared was sonicated or shaken to improve their uniformity. The solution was filtered to remove beeswax impurities (Pohl, 2009). However, matrix error is the most common problem in such cases. There are many other methods reported for digestion of the honey samples. The digestion method used depends upon type of material, instrument for analysis, accuracy and availability. The method used in the study is described below.

Wet-digestion of honey sample was performed using an oxidizing-acidic mixture of 2:1:0.5, HNO_3: H_2O_2: H_2SO_4. Initially 5.0 g of honey sample was weighed into 100 mL conical flask to which 20 mL Conc. HNO_3 was added and left for 10 minute. Then heated the mixture contents on hot plate at 85°C until 1/3 of the volume reduction. The solution was cooled to room temperature and then 10 mL H_2O_2 was added and heated again. After 10 minute 5 mL Conc. H_2SO_4 was added to the cooled digest and heated until clear solution was obtained. The solution was quantitatively transferred to 50 mL volumetric flask and made up to the mark with 0.1 N HNO_3. A sample blank was processed in the same way with each batch of the samples. The sample solution then analysed on atomic absorption spectrophotometer for the metal concentrations (Tuzen *et al*., 2007).

2.3 Measurements of Selected Metals

Analysis of honey is important regarding the general safety and the significance of honey. Although, determinations of essential and toxic trace metals in honey are necessary for human health and environmental monitoring, but it is difficult and challenging due to the complex organic matrix. Atomic spectrometric methods, including flame atomic absorption spectrometry (FAAS), electrothermal atomic absorption spectrometry (ETAAS) and inductively coupled plasma optical emission spectrometry (ICP-OES) are the important methods in analysing honey for the presence of metals.

The main advantages of FAAS are low operational costs and good analytical performance. Before determination of the metals in honey using FAAS, the samples are usually mineralized in order to avoid potential matrix-related interferences. In this case, aqueous external standard solutions and simple linear regression can be used for calibration and quantification, respectively. Otherwise, multiple linear regression can be used to minimize the effects of interferences in the flame. FAAS is commonly used for quantitative analyses of alkali and alkaline-earth metals as well as transition metals which are the most abundant in honey. The high concentrations of these metals allow the sample solutions to be diluted even 50–500 times to match the dynamic ranges of calibration lines. This practice also minimizes or completely eliminates the possible chemical interferences. In addition to major metals, minor and some trace metals can also be determined by FAAS. The sample solutions are usually not diluted due to low concentrations of these metals. In some cases, the metals are initially enriched before analysis (Poul, 2009). The digested samples was analyzed quantitatively for K, Na, Ca, Mg, Sr, Fe, Zn, Cu, Co, Mn

Cr, Cd and Pb on Flame Atomic Absorption Spectrophotometer (Shimadzu AA-670, Japan), technical features of which are mentioned below.

The Shimadzu atomic absorption spectrophotometer (AA-670, Japan) was used for analysis in the present study. Optimum analytical conditions for the analysis of selected essential and toxic metals on the instrument using air-acetylene flame are shown in Table 2. The salient features of instrument include high speed, dual frequency. It has the capacity to operate automatically in background compensation mode thus correcting all fluctuations in the observed signal arising from sources other than the sample. Other features of the equipment include automatic operation conditions, automatic recording of the data, including the calibration curve and précised data processing to ensure high precision and accuracy. Lamp position, detector gain and beam balance, are all adjusted automatically with ease to exclude deviant data in repeated analytical modes. The digitalized output can be held for transient signals in the memory storage for further reproduction.

Table 2. Optimum analytical conditions for the analysis of selected essential and toxic metals on Shimadzu AA-670 (Japan) using air-acetylene flame

Metal	Wavelength (nm)	HC lamp current (mA)	Slit width (nm)	Fuel-gas flow rate (L/min)	1% Absorption concentration (ppm)
K	766.5	5.0	0.5	1.9	0.04
Na	589.0	6.0	0.5	1.6	0.02
Ca	422.7	6.0	0.5	2.0	0.08
Mg	285.2	4.0	0.5	1.6	0.007
Sr	460.7	4.0	0.5	1.6	0.10
Fe	248.3	8.0	0.2	2.0	0.10
Zn	213.9	4.0	0.5	2.0	0.02
Cu	324.8	3.0	0.5	1.8	0.09
Co	240.7	6.0	0.2	2.2	0.20
Mn	279.5	5.0	0.4	1.9	0.05
Cr	357.9	5.0	0.5	2.6	0.09
Cd	228.8	4.0	0.3	1.8	0.02
Pb	217.0	7.0	0.3	1.8	0.20

2.4 Phytochemical Analysis

2.4.1 Extraction Methodology

The honey samples were extracted for phytochemical and antioxidant assays using reported methods (Beretta *et al*., 2005; Meda *et al*., 2004).). About 5.0 g of honey sample was weighed into conical flask and then dissolved in 20 mL methanol (80%). Solution was then filtered into 50 mL volumetric flask using Whattman filter paper (No. 41). The solution was made up to the mark with methanol and then used for different phytochemical and antioxidant assays.

2.4.2 Total Phenolics Content

The folin-ciocalteu method as proposed by Beretta *et al*., (2005) was used to determine the total phenolic contents. 0.2 mL honey extract was added to 2 mL of 10% folin-ciocalteu reagent and then 2 mL Na_2CO_3 was used. The mixture was vortexed for 5 min. 10% folin-ciocalteu reagent solution was prepared by transferring 10 mL of the reagent into 100 mL volumetric flask and then made the solution up to the mark with methanol. Similarly 6% Na_2CO_3 was prepared by dissolving 6 g of sodium carbonate in 100 mL water. The absorbance was measured at 761 nm after 30 minute incubation in dark. Gallic acid stock solution of 500 ppm was prepared by dissolving 50 mg of Gallic acid in 100 mL of water. Working standards of (0-25 mg/L) were prepared by serial dilution to draw the calibration lines. The absorbance of sample was then compared with the calibration standards. Total phenolic content were expressed as Gallic acid equivalents in mg per 100 g of the product (mg GAE/100 g), on fresh weight basis.

2.4.3 Total Flavonoid Content

Flavonoid contents in honey were estimated by using $AlCl_3$ method ((Meda *et al*., 2004). 5 ml of 2% aluminium chloride in methanol was mixed with same volume of honey extract. 2% aluminium chloride was prepared by dissolving 2 g of aluminium chloride in 100 mL of water. The absorbance was measured at 415 nm after 10 minute keeping the solution in dark. Quercetin solution (100 ppm) was prepared by dissolving 25 mg of quercetin in 250 mL of water. Quercetin standards (0-35 mg/L) were prepared for calibration line. The total flavonoids content were expressed as of quercetin equivalents in mg per 100g of product on fresh weight basis (mg QE/100 g)

2.5 Antioxidant Potential (DPPH Assay)

The scavenging activity of honey samples was evaluated using 2, 2-diphenyl-1-picryhydrazyl (DPPH) radical using method described by Meda *et al*., (2005) with some modification. 1.5 mL of the honey sample (methanolic extract) was added to 3 mL of DPPH solution (0.02 mg/mL in methanol). The mixture was incubated for 15 minute at room temperature and then absorbance was measured at 517 nm. Quercetin 100 ppm solution was prepared by dissolving 25 mg of Quercetin in 250 mL water. Similarly 100 ppm solution of ascorbic acid was prepared by dissolving 10 mg of ascorbic acid in 100 mL of water. Quercetin (0-30 mg/L) and ascorbic acid (0-30 mg/L) were used as standard for calibration line. The radical scavenging activity was calculated using the following equation:

$$\text{DPPH Scavenging Activity (\%)} = (A_B - A_S)/ A_B \times 100$$

where, A_B. = absorbance of DPPH solution without honey

A_S = absorbance of honey sample with DPPH solution

2.6 Statistical Analysis

Statistical analysis was applied to the metals data employing both univariate and multivariate models. Univariate analysis consists of basic statistical parameter, including minimum, maximum, mean, median, standard deviation (SD), standard error (SE), kurtosis, skewness and correlation analysis. They showed the distribution pattern and mutual correlation of trace metal levels. The selected trace metal concentrations were further subjected to multivariate analysis composed of Principal Component Analysis (PCA) and Cluster Analysis (CA). The multivariate methods are used to categorize the link among the measured variables.

Principal component analysis (PCA) is a widely applied multivariate data procedure. Graphical output from the method provides information about structure and the relationships between variables comprising the data set. The main objective of PCA is to reduce the dimensionality of data-sets consisting of a large number of interrelated variables. This reduction is achieved by transforming the original variables into a new set of variables, the principal components (PCs). The PCs are then ordered such that to present in all of the original variables. In this case correlated variables are transformed into a set of uncorrelated variables. Cluster analysis composed of several statistical techniques intended to classifying data by determining underlying data structures or groups For a

given series of observations, the purpose of cluster analysis is to form groups in the data in such way that the observations are as similar as possible within groups, but as dissimilar as possible outside groups (Joliffe, 1986)

2.7 Health Risk Assessment

In this study, the human health risks caused by exposure to the heavy metals were assessed (Guo *et al*., 2016). The health risk index (HRI) of the selected metals through consumption of honey was calculated using following equation ((Yasmeen et al., 2016)):

$$HRI = \Sigma_n(C_n \times Dn)/RfD \times Bw$$

where C_n represent the average metal concentration in honey on fresh weight basis (µg/g), Dn denote the average daily intake rate of fruit in whole year, RfD is the safe level of exposure by oral for life time, Bw is average body weight (70 kg for adult).

Non carcinogenic risk due to selected metals was characterized by target hazard quotient, and hazard index. Target hazard quotient was calculated by following equation:

$$THQ = (EFr \times EDtot \times I \times C \times 10^{-3})/(RfDo \times BWa \times ATn)$$

where EFr is the exposure frequency (350 days/year); EDtot is the exposure duration (30 years), C is the amount of metal in honey (µg/g); BWa is the average body weight (70 Kg, adult) and ATn is the average exposure time in days (EDtot/year × 365 days/years).

A THQ below 1 means the exposed population is safe from harmful effects; whereas a THQ above 1 means that there is a chance of adverse effects, with an increasing probability as the value increases. The hazard index (HI) was used to estimate total non-carcinogenic risks of multiple metals on the assumption of dose additively (Ru *et al*., 2013).

$$HI = HQ1 + HQ_2 + \ldots + HQn$$

The lifetime cancer risk of metal was also computed as suggested by the US EPA (US EPA, 2005):

$$TCR = (EFr \times EDtot \times CPS_o \times I \times C \times 10^{-3}) / (BWa \times ATn)$$

where CPS_o is the carcinogenic potency slope, oral $(\mu g/g/day)^{-1}$, ATc is average time, carcinogens (70 × 365 days). Since CPS_o value is not available for all trace metals, so the TCR of Cd, Cr and Pb were calculated to indicate the life time carcinogenic risk.

Chapter 3

Results and Discussion

3.1 Distribution of Selected Metals in Honey

The distribution of selected essential and toxic metal levels in various honey samples collected during the present study are shown in Table 3, in terms of basic statistical parameters. Most of the metals exhibit broad range in honey samples; their variations were spread over several orders of magnitude as shown by the minimum and maximum levels. On the average basis, highest concentration in honey samples was shown by K (210.3 µg/g), followed by significant contributions of Na (80.62 µg/g), Fe (60.33 µg/g), Co (40.20 µg/g), Cr (38.90) and Pb (27.76 µg/g). However, comparatively lower concentrations were noted for Sr (1.881 µg/g), Cu (3.570 µg/g), Mn (5.611 µg/g) and Cd (7.999 µg/g). Most of the metal measured in the present study exhibited diverse and random distribution as manifested by divergent mean and median levels as well as considerably elevated SD and SE values. Highest dispersion as shown by SD and SE values was noted for K, followed by Na, Co, Cr and Pb. Similarly, majority of the selected metals revealed asymmetric distribution as shown by considerably higher kurtosis and skewness values, which were for highest for Co, Mg and Mn. Nevertheless, some of the metals (Fe, Cr and Sr) revealed relatively symmetric distribution in honey samples. Overall, most of the metals showed non-Gaussian and asymmetric variations in various honey samples analysed in the present study.

Average concentrations of the essential and toxic metals were also compared and the results are shown in Figure 1, for comparative assessment. On the average scale, highest contribution was noted for K while the least contribution was noted for Sr. On comparative basis the average metals levels in various honey samples showed following decreasing order; K > Na > Fe > Co > Cr > Pb > Zn > Ca > Mg > Cd > Mn > Cu > Sr. Similarly, quartile distribution of the essential and toxic metals measured in the honey samples are shown in Figure 2. Most of the metals showed broad dispersion in the honey samples. Among the selected metals, very broad and asymmetric distributions were noted for Mn, Cu, Pb, Co, Na, Ca, Sr and Cr while relatively symmetric variations were fond for K, Mg and Zn. Nonetheless, comparatively narrow distribution and lower variations were observed in case of Fe and Cd in the honey samples.

Table 3. Statistical distribution parameters for selected essential and toxic metal levels (µg/g, fresh weight) in honey

	Min	Max	Mean	Median	SD	SE	Kurtosis	Skewness
K	12.94	760.9	210.3	127.0	211.6	44.12	1.557	1.499
Na	1.996	280.9	80.62	57.87	76.32	16.27	2.272	1.532
Ca	0.790	30.20	12.86	8.317	10.94	2.387	-1.566	0.382
Mg	0.568	38.82	8.985	6.735	8.398	1.751	6.546	2.219
Sr	0.146	5.133	1.881	1.106	1.599	0.377	-0.862	0.722
Fe	10.20	154.1	60.33	55.85	41.45	8.643	0.053	0.824
Zn	2.904	77.97	24.21	18.74	19.54	4.263	1.959	1.467
Cu	0.078	10.37	3.570	3.531	2.508	0.547	1.207	0.947
Co	2.770	216.4	40.20	23.29	53.20	11.34	6.486	2.568
Mn	0.080	26.80	5.611	2.945	6.490	1.451	5.180	2.077
Cr	2.444	88.80	38.90	34.44	24.96	6.053	-0.495	0.545
Cd	1.558	22.07	7.999	6.452	5.859	1.566	0.959	1.036
Pb	0.703	88.63	27.76	20.98	22.61	5.057	1.877	1.361

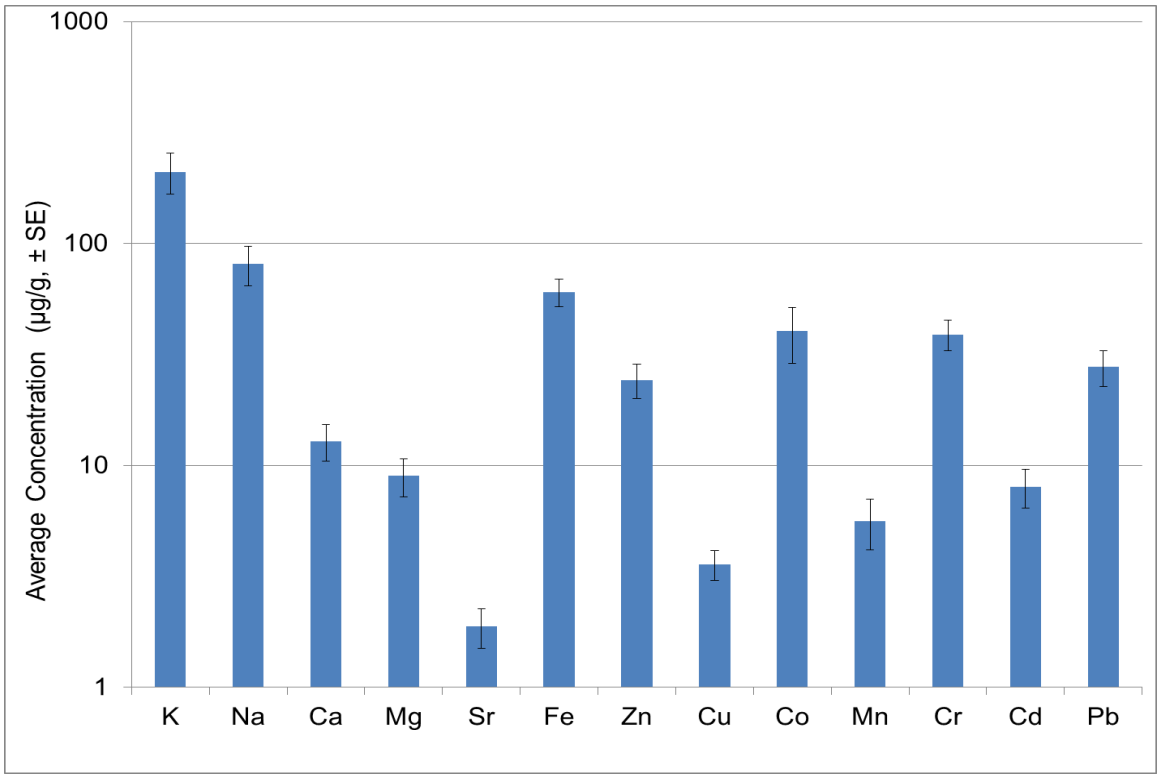

Figure 1. Comparison of the average concentrations of selected essential and toxic metals (µg/g, fresh weight) in honey

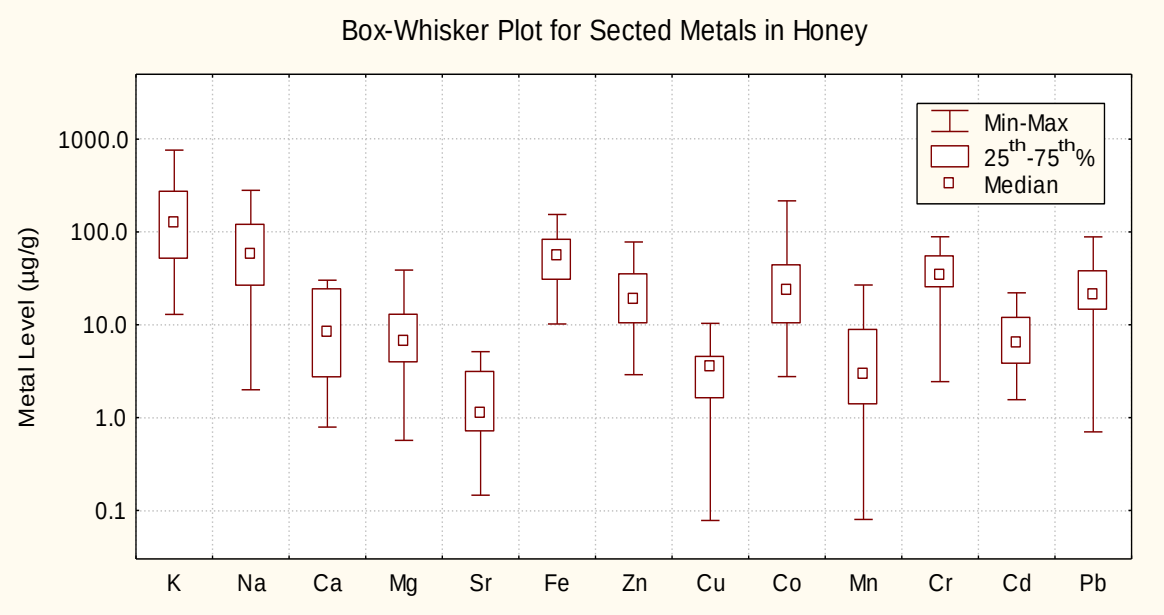

Figure 2. Quartile distribution of selected essential and toxic metal levels (µg/g, fresh weight) in honey

3.2 Distribution of Phytochemical Contents in Honey

Distribution of total phenolic contents (mg GAE/100 g, FW) in the honey samples is shown in Figure 3 for comparative assessment. Mostly random variations were observed for the phenolic contents in honey samples. Highest phenolic contents were observed in the branded honey sample M-102 (325 mg GAE/100 g, FW), followed by another branded sample M-106 (nearly 300 mg GAE/100 g, FW), and some unbranded samples V-205 and V-203 (above 200 mg GAE/100 g, FW). However, the least phenolic contents were found in one of the branded honey sample M-108 (less than 50 mg GAE/100 g, FW). Most of the honey sample (more than 80%) showed significant amount of phenolic contents (> 100 mg GAE/100 g, FW) thus indicating its nutritional significance.

Comparative distribution of the flavonoid contents (mg QE/100 g, FW) in the honey samples is depicted in Figure 4. Most of the hone samples exhibited relatively lower flavonoid contents. Overwhelmingly elevated flavonoid contents were observed in couple of branded honey samples; M-102 (95 mg QE/100 g, FW) and M-106 (83 mg QE/100 g, FW), followed by an unbranded sample V-208 (51 mg QE/100 g, FW), while least contribution of the flavonoids were found in unbranded honey sample V-213 (7 mg QE/100 g, FW) and V-212 (11 mg QE/100 g, FW). Majority of the honey samples exhibited flavonoid contents in the range of 15 to 25 mg QE/100 g (FW). Major contributions of the phenolic and flavonoid contents were found in the same samples.

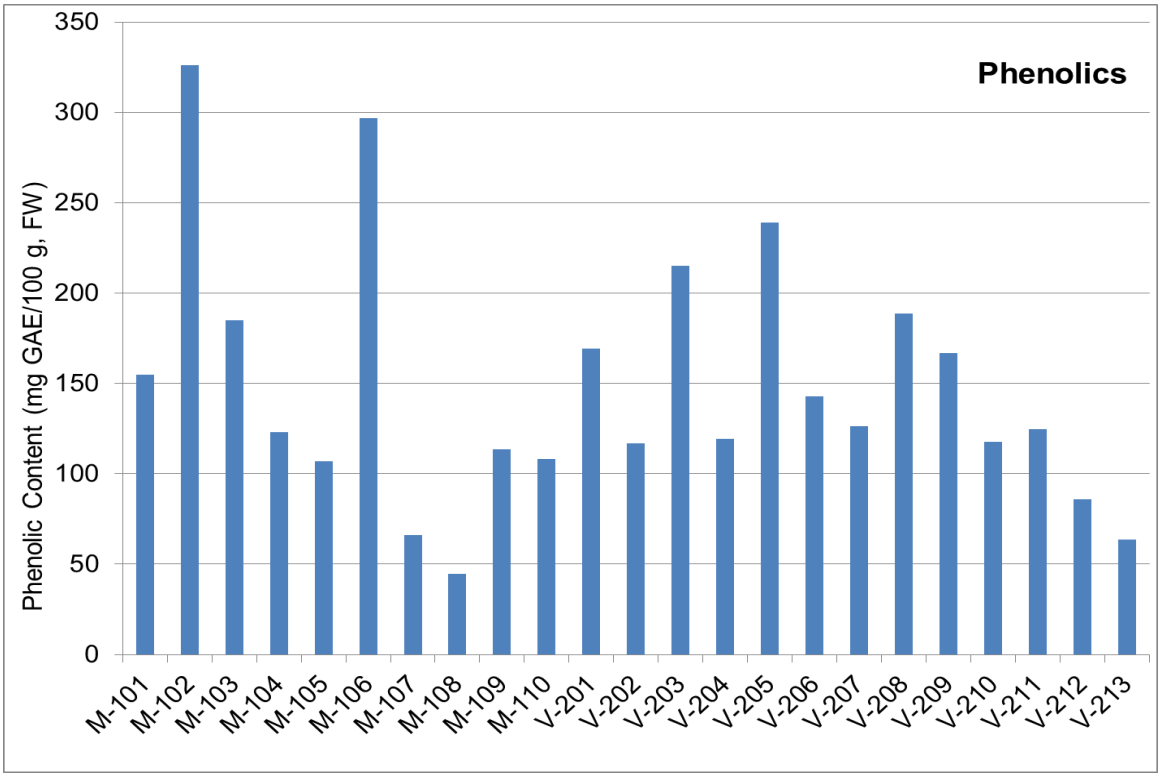

Figure 3. Comparative distribution of the phenolic contents (mg GAE/100 g, FW) in various honey samples

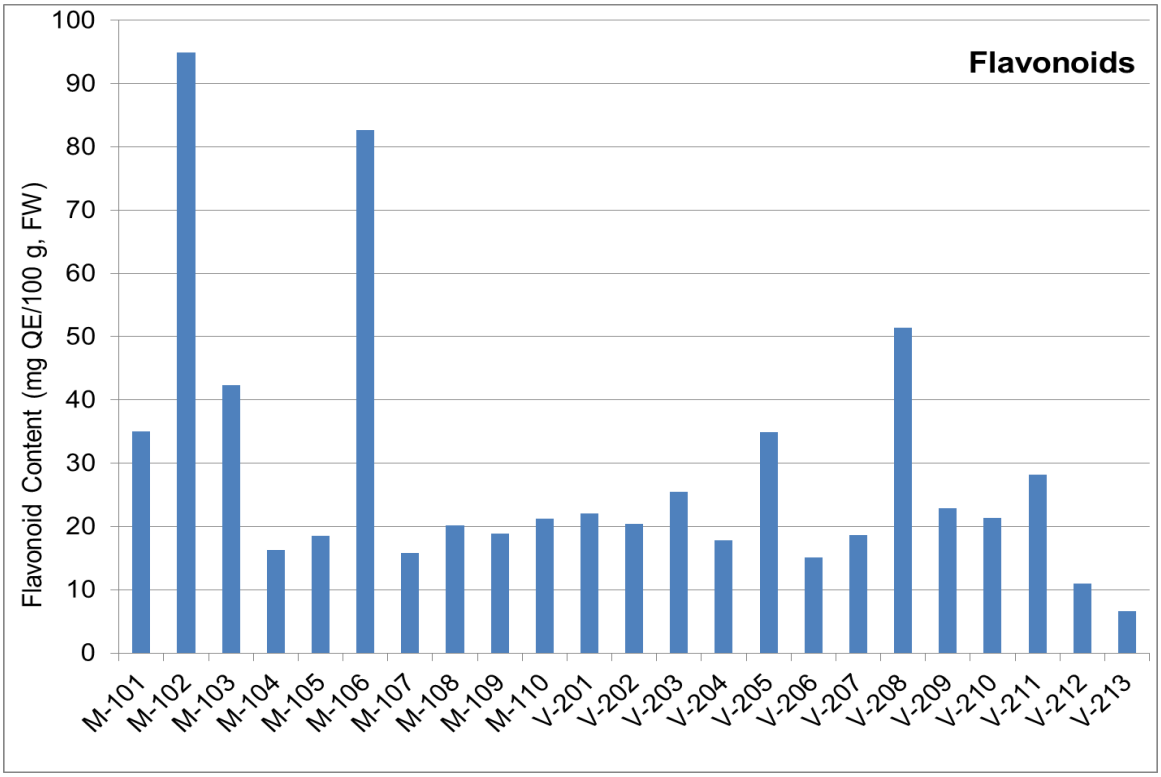

Figure 4. Comparative distribution of the flavonoid contents (mg QE/100 g, FW) in various honey samples

3.3 Distribution of DPPH Scavenging Activity in Honey

Figure 5 shows the comparative distribution of DPPH scavenging activity (%) in various honey samples. In most of the cases significantly elevated scavenging activity was observed in the honey samples. Highest scavenging activity was noted in a branded honey sample M-106 (92%), followed by another branded sample M-102 (91%) and an unbranded sample V-205 (89%). These honey samples also exhibited elevated total phenolic and flavonoid contents thus manifesting their contributions toward antioxidant capability of honey. Generally, the phenolics and flavonoids are well known for their antioxidant potential in natural products and they are considered the major phytochemical towards this effect. All branded honey samples showed excellent DPPH scavenging activity (>70%) whereas almost half of the unbranded samples exhibited ≤ 60 DPPH scavenging activity. Therefore the unbranded samples were not considered as efficient antioxidants. Lowest DPPH scavenging activity was observed in unbranded samples V-212 and V-213 (both < 25%). Overall, almost comparable/higher free radical scavenging activity was noted in about 60% of the honey samples.

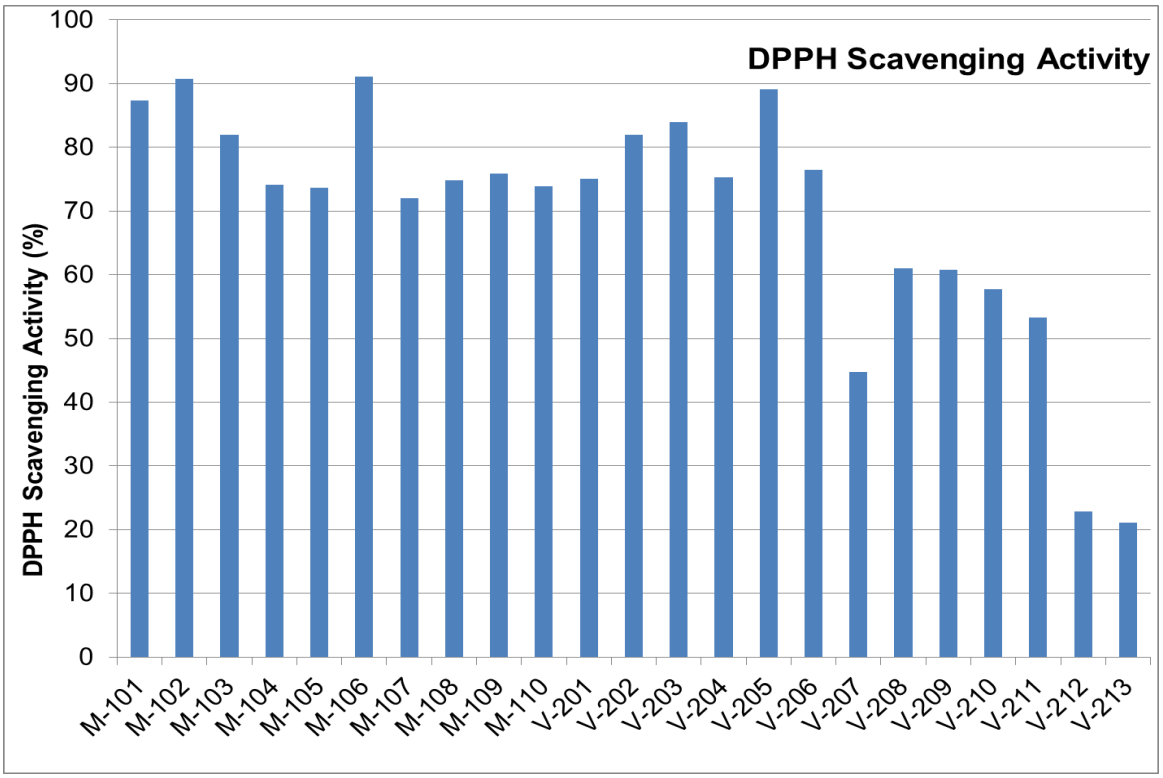

Figure 5. Comparative distribution of the DPPH scavenging activity (%) in various honey samples

3.4 Correlation Study of Selected Metals in Honey

The correlation coefficients among the selected essential and toxic metals in the honey samples were calculated in order to find out their plausible mutual associations. The results of metal-to-metal correlation (*r*-values) for selected essential and toxic metals in various honey samples are shown in Table 4, in which the significant correlations are indicated by bold values. A number of significantly strong correlations were observed among; Sr-Cr (r = 0.761), K-Mg (r = 0.708), Mn-Cr (r = 0.673), Ca-Sr (r = 0.653), Fe-Mn (r = 0.603), Ca-Cu (r = 0.603), Zn-Cr (r = 0.575), Cu-Cd (r = 0.574), Fe-Cr (r = 0.549), Sr-Fe (r = 0.500), Na-Zn (r = 0.481), Sr-Mn (r = 0.478), Co-Cr (r = 0.465), Ca-Mn (r = 0.448), Fe-Zn (r = 0.431), Ca-Cr (r = 0.411), Ca-Fe (r = 0.396), Zn-Cu (r = 0.370), Na-Cd (r = 0.348), Sr-Pb (r = 0.333), Zn-Co (r = 0.322) and Zn-Mn (r = 0.309). In addition, significant inverse correlation was observed between Na and Pb (r = -0.386) and between Mg and Fe (r = -0.307) suggesting their opposing variations. The correlation study manifested mutual associations among most of the selected metals in various honey samples; such relationships are difficult to interpret and multivariate statistical methods are therefore employed to identify their grouping and source apportionment in various environmental segments. This aspect would be considered in impending section.

3.5 Correlation Study of Phytochemicals with Antioxidant Activity

Mutual correlation of the phenolic contents and antioxidant activities of various honey samples is shown in Figure 6. Generally, elevated DPPH scavenging activity was observed in the honey samples having considerably higher phenolic contents. The linear regression showed a significantly strong relationship between the phenolic contents and DPPH scavenging activity with the coefficient (r) value of 0.652 (R^2 = 0.425). This trend showed the strong dependence of the antioxidant property of honey on its phenolic contents. Similarly, mutual relationship of the flavonoid contents and DPPH scavenging activity in various honey samples is shown in Figure 7. The increasing trend of the scavenging activity with the increase in flavonoid contents was not as pronounced as was in the previous case but still a statistically significant correlation was observed between the flavonoid contents and DPPH scavenging activity (R^2 = 0.237). The correlation study thus indicated significant contributions of the phenolic and flavonoid contents towards free radical scavenging activity in the honey samples.

Table 4. Correlation coefficient (r)* matrix for selected essential and toxic metals in honey samples

	K	Na	Ca	Mg	Sr	Fe	Zn	Cu	Co	Mn	Cr	Cd	Pb
K	1.000												
Na	-0.231	1.000											
Ca	-0.082	-0.163	1.000										
Mg	**0.708**	-0.048	-0.079	1.000									
Sr	-0.171	-0.123	**0.653**	-0.217	1.000								
Fe	-0.096	0.005	**0.396**	**-0.307**	**0.500**	1.000							
Zn	-0.222	**0.481**	0.245	-0.208	0.121	**0.431**	1.000						
Cu	0.074	-0.033	**0.603**	0.028	0.202	0.225	**0.370**	1.000					
Co	0.025	-0.035	-0.175	-0.101	-0.121	0.256	**0.322**	0.067	1.000				
Mn	-0.089	0.058	**0.448**	-0.022	**0.478**	**0.603**	**0.309**	0.107	-0.014	1.000			
Cr	-0.120	0.180	**0.411**	-0.243	**0.761**	**0.549**	**0.575**	-0.007	**0.465**	**0.673**	1.000		
Cd	-0.137	**0.348**	-0.219	-0.135	-0.280	0.009	0.220	0.296	**0.574**	-0.277	-0.081	1.000	
Pb	-0.154	**-0.386**	0.114	0.028	**0.333**	-0.050	-0.073	0.001	-0.283	-0.283	-0.276	-0.263	1.000

*r-values shown in bold are significant at $p < 0.05$

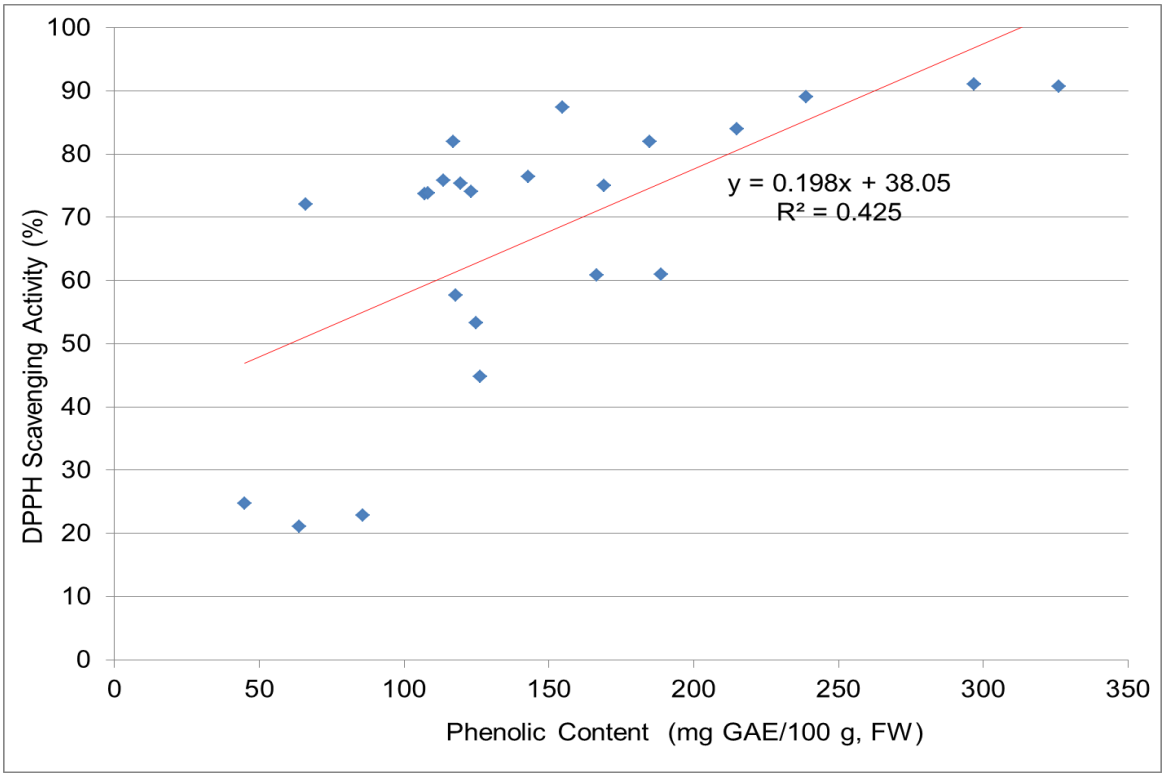

Figure 6. Mutual correlation of phenolic contents and DPPH scavenging activity in various honey samples

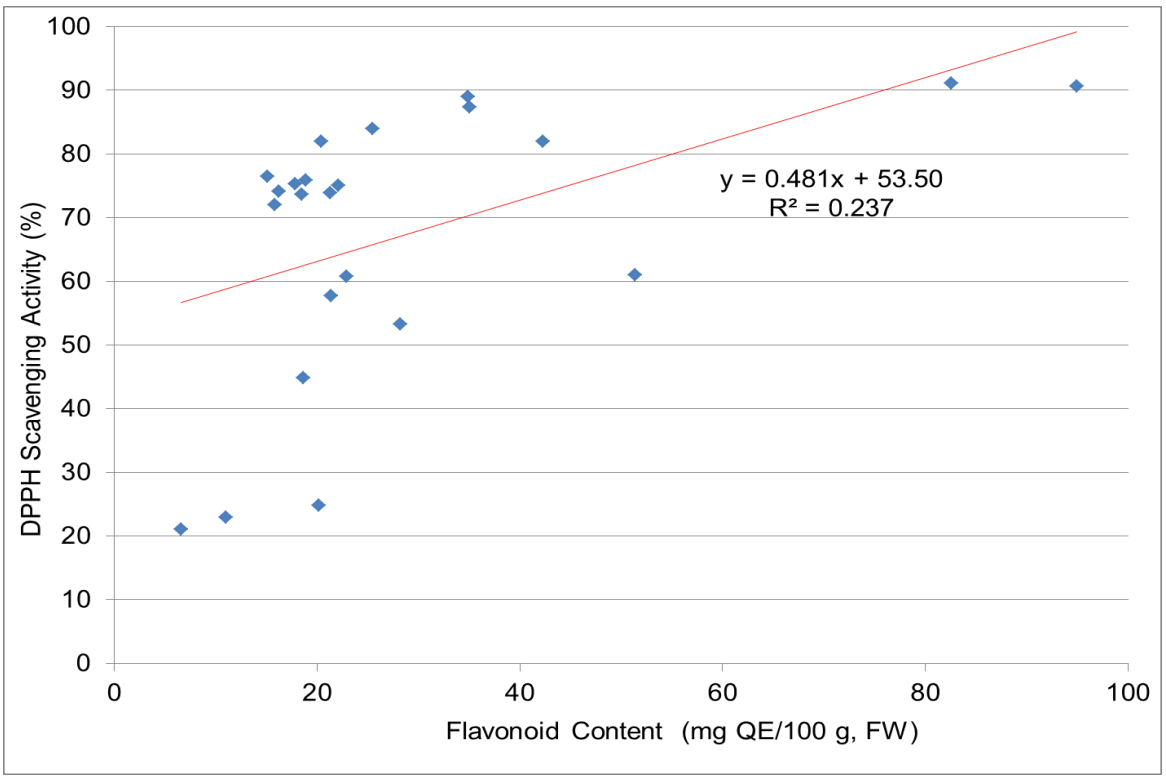

Figure 7. Mutual correlation of flavonoid contents and DPPH scavenging activity in various honey samples

3.6 Correlation Study of Phytochemical Contents and Antioxidant Activity with Selected Metals in Honey

Table 5 summarizes the correlation coefficient matrix for the phytochemical constituents and antioxidant activity with the selected essential and toxic metals in honey. Significantly strong correlations were observed for K with phenolics (r = 0.659), flavonoids (r = 0.407) and DPPH scavenging activity (r = 0.455). Similarly, Mg also showed strong and significant correlations with phenolics (r = 0.684), flavonoids (r = 0.591) and DPPH scavenging activity (r = 0. 376). Among rest of the metals, Zn was significantly correlated with flavonoids (r = 0.303). Nonetheless, Cd exhibited significantly inverse relationships with flavonoids (r = -0.414) and phenolics (r = -0.351). Rest of the metals (Na, Ca, Sr, Fe, Cu, Co, Mn, Cr and Pb) showed statistically insignificant correlations with the phytochemicals and free radical scavenging activity in honey. The correlation study thus manifested valuable role of K, Mg and Zn in the honey samples as shown by their significant associations with phytochemicals and antioxidant potential; however Cd exhibited negative association as it is a well-known carcinogenic metal which promotes ROS and free radicals in the biological systems.

Table 5. Correlation coefficient (r)* matrix for phytochemical constituents and antioxidant activity with selected essential and toxic metals in honey

	Phenolics	Flavonoids	DPPH
K	**0.659**	**0.407**	**0.455**
Na	0.072	0.075	-0.030
Ca	-0.194	0.174	-0.094
Mg	**0.684**	**0.591**	**0.376**
Sr	-0.189	-0.089	0.049
Fe	-0.119	-0.180	-0.234
Zn	0.221	**0.303**	0.085
Cu	-0.154	0.210	-0.029
Co	0.034	-0.113	0.101
Mn	0.150	0.223	-0.267
Cr	-0.009	-0.023	-0.103
Cd	**-0.351**	**-0.414**	-0.077
Pb	-0.118	-0.002	-0.109

*r-values shown in bold are significant at $p < 0.05$

3.7 Comparative Assessment of Selected Metals in Honey

The concentrations of selected essential and toxic metals measured in the honey samples were compared for their relative evaluation in various types of honey. Comparative assessment of K level in various honey samples is shown in Figure 8. Elevated level of K was noted in an unbranded honey sample V-205 followed by a branded honey sample M-106 and couple of unbranded samples V-203 and V-206. However, least concentration was found in the unbranded honey sample V-212 and V-213. The comparative study showed relatively higher K levels in honey samples M-102, V-201, V-209 and V-211 than rest of the samples.

Comparative assessment of the concentration of Na in various honey samples is shown in Figure 9. Highest Na level was found in one of the unbranded honey sample V-201, followed by a branded honey sample M-103. Minimum level of Na was observed in an unbranded sample V-102. Nevertheless, moderately higher and comparable levels of Na were noted in the sample M-102, M-105, V-208, and V-213. Figure 10 depicts the comparison of Ca levels in various honey samples. An unbranded honey Sample V-208 showed highest contribution of Ca while least Ca contents were observed in sample V-202 and V-212, respectively. More or less similar concentration of Ca was observed in the honey samples M-109, M-110 and V-207 during the present study.

Comparative measurement of Mg levels in various honey samples is summarized in Figure 11. Highest Mg level was observed in a branded honey sample M-106, followed by an unbranded sample V-209 while least concentration of Mg was shown by the unbranded sample V-212. In most of the honey samples, relatively lower concentration of Mg was found in the present investigation. Comparative appraisal of Sr levels in various honey samples included in the present study is shown in Figure 12. An unbranded honey sample V-208 showed highest concentration of Sr while least concentration of Sr was found in the honey sample V-213. Some of the honey samples including M-106, M-109 and M-110 showed moderately higher Sr levels compared to the other honey samples.

Comparative evaluation of Fe levels in various honey samples is shown in Figure 13. Again an unbranded sample V-208 exhibited highest Fe level among all honey samples, followed by the honey sample V-207 while some branded honey samples (M-101 and M-106) showed lowest Fe levels. Comparison of Zn levels in various honey samples is shown in Figure 14. A branded honey sample M-103 displayed highest concentration of Zn while another sample M-106 exhibited the lowest level of Zn.

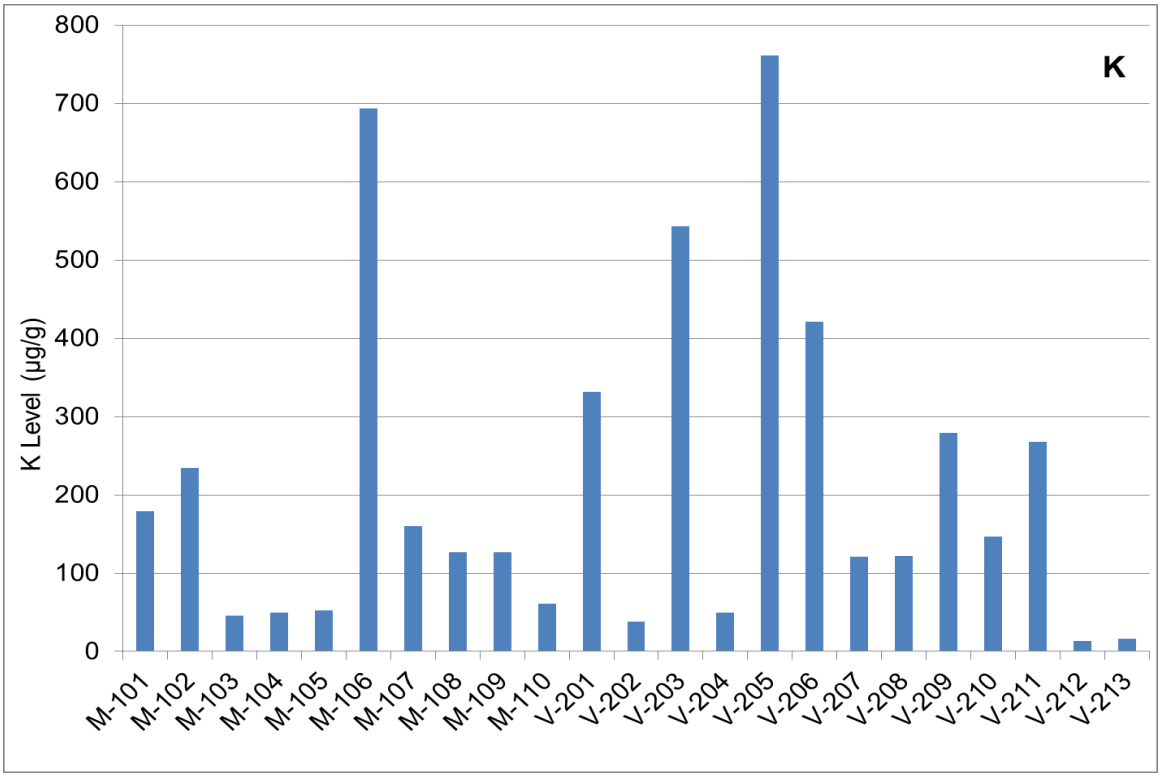

Figure 8. Comparative assessment of K level (µg/g, FW) in various honey samples

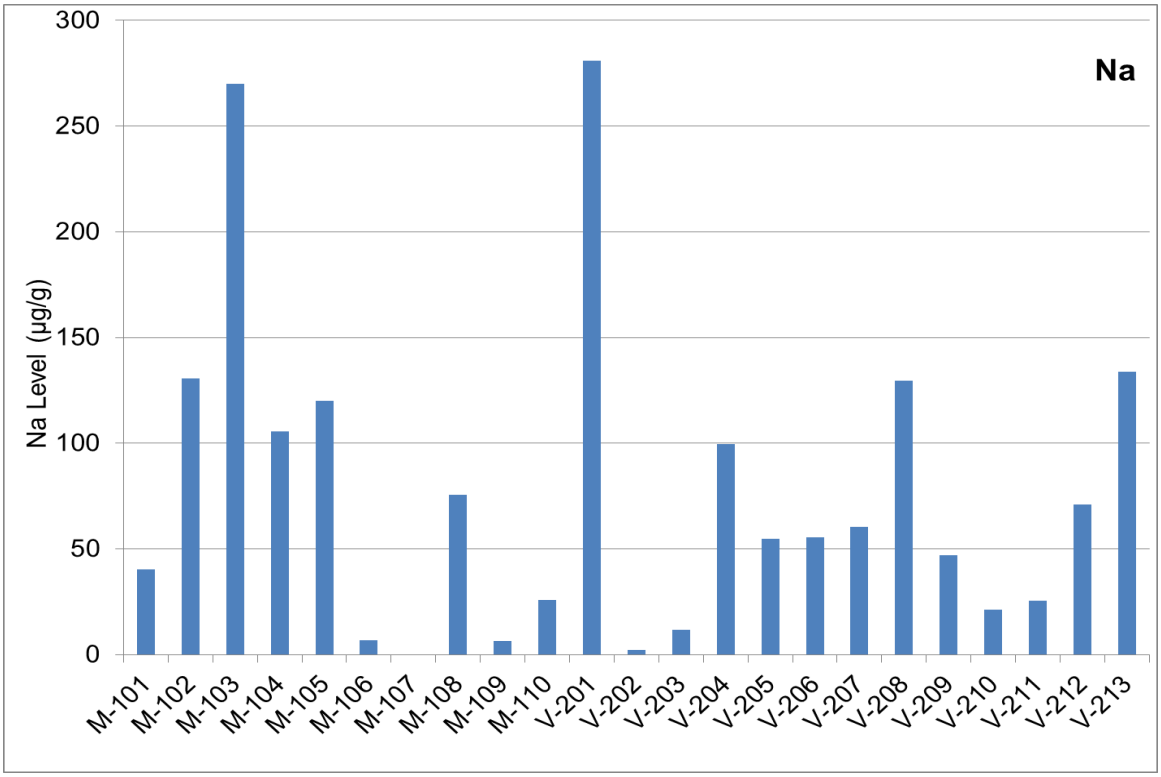

Figure 9. Comparative assessment of Na level (µg/g, FW) in various honey samples

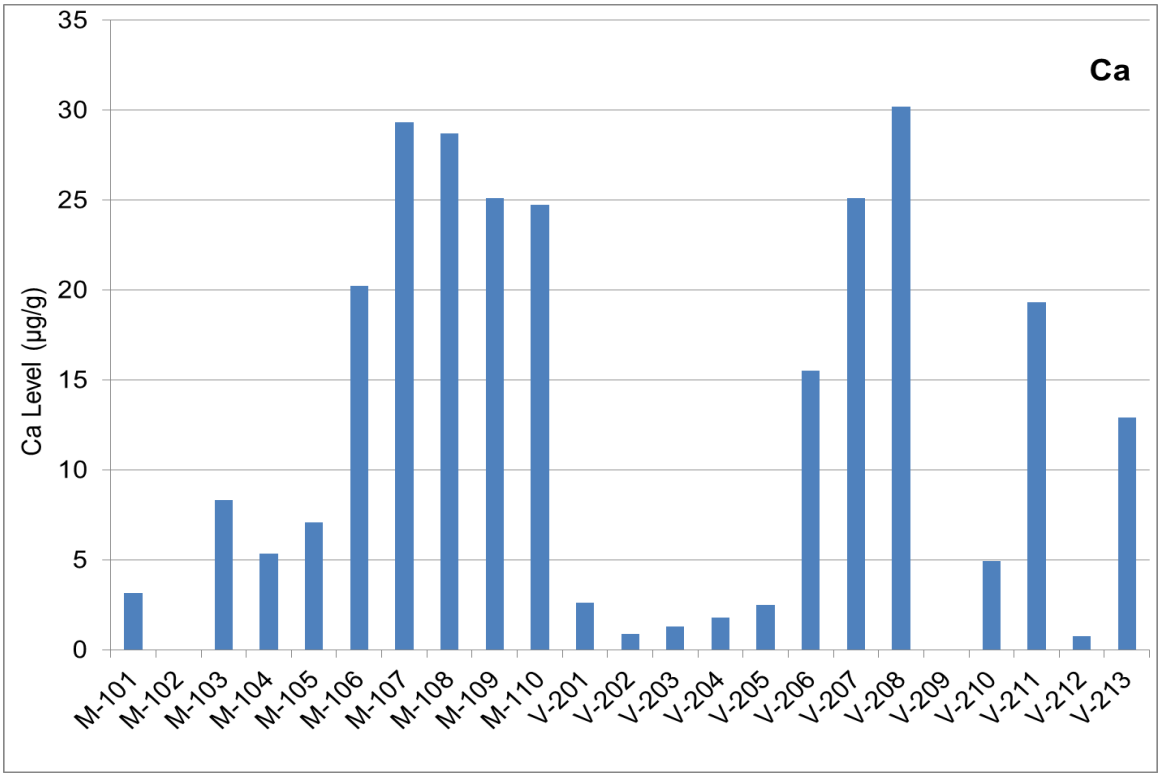

Figure 10. Comparative assessment of Ca level (µg/g, FW) in various honey samples

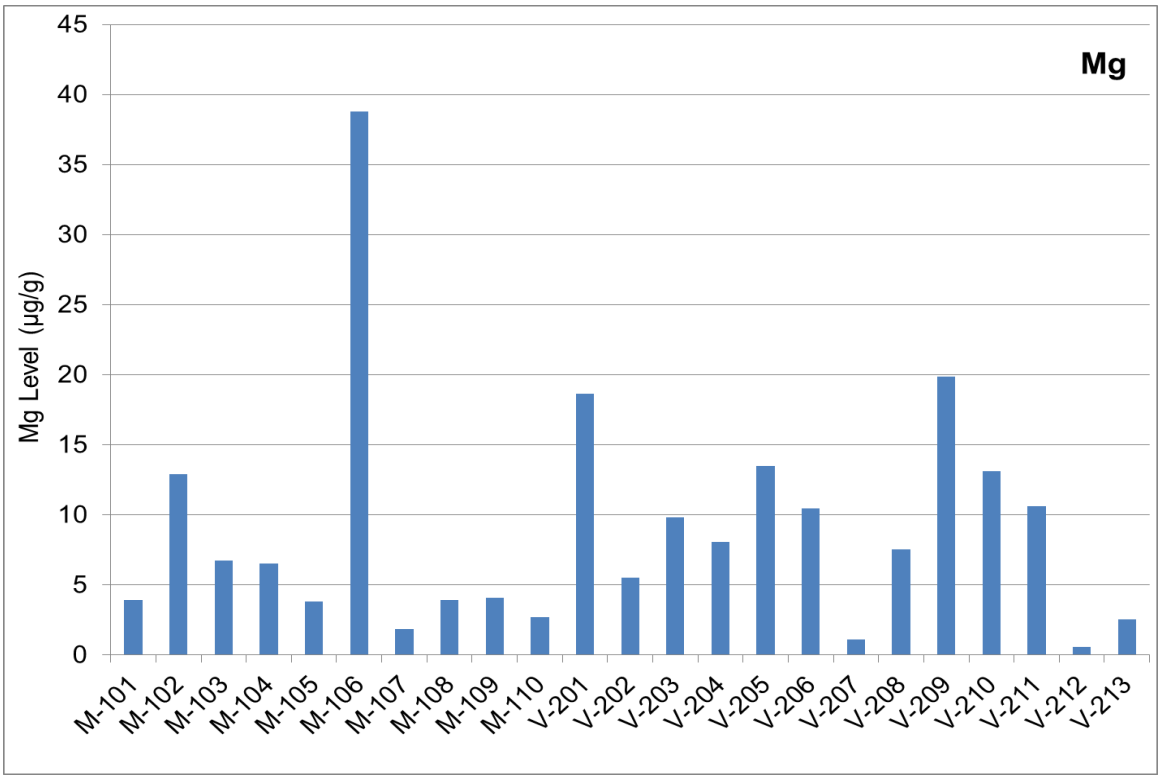

Figure 11. Comparative assessment of Mg level (µg/g, FW) in various honey samples

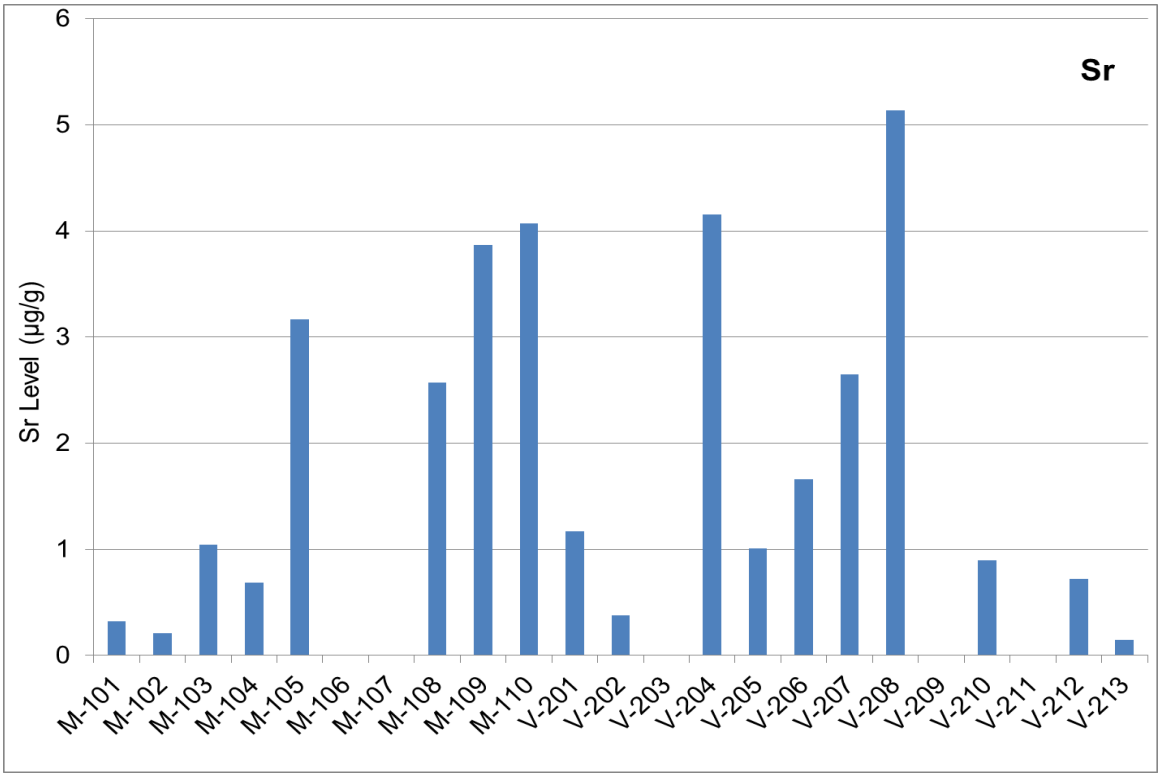

Figure 12. Comparative assessment of Sr level (µg/g, FW) in various honey samples

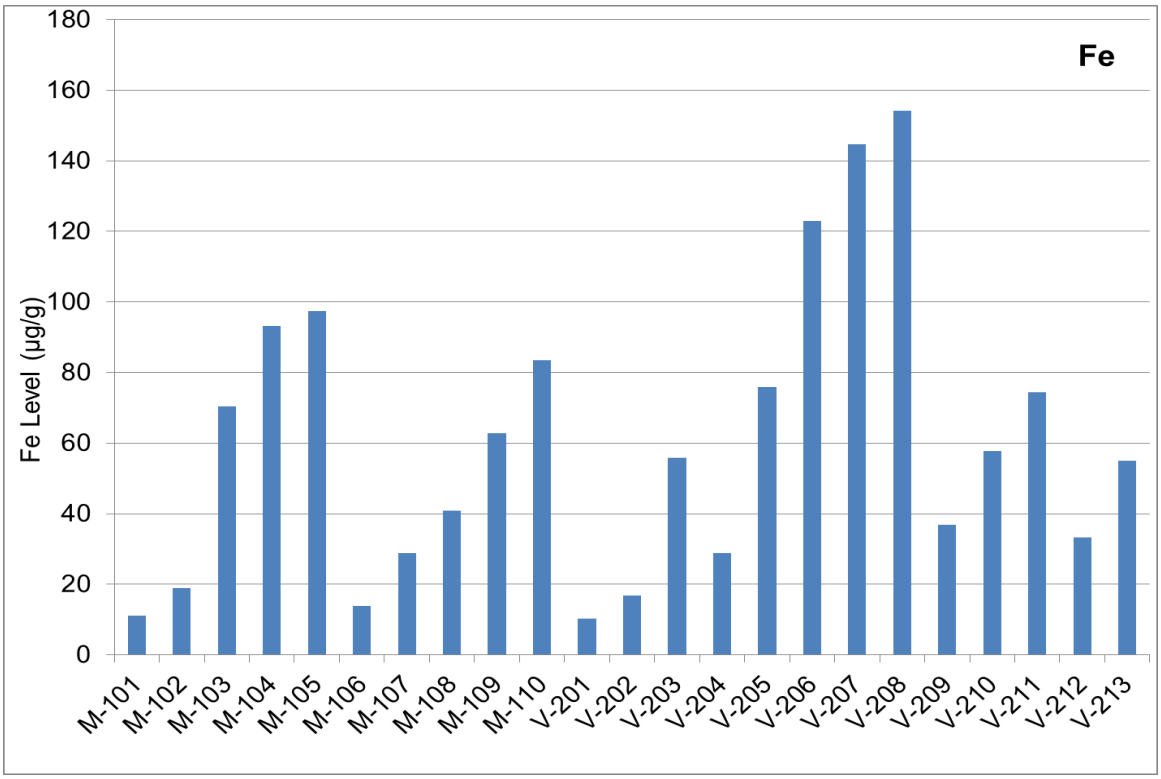

Figure 13. Comparative assessment of Fe level (µg/g, FW) in various honey samples

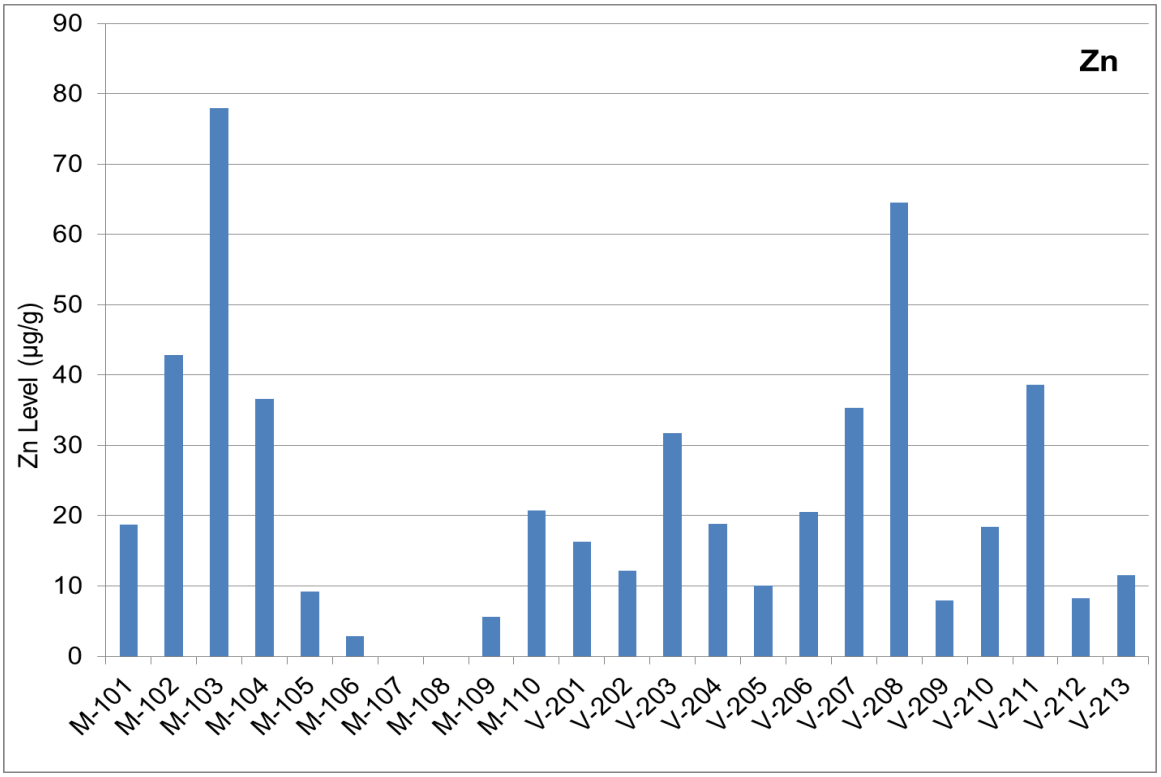

Figure 14. Comparative assessment of Zn level (µg/g, FW) in various honey samples

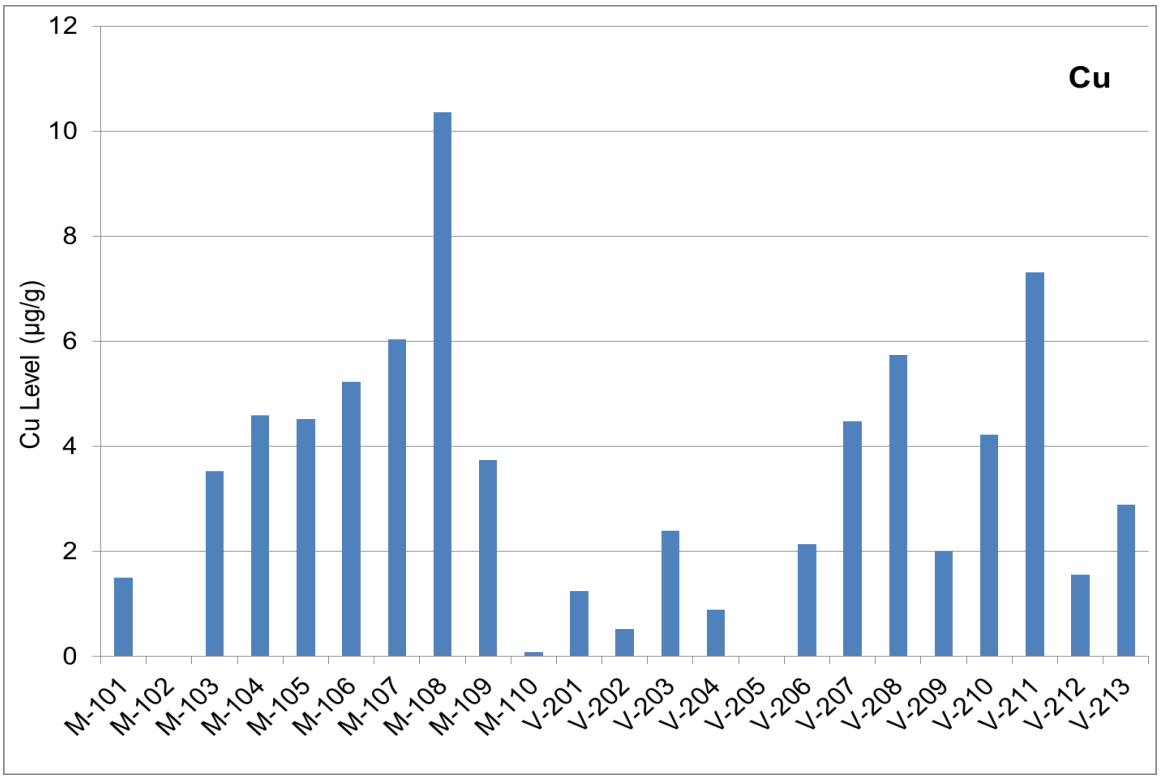

Figure 15. Comparative assessment of Cu level (µg/g, FW) in various honey samples

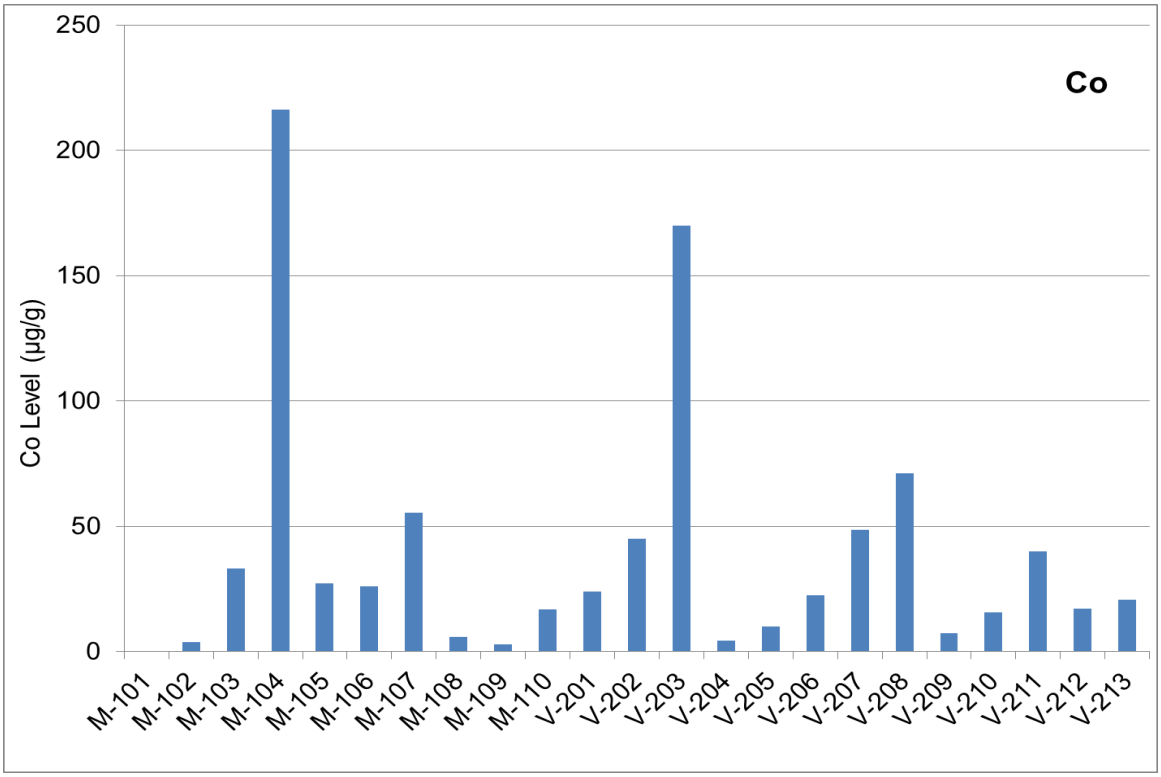

Figure 16. Comparative assessment of Co level (µg/g, FW) in various honey samples

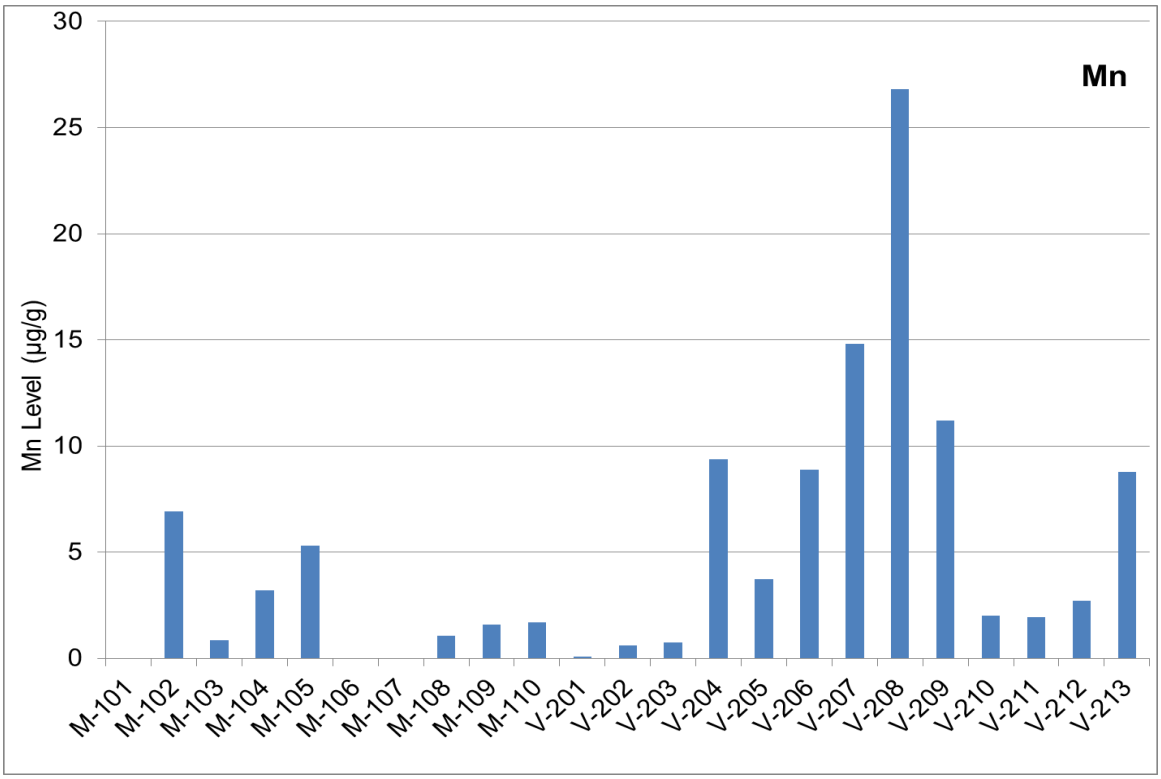

Figure 17. Comparative assessment of Mn level (µg/g, FW) in various honey samples

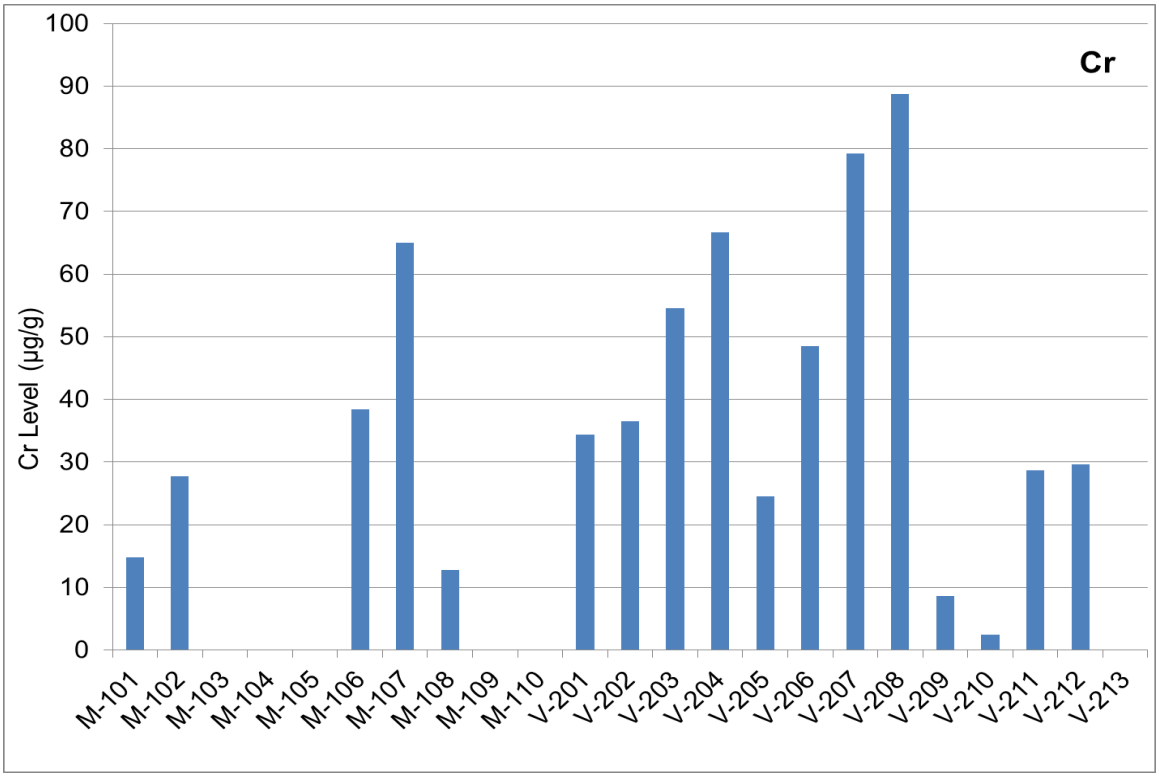

Figure 18. Comparative assessment of Cr level (µg/g, FW) in various honey samples

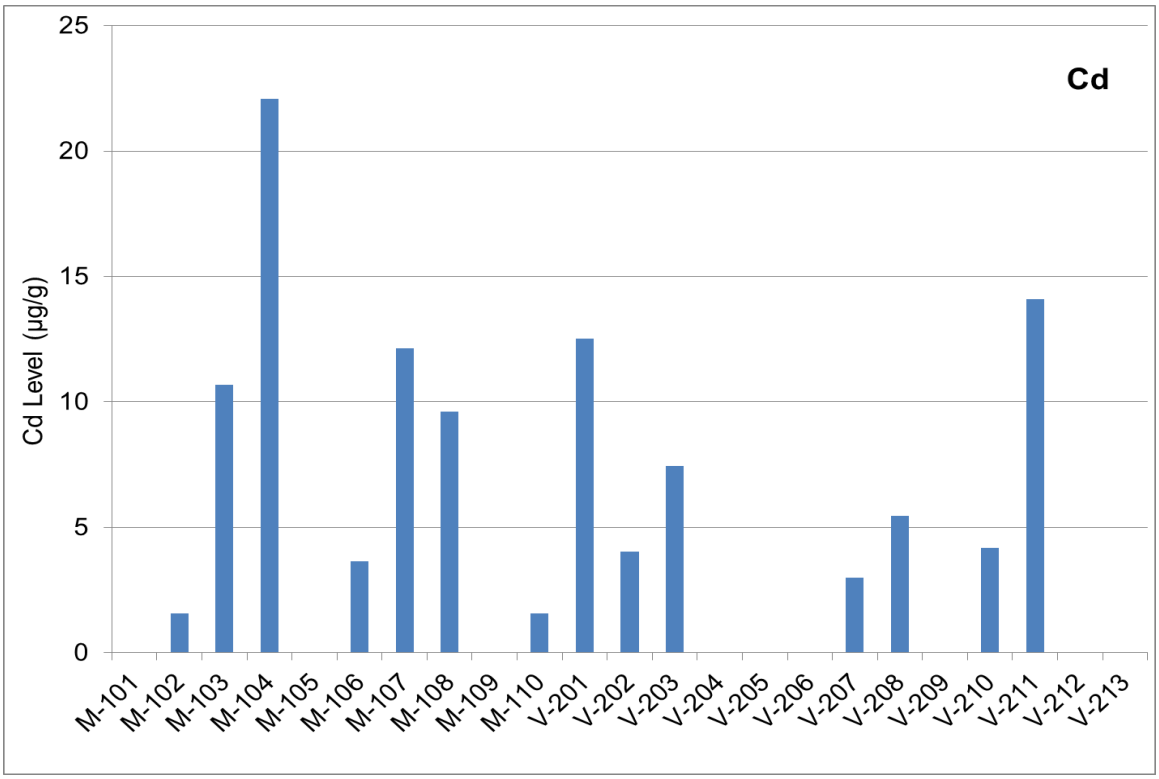

Figure 19. Comparative assessment of Cd level (µg/g, FW) in various honey samples

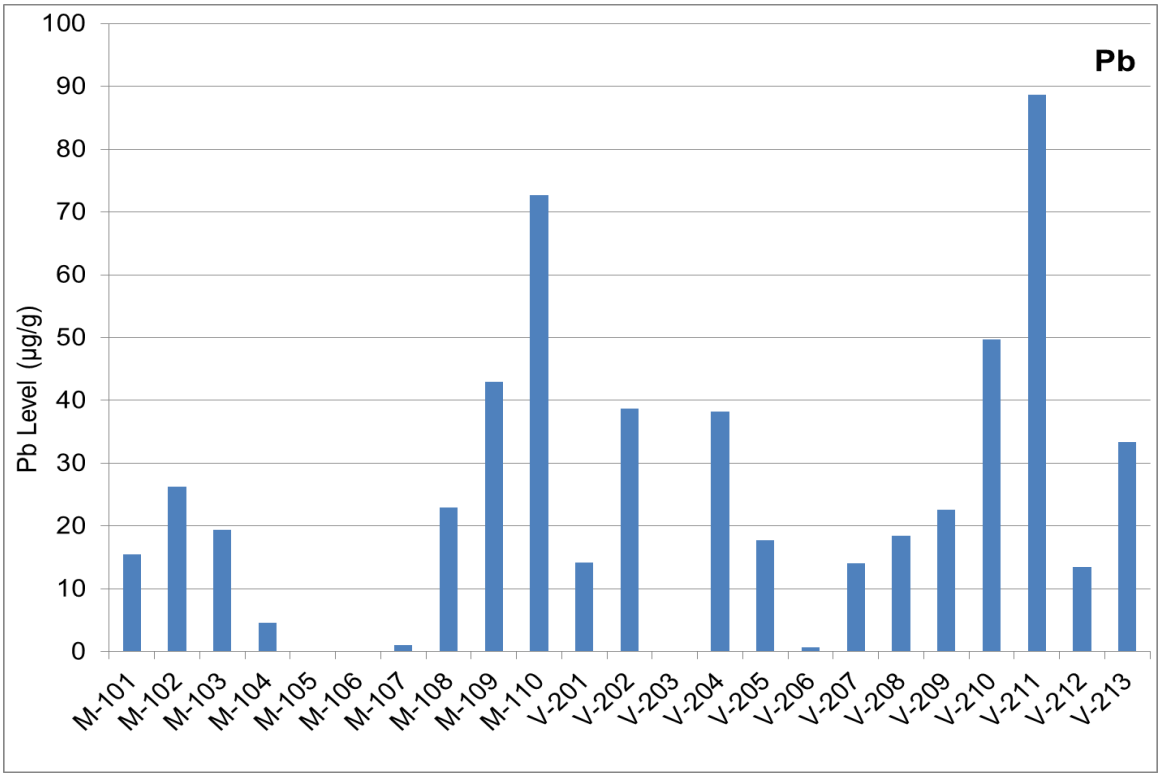

Figure 20. Comparative assessment of Pb level (µg/g, FW) in various honey samples

Figure 15 shows the measured levels of Cu in various honey samples for comparative assessment. A branded honey sample M-108 showed highest concentration of Cu while the lowest concentration of Cu was observed in the sample M-110. In a couple of samples, the concentration of Cu was below the detection limit of the instrument. Overall, the comparative study revealed relatively higher contributions of Cu in the branded honey samples compared with the unbranded honey samples. Figure 16 summarizes the results for Co levels in various samples analysed in the present study. Overwhelmingly higher contribution of Co was found in a branded honey sample M-104, followed by and unbranded honey sample V-203. Rest of the samples showed considerably lower levels of Co while the least concentration was found in the honey sample M-109.

Comparative assessment of Mn levels in various honey samples is presented in Figure 17. Predominantly higher concentration of Mn was found in the unbranded samples V-208 and V-207, followed by almost comparable Mn levels in V-209, V-204, V-206 and V-213. It is interesting to note that significantly higher contribution of Mn was found in the unbranded honey samples compared with the branded honey samples which showed very lower Mn levels in the present study. Figure 18 depicts the comparison of Cr levels in various honey samples. Highest concentration of Cr was shown by an unbranded honey

sample V-208, followed by the honey samples V-207, V-204 and M107. However the lowest concentration was measured in the honey sample V-210. In the present study, relatively higher Cr levels were found in the unbranded honey samples than the branded honey samples; nonetheless the Cr levels were noted to be lower than the detection limit of the instrument in about half dozen of the honey samples.

Comparative evaluation of Cd levels in various honey samples is illustrated in Figure 19. Highest concentration of Cd was observed in one of the branded honey samples M-104, followed by almost comparable contributions of Cd in the honey samples V-211, V-201, M-107 and M-103. Appreciably lower concentration of Cd was measured in the honey samples M-102 and M-110. Nevertheless, Cd concentration was found to be less than the detection limit in significant number of samples (n = 9) in the current study. Figure 20 shows the comparison of Pb levels in various honey samples. Highest concentration of Pb was observed in an unbranded honey sample V-211, followed by a branded honey sample M-110. Significantly elevated and almost comparable contribution of Pb was found in the honey samples V-210, M-109, V-202, V-204, and V-213. However, least concentration of Pb was noted in the honey sample V-206 compared with the other samples.

3.8. Multivariate Analysis of Selected Metals in Honey

Multivariate statistical methods were used for the source apportionment and identification of the metals in the present study. The principal component analysis (PCA) of the selected essential and toxic metals in various honey samples extracted by using varimax normalized rotation on data set is shown in Table 6. Five principal components were extracted with eigen values greater than 1, cumulatively explaining 76.78% of the variance of data. The variables with higher principal component (PC) loadings are those which contribute most to explain the variance and origin of each component. First PC showing the highest percentage of the total variance (26.22%) indicated considerably higher loadings for Cr, Mn, Fe and Sr, together with significant contributions of Ca and Zn. In the second PC, Co and Cd showed their elevated loadings whereas PC 3 revealed higher loadings for K and Mg. Similarly, PC 4 showed elevated loadings for Cu and Ca and PC 5 showed higher loading for Na, Pb and Cu. Principal component analysis provided the quantitative information about the origin of these metals in honey; PC 1, PC 3 and PC 4 showed the natural contribution of the metals in honey samples, while PC 2

showed the contribution from anthropogenic activities. PC 5 showed mixed contributions both from natural as well as anthropogenic activities. The cluster analysis of selected essential and toxic metals is shown in Figure 21. Two strong and distinctly separate clusters were observed for selected metals in the honey samples; first cluster comprised of Na, Cd, K, Mg and Pb while the second mutual cluster was composed of Ca, Fe, Cu, Sr, Zn and Mn, which were weakly linked with Co and Cr. First cluster was mostly contributed by the anthropogenic activities such as automobile emissions, industrial emissions, agricultural activities, fertilizers and excavation activities as well as some natural lithogenic contributions. Second cluster was mostly derived from natural sources and the anthropogenic contributions were inconsequential and not significantly affecting their distribution in the honey samples. Overall, the multivariate study revealed diverse apportionment of the metals in honey samples collected during the present study.

Table 6. Principal component analysis of selected essential and toxic metals in honey

	PC 1	PC 2	PC 3	PC 4	PC 5
Eigen value	3.408	2.281	1.665	1.446	1.180
Total Variance (%)	26.22	17.55	12.81	11.12	9.081
Cumulative Eigen value	3.408	5.690	7.355	8.801	9.982
Cumulative Variance (%)	26.22	43.77	56.58	67.70	76.78
K	0.036	-0.075	0.910	0.001	0.134
Na	-0.060	-0.036	0.169	0.009	0.914
Ca	0.472	-0.224	0.035	0.724	-0.121
Mg	0.161	0.156	0.867	-0.058	-0.020
Sr	0.677	-0.219	0.152	0.287	-0.253
Fe	0.744	0.224	0.172	0.211	-0.010
Zn	0.340	0.217	0.252	0.317	0.534
Cu	0.028	0.172	-0.095	0.912	0.048
Co	0.182	0.922	-0.006	-0.086	-0.006
Mn	0.823	-0.171	-0.094	0.043	0.215
Cr	0.854	0.141	0.093	-0.058	0.152
Cd	-0.275	0.741	0.122	0.224	0.272
Pb	0.178	0.279	-0.275	-0.242	0.596

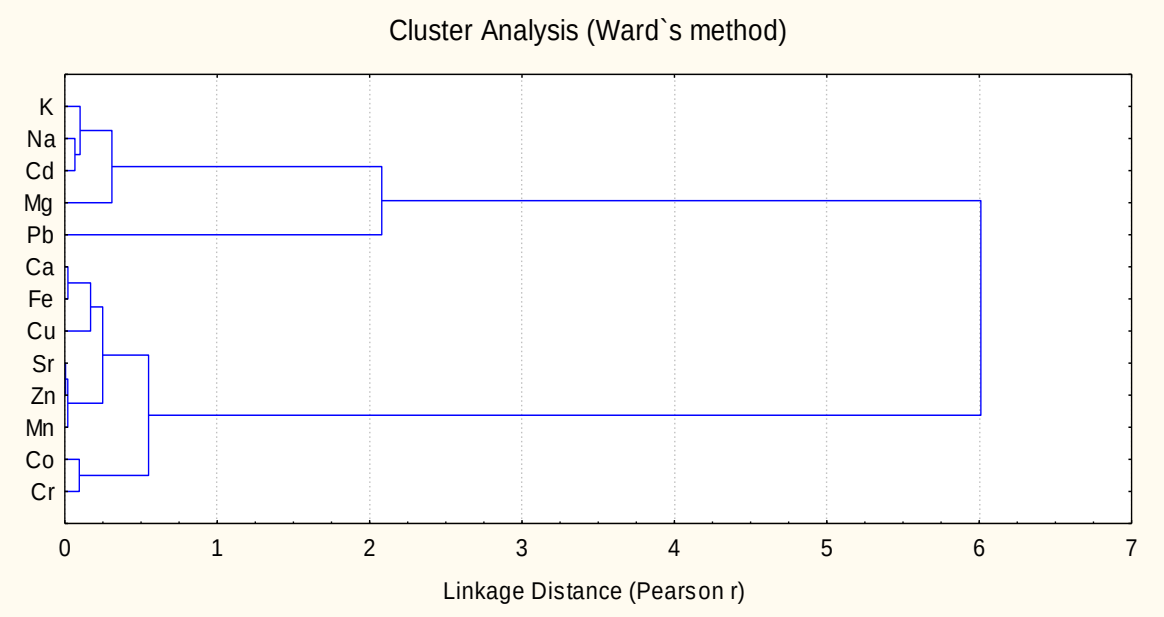

Figure 21. Cluster analysis of selected essential and toxic metals in honey

3.9 Health Risk Assessment of Selected Metals in Honey

In this study potential health risk associated with the metal contents in various honey samples was evaluated. Health risk assessment involve four steps which include average daily intake of the metal (DIM), health risk index (HRI), hazard quotient/index (HQ/HI) and carcinogenic risk (CR). Health risk index (HRI) was calculated to approximate the toxic risks posed by individual metals via honey consumption and is shown in Figure 22. Health risk index value below one is considered as safe and above one means there can be chances of adverse effect. The HRI values for Cr, Cd and Pb shown in Figure 22 are higher than 1 thus they indicated probable health risk associated with the consumption of honeys. Rest of the metals showed HRI values below unity demonstrating no significant adverse health effects. Results for hazard quotient and hazard index of the metals in honey are shown in Figure 23. Hazard index is summation of hazard quotients for all the metals to which individual is exposed. Hazard quotient and hazard index values were significantly less than 1 thus demonstrating no adverse non-carcinogenic health risk associated with the metal levels in honey. Figure 24 depicts the carcinogenic risk for Pb, Cd and Cr in honey samples. Generally, the safe limit for CR is $1x\ 10^{-4}$ (one out of 10,000). The results of the present study indicated that no carcinogenic risk was associated with the consumption of the honeys as the CR values calculated for Pb, Cd and Cr were far less than the safe limit. Therefore, the consumption of honey was considered safe with respect to the selected metal contents.

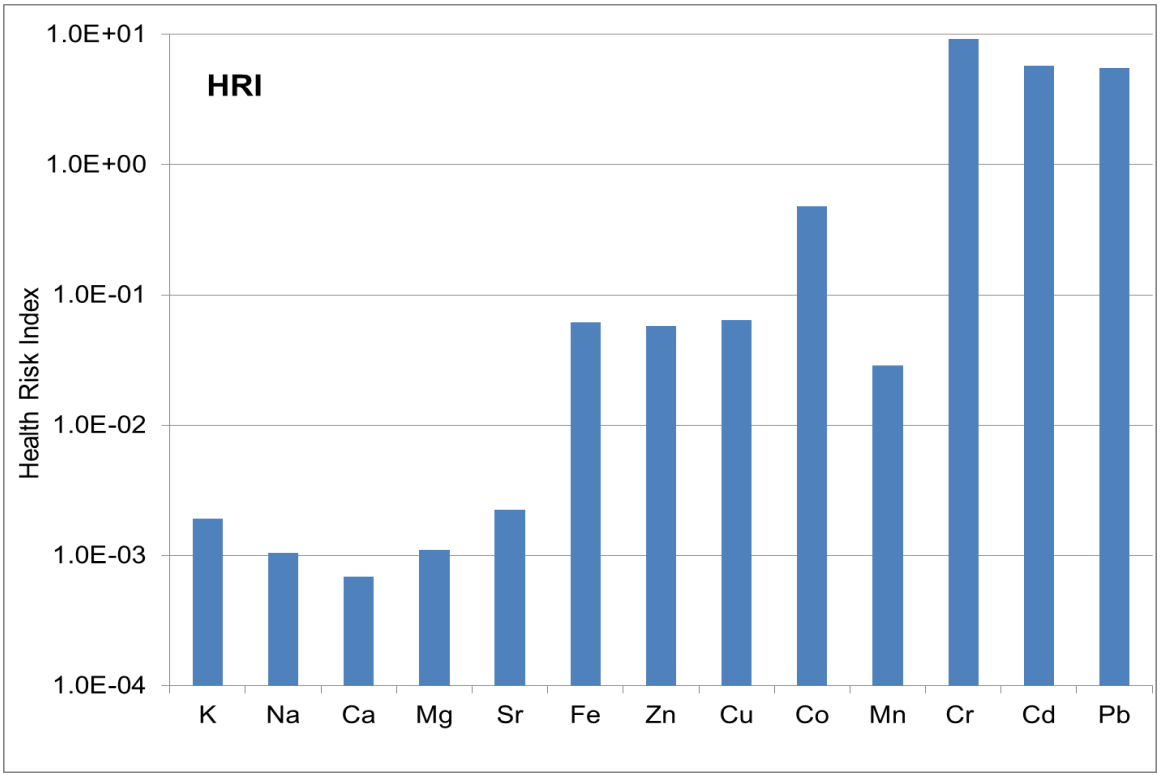

Figure 22. Health risk index for selected metal contents in honey

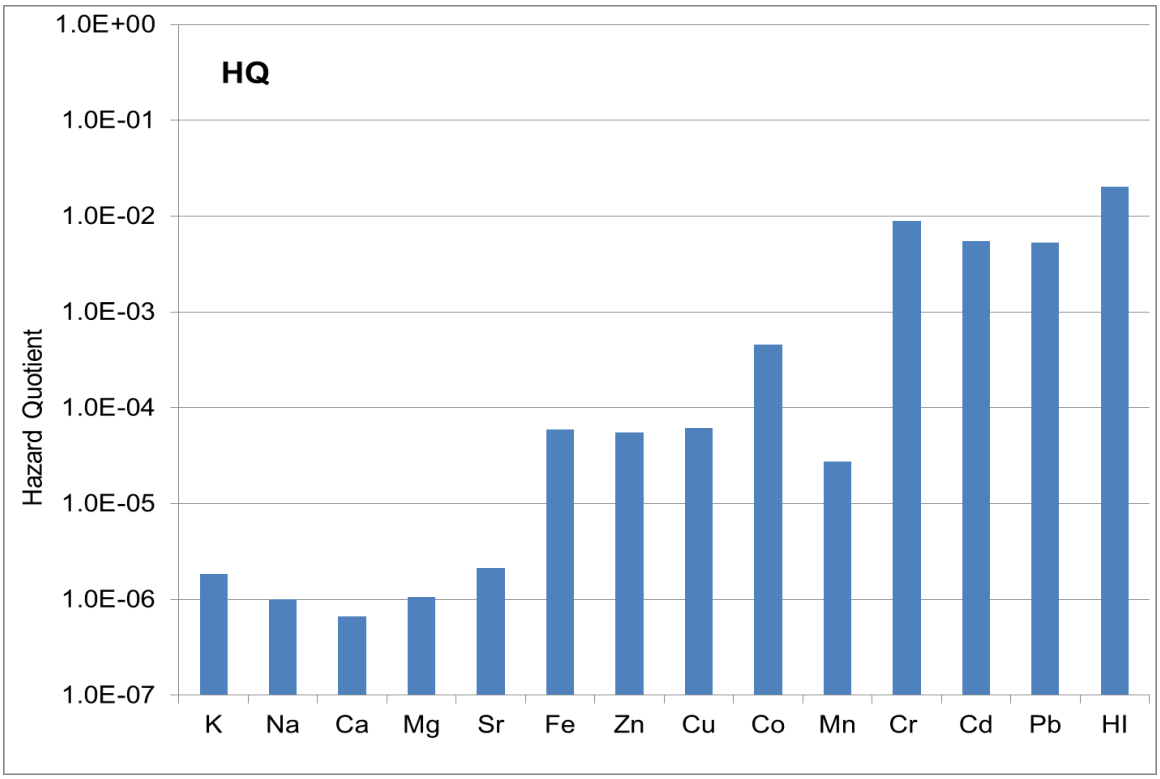

Figure 23. Hazard quotient/index for selected metal contents in honey

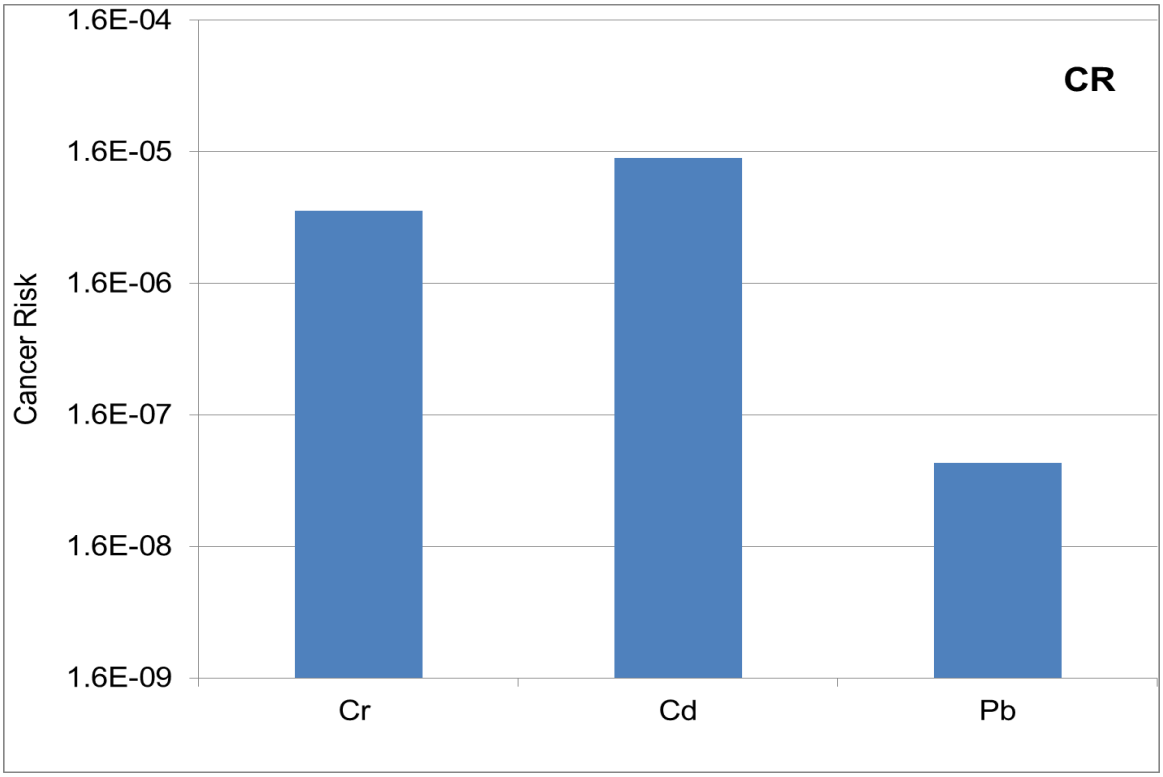

Figure 24. Carcinogenic risk for selected metal contents in honey

3.10 Comparison of the Present Metal Levels with Reported Levels in Literature

The average metal levels in honey sample collected during the present study were compared with the reported levels in literature around the world as shown in Table 7. The average content of K in the present study was significantly lower than the reported levels in honey from other countries including Hungary, New Zealand, Italy, Macedonia, Spain, Ireland and Malaysia. Sodium level in the present study was found to be higher than those reported for honey samples from New Zealand, Macedonia, and Brazil, whereas it was comparable with the honey levels from Italy, Spain and Ireland. Average Ca and Mg levels in the current study were significantly lower than the reported levels in honey from other countries mentioned in the Table. Mean concentration of Sr in the present study was almost comparable with the reported level in honey from Italy but higher than those of Spain. The average levels of Fe and Zn in the present study were considerably higher than the reported level given in the Table except Malaysia. Similarly, average concentrations of Cu, Co, Mn, Cr, Cd and Pb in the present study were considerably higher than the reported levels from other regions of the world as shown in Table 7.

Table 7. Comparison of the present average metal levels (µg/g, FW) in honey with the reported levels in literature around the world

Location	K	Na	Ca	Mg	Sr	Fe	Zn	Cu	Co	Mn	Cr	Cd	Pb	Ref.
Pakistan	210.3	80.62	12.86	8.985	1.881	60.33	24.21	3.570	40.20	5.611	38.90	7.999	27.76	Present study
Turkey	296	118	51	33		6.6	2.7	1.8	1.0	1.0				Yılmaza and Yavuzb, 1999
Malaysia	1349.3	236.80	183.67	64.46		162.31	43.88							Moniruzzaman *et al*., 2014
Egypt				0.63		34	6.09	1.38	0.08		0.65	0.005	1.05	Rashed *et al*., 2009
Macedonia	985	30	40	18		1.2	2.3	0.70		1.8		0.0036		Stankovska, 2008
Spain	1124	76	169	39	0.6		3.9			3.4				Fernandez-Torres *et al*., 2004
Spain	1778	279	113	136	0.41	9.19	5.65							Terrab *et al*., 2005
Italy	1195	96.6	257	56.7	1.43	3.07	1.82	0.906	0.011	1.54		0.0039	0.076	Pisani *et al*., 2008
Italy	472	96	47.7	37		4.5	3.1			3.0				Conti, 2000
Hungary	372		47.9	16.3		0.760	2.32	0.189		1.03	13.3			Czipa *et al*., 2015
Switzerland						1.390	1.041	0.656		2.063	0.005	0.003	0.041	Bogdanov *et al*., 2007
Ireland	566	98	111	31		8	5			4				Downey *et al*., 2005
New Zealand	1050	23.9	50.92	24.8		1.71	1.18	0.25		1.04	0.37	0.149	0.017	Vanhanen *et al*., 2011
Brazil	310.30	15.06	62.00	13.53		1.58	0.56	0.43		0.80	77.17			Liberato *et al*., 2013

3.11 Salient Findings

Based on the observations presented in preceding sections, following salient findings emerged from the present study:

- Most of the metals showed predominantly random and asymmetric distribution in the honey samples. Overall the average metal levels in honey showed following order: K > Na > Fe > Co > Cr > Pb > Zn > Ca > Mg > Cd > Mn > Cu > Sr.
- The correlation study showed strong relationships between K-Mg, Na-Zn, Ca-Sr, Ca-Cu, Fe-Mn, Sr-Cr, and Mn-Cr while Na and Mg showed inverse relationship with Pb and Fe, respectively.
- Relatively higher phytochemical contents and DPPH radical scavenging activity was shown by most of the honey samples.
- Total phenolic contents, flavonoid contents and DPPH radical scavenging activity showed strong correlations with K, Mg and Zn, while Cd showed inverse relation.
- Significant anthropogenic contributions of some metals were shown by the principal component analysis and cluster analysis.
- Most of the honey sample showed elevated concentrations of essential metals particularly K, Na and Fe whereas some of the honey samples showed higher levels for toxic metals such as Cd, Cr and Pb.
- The health risk index (HRI) values of most of the metals were less than unity which is considered safe for human consumption. However, HRI values of Cr, Cd and Pb were greater than 1 which indicated some health concerns.
- Target hazard quotient (THQ) and hazard index (HI) values were less than unity, so no non-carcinogenic health risk is associated with the consumption of honey.
- The target cancer risk (TCR) values for Cr, Cd and Pb was significantly less than the acceptable risk limit (1×10^{-4}), indicating no lifetime carcinogenic risk associated with these metals in honey.

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