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DEPARTMENT OF CHEMISTRY

MSc THESIS ON

INVESTIGATION OF PHYSICOCHEMICAL AND BIOCHEMICAL PROPERTIES OF PURE AND ADULTERATED HONEY WITH SUGAR

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FEBRUARY, 2021

JIMMA, ETHIOPIA

INVESTIGATION OF PHYSICOCHEMICAL AND BIOCHEMICAL PROPERTIES OF PURE AND ADULTERATED HONEY WITH SUGAR

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MSc THESIS APPROVAL SHEET

We, the under signed, member of the Board of Examiners of the final open defense by **Amanu Abamecha** have read and evaluated his thesis entitled **"Investigation of Physicochemical and Biochemical Properties of Pure and Adulterated Honey with Sugar"** and examined the candidate. This is therefore to certify that the thesis has been accepted in partial fulfillment of the requirement for the degree of Master of Science in Chemistry **(Analytical)**

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Acronyms/abbreviations

CAC	Codex alimentarius commission
USDA	United states departments of agriculture
AOAC	American official association of chemists
CSA	Central statistics authority
HMF	Hydroxyl-methyl-furfural
HFCS	High fructose corn syrup
GC	Gas chromatography
HPLC	High pressure liquid chromatography
NMR	Nuclear magnetic resonance
UV-Vis	Ultraviolet-visible
DPPH	1,1-Di-phenyl-2-picrylhydrazyl
FRAP	Ferric reducing antioxidant
ORAC	Oxygen radical absorbance capacity
MC	Moisture content
LA	Light amber
FA	Free acidity
Am	Amber
TPC	Total phenolic content
GAE	Gallic acid equivalents
IC_{50}	Inhibition concentration of 50% DPPH
RSA	Radical scavenging activity
EC	Electrical conductivity
RS	Reducing sugar
SC	Sucrose content
AC	Ash content

Abstract

Honey is a natural sweet substance produced by honey bees and honey dew from the nectar of plant flowers. It contains higher amount of glucose and fructose. The impacts of adulteration of honey by sugar were aimed to be analyzed. The physicochemical and biochemical parameters were investigated using standard analytical methods after collecting the sample by purposive sampling techniques. The obtained results showed 68.56 ± 0.12 and 102.66 ± 0.11(Pfund, mm) for colors, 19.62 ± 0.07 and 19.44 ± 0.10 (%) moisture, 180.94 ± 2.44 and 778.78 ± 1.62 (µS/cm) electrical conductivity, 0.36 ± 0.00 and 0.41 ± 0.00(%) ash, 3.96 ± 0.01 and 4.08 ± 0.08 pH, 42.50 ± 0.50 and 38.66 ± 0.58 (meq/kg) acidity. And, 61.41 ± 0.11 and 62.83 ± 0.30 (%) reducing sugar, 4.23 ± 0.02 and 4.65 ± 0.04(%) sucrose, 42.33 ± 1.05 and 34.73 ± 2.74 (mg/Kg) hydroxyl-methyl-furfural, 28.48 and 30.46 (IC_{50}, mg/ml) antioxidant activity and 145.00 ± 01.40 and 149.40 ± 1.54 (mg.GAE/100g) total phenolic were analyzed for light amber and amber honey samples respectively. The adulteration of the honey samples were showed decrement in per gram of the samples of physicochemical and biochemical properties. This intern reduces the aroma, taste, essential minerals and protective activity in honey; but adulterating honey with sugar showed positive impact by reducing Hydroxyl-methyl-furfural content. Analysis was performed in triplicate, and the results were expressed as Mean ± SD. The significance difference was tested by ANOVA at ($p \leq 0.05$); interpreted and compared with values reported in literatures and international standards such as codex Alimentarius commission.

Keywords: Honey, Adulteration, Physicochemical properties, Biochemical properties, Antioxidant activities

1. Introduction

Honey is a natural sweet substance produced by honey bees (*Apismelifera*) and honey dew. Honey is produced from nectars of flowers. Nectar is a liquid containing high sucrose which is produced in plants gland called nectarines [1]. About 80% - 95% of nectar is water and (5% - 20%) is sucrose. Honey contains fructose and glucose (95 – 99%) of the dry matter. Bees collect nectars and transform by addition of some specific substances of their own to make honey. Honey is a natural product with complex chemical composition, mineral substances, and other compounds such as proteins, minerals, enzymes, vitamins, hydroxyl-methyl-furfural (HMF), volatile compounds, flavonoids and phenolic acids. The chemical composition and other properties can be varied due to their seasonal variations, botanical origin, human activities and environmental factors [2].

The environmental conditions of Ethiopia's are conducive for the growth of over 7000 species of flowering plants. Ethiopia is known to be the largest honey producer in the world. It was reported by Gemeda and coworkers that in Ethiopia the production of honey is thought to be 24,600 to 43,000 tons per year [3, 4]. Exclusively, honey is almost used for local consumption (80%) such as honey beer locally called '*tej*' and juice in Ethiopia. According to the ministry of agriculture and the central statistics authority (CSA) report of Ethiopia, honey production has been growing at about 12% over the decades. In Ethiopia, the production and supply of honey and beeswax by regions has also shown that Oromia (41%), Amhara (22%), SNNPR (21%) and Tigray (5%) [5].

Honey is higher in its antimicrobial activity than commonly used antibiotics [1]. Thus; antimicrobial activity of honey is different in mechanism from antibiotics, by inhibiting intracellular metabolic pathways [6]. Honey has been used to treat ailments including gastric disturbances, ulcers, wounds and burns. Honey syrup supports health and recovery. The remarkable effect of honey cleaning up of wounds is due to a combination the osmotic outflow and a bioactive effect of honey [7].

The quality and properties of honey are related to honey maturity, production methods, processing, storage conditions, climatic conditions as well as the nectar source of the honey. Improper handling of honey can cause quality deterioration of honey that are caused by heating

at high temperatures, high moisture content, adulteration, poor packaging and poor storage conditions. The characteristic quality of honey can be altered by adulteration [8]. Honey adulteration is done either through indirect adulteration by feeding honey bees with industrial sugars at the stage when broods become naturally available or direct adulteration by addition of foreign substances to honey resulting serious problem to occur [9]. These are sucrose syrups from sugar beet, high fructose corn syrup (HFCS), maltose syrup and industrial sugar are some of the adulterants. Cane sugar is the common honey adulterant in Ethiopia [10]. The adulterated honey Change in its properties like enzymatic activity, color, viscosity, refractive index, and electrical conductivity, contents of some specific compounds like hydroxyl-methyl-furfural (HMF), glucose, fructose, sucrose, maltose, and ash content are some of the observed parameters [11].

Testing quality of honey is needed to reveal authenticity of the product by identifying adulteration to address market needs [8]. The physicochemical and biochemical properties of honey are affected by adulteration, so the determination of selected physicochemical and biochemical properties; pH value, total acidity, sugar content, moisture content, ash content, hydroxyl-methyl-furfural (HMF) and electrical conductivity, and phenolic content allow as to know the extent of adulteration of common adulterants like sugar [12, 13]. However; quality of honey significantly affected by adulteration of honey by sugar, no detailed study has been conducted on the effects of intentional adulteration of honey with various sugar ratios on its physicochemical and biochemical properties. Therefore; this study initiated to investigate the impact of adulteration by sugar on physicochemical and biochemical properties of pure (unadulterated) honey.

1.1 Statement of the problem

South western region of Ethiopia is the well-known hub of honey production due to the potential and diversified botanical, floral and geographical conditions to harvest varieties of honey types. Jimma town is where these products become destination from different part of the region for large commercial activities. Honey largely consumed locally as food (juice), honey beer (*tej*) and for therapeutic purposes of some disease. Ethiopia is also striving to enter the global market by exporting pure honey even though, awareness and practice to maintain honey quality is compromising. Recently, intentional adulteration is a serious problem; reducing its nutritional composition and medicinal use, altering its characteristic effect on physicochemical and biochemical properties of honey. Most honey consumers in Ethiopia suspect that honey venders intentionally adulterate honey by adding sugar. However, no sufficient scientific justification has been reported to what extent the rumors of sugar intentional adulteration affect the physicochemical and biochemical properties of honey. That is why this study was aimed to evaluate the effects of adulterating honey with different amount of sugar on physicochemical and biochemical properties of honey.

1.2 Objectives of the study

1.2.1 General objectives of the study

- The main objective of this study was to investigate physicochemical and biochemical properties of pure (unadulterated) and adulterated honey with various amount of sugar.

1.2.2 Specific objectives of the study

- To determine physicochemical parameters such as moisture content, electrical conductivity, ash content, pH value and acidity of the pure (unadulterated) honey and intentionally adulterated honey with various amount of sugar.
- To analyze biochemical properties including, sugar content, hydroxyl-methyl-furfural (HMF), total phenolic content and antioxidant activities of the pure (unadulterated) honey and intentionally adulterated honey with various amount of sugar.
- To compare the obtained results with other reports and international honey quality standards.

1.3 Significance of the study

The result obtained from this study was crucial to support enforcement on preventing adulterated honey production and it could be used as an additional source of information for researchers who wants to conduct their research on adulteration of honey. Additionally, it could give information for stake holders and institution whose working on problems related to adulteration of honey.

2. Literature review

2.1 Honey composition and its diversity

Honey is a natural sweet substance produced by honey bees and honey dew. Honey bees collects nectar of some plants which contain (80– 95%) water and (5– 20%) sucrose and combine with some specific substances. Mature honey bees transport the nectar to the hive, a protein enzyme in their stomach, called invertase breaks the sucrose in to two simpler sugars fructose and glucose. Dry matter of honey contains fructose and glucose (95 – 99%) and fructo-oligosaccharides (4 – 5%) and trace amounts of sucrose, maltose, isomaltose, maltotroise, melazitose, melibose, nigerose, turanose and panose. And additionally, it contains substances like minerals, enzymes, vitamins, amino acids, nitrogenous compounds, phenol, flavonoids and antibiotic rich inhibine. It has been reported that honey contains around 200 substances [1, 2, 14 – 16].

Honey diversity is the most important to describe the properties of chemical composition of honey. Biodiversity is one of the major sustainable functioning of ecosystem. Complex landscape enhances resource diversity or honey diversity. Honey diversity can result in increased consumer community diversity and broader ecosystem function. *Apismellifera* of honey bees are native subspecies in Africa [16 – 18].

Honey is graded according to its color and optical density by united states department of Agriculture (USDA) standards, on scale called the pfund scale, which ranges from 0 – 114, colors from “water white” to “dark amber” honey.

Table 1: Color Designation of honey [19]

USDA color standard	Pfund scale (mm)
Water white	≤ 8
Extra white	>8 and ≤ 17
White	>17 and ≤ 34
Extra Light Amber	>34 and ≤ 50
Light Amber	>50 and ≤ 85
Amber	>85 and ≤ 114
Dark Amber	>114

Honey can be classified depending on floral (flower nectars), regional locations or blended after collection. Also, honey depends on its packaging and processing used. Bottling honey in a liquid form is familiar; but commercially subjected to various processing methods. These are, crystallizing (granulating and candying) and Pasteurizing at 72^{o}c which can destroy yeast cells. Excessive heat expose to the increment of amount of hydroxyl-methyl-furfural (HMF), affects appearance, taste and fragrance; also, it reduces enzyme (e.g diastase) activity. The complex mixture, very great variation in composition and characteristics is due to its climatic, geographical and botanical origin. Also, it depends on the floral origin or the nectar utilized by bees [1, 4, 11, 19].

Ethiopia is the largest honey producer in Africa; thus, fourth in beeswax and tenth in honey production in the world. The countries' environmental conditions are conducive for the growth of over 7000 species of flowering plants. According to Gemeda and coworkers, 24,600 to 43,000 tons of honey produced per year in Ethiopia [3].

2.2 Medicinal and Nutritional Uses of Honey

Honey possesses higher antimicrobial activity than commonly used antibiotics. Honey has been used to treat ailments including gastric disturbances, ulcers, wounds, and burns. Honey syrup supports health and recovery. The remarkable effect of honey cleaning up of wounds is due to a combination the osmotic outflow and a bioactive effect of honey. The enzyme glucose oxidase of honey provides glucose to leucocytes, which is essential for respiratory burst to produce hydrogen peroxide leading to antibacterial activity of macrophages. Osmotic outflow after the application of honey assists in lifting dirt and debris from the bed of the wound. Honey does not lead to the development of antibiotic resistant bacteria, and can be used continuously. On burns, it has an initial soothing and rapid healing effects later. It is a wound barrier against tumor implantation in laparoscopic oncological surgery. Honey has a supportive effect on patients who have undergone a cancer radiation therapy by reducing the incidence of radiation mucositis. Additionally, honey intake increases heart frequency and level of glucose in blood during athletic performance. Honey is used in cooking, baking, as spread on bread, and addition to various beverages. In Ethiopia honey is almost used for local consumption to a very large extent (80%) for brewing of local beverages known as honey beer, locally called '*tej*' and juice [1, 3, 6, 7, 11].

2.3 Adulteration and its impact

Adulteration is a result of reduction in nutrition and medicinal value. Testing quality of honey is needed to reveal authenticity of the product by identifying the presence of artificial components or adulteration to address market needs. Adulteration alters quality characteristics of honey. Honey can be adulterated by addition of substances like sugar, inverted beet syrup, maltose syrup, and fructose corn syrup and higher fructose corn syrup. Changes those can occur due to adulteration are enzymatic activity, electrical conductivity, contents of specific compounds like HMF, glucose, fructose, sucrose, maltose, isomaltose, proline, and ash when compared with unadulterated honey. Some parameters like HMF may be ambiguous because HMF and enzymatic activity vary in different honeys and can be affected by heat [8, 11].

Adulterant is any material which could be added for making the food unsafe, substandard, and misbranding. Honey adulteration is done by indirect adulteration by feeding honey bees with industrial sugars at the stage when broods become naturally available. Due to direct adulteration by addition of foreign substances to honey, a serious problem is occurring now a day's. Honey adulteration occurs by direct addition of sucrose syrups that are produced from sugar beet, high-fructose corn syrup (HFCS), maltose syrup or by adding industrial sugar (glucose and fructose), syrups obtained starch by heat, enzyme or acid treatment, or by feeding the bee colonies excessively with these syrups during the main nectar period. Cane sugar is also commonly used adulterant in honey in Ethiopia. According to Kong and coworker's antioxidant activity of several samples of brown sugar made of sugarcanes and suggested that a number of phenolic acids and flavonoids accounted for at least partially the observed antioxidant activity. Phenolic substances in sugarcane juice may have biological activities [9, 10, 20].

One of the indicators for the degradation of quality of honey is its color. The color of honey is a sensory parameter that varies between different types of honey and dependent on mineral content and polyphenols content. The dark and amber honeys have higher content of minerals when compared with light-colored honey. Transition metals influence for color formation by forming complexes with some organic compounds. The color of honey ranges from 0 – 114, colors from "water white" to "dark amber" on the pfund scale according to United states department of Agriculture (USDA) standards. Adulteration changes the color of honey depending on the adulterant materials. It is studied in Oromia that when honey is adulterated by molasses using

heat the color changes to black or looks like coffee. Honey separately in Arsi zone, due to addition of adulterants like sugar, ripened banana, wheat flower, potato, maize flower, pollen, empty combs, melted candy, molasses and hot water the color changed to yellow, brown yellow and brown on physical observation. The color of honey can be measured by the method of visual Lovibond comparator [1, 11, 13 – 15].

Adulteration can alter refractive index and water activity due to variation in moisture content. The refractive index of a substance is a ratio of the velocity of light in the substance to that in air. It is used to determine moisture content of honey. Adulteration causes problems in human's daily life unsafe and unhygienic for use. Honey adulteration is a serious problem causing serious diseases like cancer, diarrhea, asthma, ulcers. So, it affects producers/farmers, processers, or manufacturers, consumers and government [1, 10, 11].

Honey can be detected by physical (traditional analysis) ways commonly, but there are several analytical methods; using differential calorimetry, carbon-isotope, GC, HPLC, NMR, UV-Vis, Vis-NIR, FTIR, Raman and soon, but the spectrometric techniques are preferred since they are commonly used for food authentication. The formers including chromatographic methods are expensive, time consuming, destructive and require skilled operator. The NIR spectra of any sample are influenced by its physicochemical properties and pose some problems in evaluating the important aspects of the sample. Both pure and adulterated honey samples can be analyzed in the range of 400 – 2500nm with spectral resolution of 0.5 nm, which contain spectral region of Vis-NIR using FOSS XDS rapid content^{TM} with XDS near infrared technology, by observing the difference between the pure and adulterated one [8, 9, 14, 21].

2.4 Physical and chemical properties of honey

2.4.1 Physical properties of honey

2.4.1.1 Electrical conductivity of honey

According to international honey commission 2019, electrical conductivity of honey is defined as that of 20% weight in volume solution in water at 20^{o}c, where 20% refers to dry matter of honey. Electrical conductivity of honey is one indicator of quality of honey. Electrical conductivity is another parameter to identify adulterated honey among the pure honey.

According to Damto and coworkers the electrical conductivity of honey is closely related to the concentration of mineral salts, organic acids, and proteins and characterized by a conductance of near zero and adulteration cause to decrease. The minerals and organic acids dissociate in aqueous solution in to ions which can conduct electricity. The bright color of honey usually points to a lower conductivity than dark color of honey. As it is mentioned in Pauliuc and coworkers, this parameter is specified by standards to be 500 to 800 $\mu S.cm^{-1}$for honeydew/mixed honey/ and <500 $\mu S.cm^{-1}$ for pure floral honey with some exception. If this value exceeds 800 $\mu S.cm^{-1}$, it is considered to be adulterated. Generally, it depends on the type of adulterant added [11, 13, 14, 22].

2.4.2 Chemical properties of honey

2.4.2.1 Moisture content

The moisture /water/ content of honey is a measure of stability and resistance to fermentation. Moisture content can be affected by climate, season and moisture content of plant nectar. It can be determined by using gravimetric method. In most cases the water content of honey varies from 15 – 20%; so quality and storage of honey depend on this. The higher the water content the higher is yeast in honey. Pure honey is suggested to have 14 – 18% water content. But content can vary due to adulteration by addition of starch (21.2 - 39.6%), addition of glucose (20.7 - 39.6%). Honey that contains water more than 18% is more likely to get fermented during prolonged storage. However, it is mentioned water content of honey to be less than 20% according to codex alimentarius 2019 for honey. Fermentation occurs when honey is harvested prematurely since it contains higher level of water [1, 4, 11, 23].

2.4.2.2 Ash and mineral content

The main minerals present in honey originate from soil and transported to trees by the roots. Minerals present in honey mainly are calcium, copper, iron, magnesium, manganese, potassium, sodium, chlorides, phosphates, silicates and sulfates. The composition of metals in honey is affected by geochemical and geological features. Soil and plants are natural resources that have a great influence on the mineral composition of honey. Other factor affecting mineral composition is polluted site where honey is originated; that is contamination by heavy metals from emissions

of gases and particles. In most cases the ash (mineral) content of a pure honey increases by adulteration depending on the type of adulterant. The ash content of pure honey is increased by 0.15% due to addition of higher fructose corn syrup 10 to 50% (w/w). Ash content can be determined method used by Lawal and coworkers [1, 11, 12, 24].

2.4.2.3 Sugar content of honey

Carbohydrate is largest content of honey. Honey carbohydrates contain 70 monosaccharide's (Fructose and glucose), 10% disaccharides, and small amount of trisaccharides and tetrasaccharides. Both fructose and glucose are reducing sugars and sucrose defined to be non-reducing sugars which can be hydrolyzed either by mineral acids or by enzyme invertase. The dry matter of honey comprises 95% of carbohydrate. Sucrose level exceeding 5% will suggest adulteration. Addition of sugar products increases sucrose content from 3.81% for pure honey to 9.8% for mixture of honey and sugar. Reducing sugars are determined by using lane-Enyon method [1, 4, 5, 11].

2.4.2.4 Acidity and pH

The acidity of honey developed due to the presence of organic acids. The increment of total acidity may mean honey had fermented at some time, and the resulting alcohol will be converted to organic acids. Total acidity is ranged between 17.97 – 49.1 meq/kg. Honey is mildly acidic, Lactonic acid and gluconic acids are those present in honey in different ranges; the pH of honey ranges between 3.4 to 6.1 and most of it has the average pH 3.9. But adulteration either increases or decreases the pH depending on the type of adulterant, so determining acidity helps to know the freshness of honey [10, 11, 13, 14].

2.4.2.5 Aroma and flavor

More than 600 volatile organic compounds (VOCs) are identified in honey. These are derived from the plant or the nectar source or transformation of plant compounds by metabolism. The aroma and flavor of honey is mainly because of these volatile organic compounds. Honey contains a wide range of phenolic acids and flavonoids those can exhibit antioxidant activity.

The aroma, color and taste of honey are due to the presence of phenolic compounds and flavonoids (flavonols, flavones and flavonones) [15, 25, 26].

2.5 Biochemical properties

2.5.1 Hydroxyl-methyl-furfural (HMF)

Hydroxyl-methyl-furfural (HMF) is a solid yellow substance that has a low melting point but is highly soluble in water. Hydroxyl-methyl-furfural (HMF) is acyclic aldehyde produced by degradation through the Millard reaction or a breakdown product of fructose /decomposition of fructose/ that is formed slowly and naturally during the storage of honey and quickly when heated. The presence of simple sugars, acids, and minerals in honey further enhance the production of HMF. Additionally, HMF can be produced from oligo-saccharides and polysaccharides that can yield hexos up on hydrolysis. Reasons for higher yields of HMF from fructose (ketose) than glucose (aldose) is the reactivity of glucose to be less than fructose with a lower enolization rate which is rate-determining step for the formation of HMF, and fructose forms equilibrium mixture of difructoses and dianhydrides, thus internally blocks most reactive groups leading to formation of by-products. But glucose forms true oligosaccharides that still contain reactive reducing groups which can possess cross-polymerization with reactive intermediates including HMF. The amount of HMF in honey is used as reference guide to the quality of honey (40 mg/kg) but differ according countries. According to codex alimentarius for honey 2019, the hydroxyl-methyl-furfural (HMF) content of honey shall not be more than 40 mg/kg after processing and/or blending. But for honey of declared origins with tropical ambient temperatures, blends of these honey shall not be contained more than 80 mg/kg of HMF. The concentration of HMF is a parameter affecting honey freshness. Fresh honey contains very small amount of HMF. While the concentration rises during processing and because of prolonged storage. Enzymes in honey are invertase, glucose oxidase, amylase and etc. The level of enzymes in honey is in small amount and sometimes used to identify its quality. Enzymes can be distorted at higher temperature (above 35^{o}c) [10, 23, 27, 28].

At high concentration, HMF has cytotoxic effects causing irritation in the mucous membranes, skin, eyes and upper respiratory tract. In addition, it causes inhibition of DNA, poly deoxynucleotidyl transferase, decreased cellular glutathione level, and hepato-toxicities and renal

toxicities; but at recommended concentration it acts as antioxidant, increase survivability under hypobaric hypoxia, anti-allergen, anti-hyperucemic, anti-sickling agent and anti-carcinogenic [29].

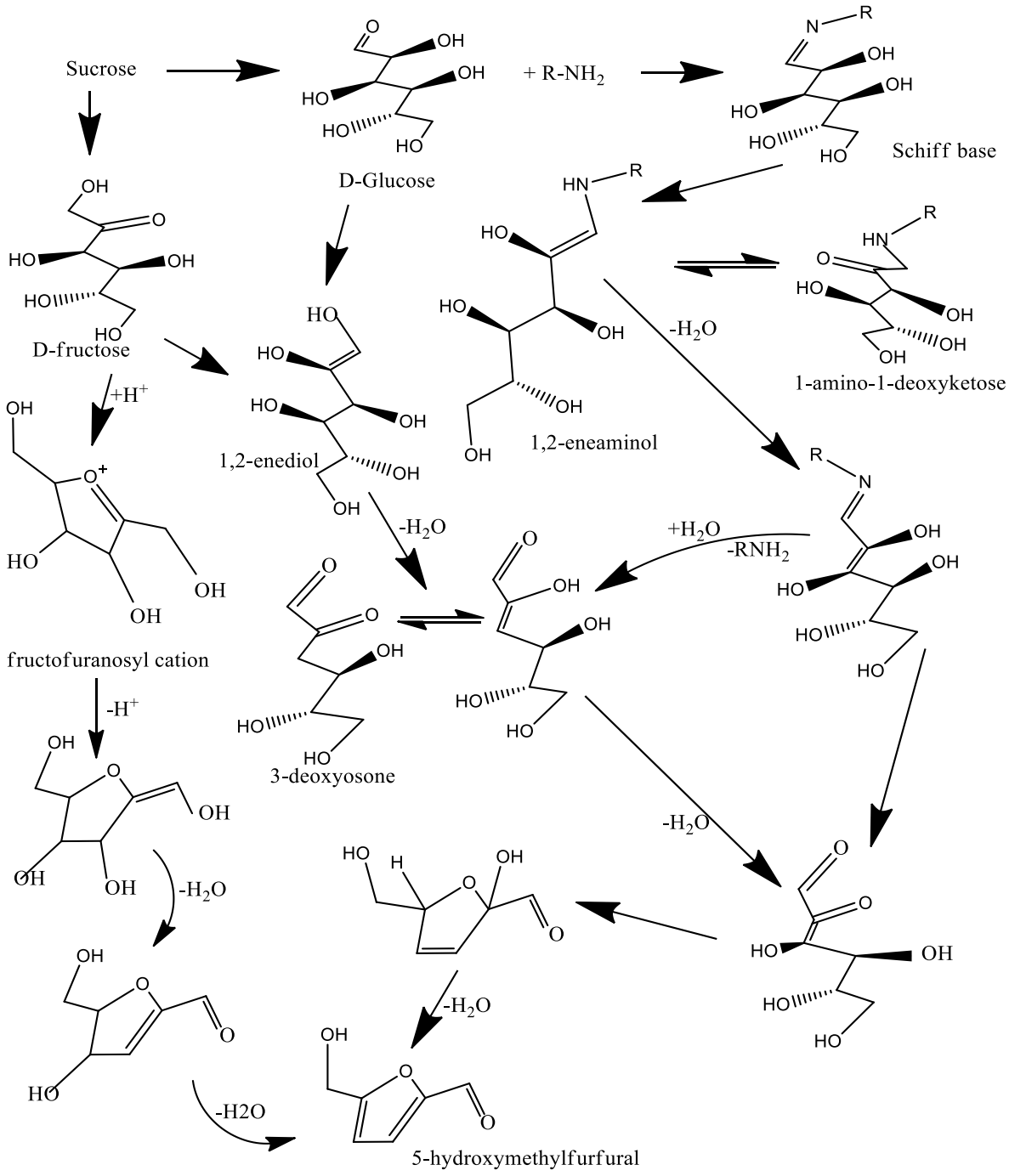

Figure 1: Scheme of formation of hydroxyl-methyl-furfural.

2.5.2 The antimicrobial activity of bee honey

Honey possesses higher antimicrobial activity (antibacterial and antifungal) than commonly used antibiotics. It enhances the healing of wounds and pressure sores. The mechanisms of antimicrobial activity of honey are different from antibiotics which destroy bacterial cell wall or inhibit intracellular metabolic pathways. Therefore; it draws moisture out of the environment, thus dehydrates bacteria. The pH range of honey (3.2 – 4.5) or lower acidity inhibits growth of most microorganisms. The enzyme glucose oxidase of honey provides glucose to leucocytes, which is essential for respiratory burst to produce hydrogen peroxide produced from glucose oxidase, is leading to antibacterial activity of macrophages. The antibacterial activity of honey is determined by the agar well diffused method with some modification as reported by Lewoyehu and coworkers [5, 6, 12].

2.5.3. Antioxidant activity of honey

Honey has been found to contain significant antioxidant activity. Antioxidant activity is a protection against oxidation of free radicals. The lack of equilibrium between the production of free radicals and the antioxidant protective activity in human body is thought to be oxidative stress. Anti-oxidative effects of honey are both as enzymatic antioxidants (glucose oxidase and catalase) and non-enzymatic antioxidants (L-ascorbic acid, flavonoids and phenolic acids). Honey is effective to increase total plasma antioxidant and inhibit the oxidation of other molecules. Phenolic compounds are those contributing antioxidant activities of honey. 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. Common antioxidants in honey are Gallic acid, caffeic acid, and chlogenic acid. Antioxidant activity of honey can be determined through DPPH, FRAP, ORAC, and TEAC; but DPPH is a quick, simple test and guarantees reliable results and only needs a UV-vis spectrophotometer. Antioxidant activity is the ability and potential of honey in reducing oxidative reactions within food systems. Notably, these oxidative reactions can cause deleterious reactions in food products (e.g. lipid oxidation in meat, and enzymic browning in fruits and vegetables) and adverse human health effects, such as chronic diseases and cancers [5, 7, 25, 29].

2.5.4 Total phenolic content of honey

The modern classification of phenolics states two types of phenolics; simple phenols and polyphenols. Simple phenols contain phenolic acids. The presence of phenolic acids, flavonoids, ascorbic acid, carotenoids, catalase, peroxidase, are constitutes phenolic contents. Phenolic compounds, nitrogen compounds (alkaloids, chlorophyll derivatives, amino acids, and amines), or carotenoids as well as ascorbic acid are natural antioxidants. Phenols are very efficient scavengers of peroxyl radicals because of their molecular structures which include an aromatic ring with hydroxyl groups containing mobile hydrogen. Moreover, the action of phenolic compounds can be related to their capacity to reduce and chelate ferric ion which catalyse lipid peroxidation. According to Cheung and coworkers, phenolic acids constitute a wide range among phenolic compounds in honey. The most dominant phenolic acid in honey is gallic acid. They are dependent on botanical origin of honey. They contribute antioxidant effects and a marker of quality of honey [14, 15, 25, 29, 30].

3. Materials and Methods

3.1 study area

The study was conducted beehive honey from Jimma city venders and surrounding honey producers with cooperation. Jimma is located south-western of Oromia Regional state, which is 342 km far from Addis Ababa. The study was covered from August, 2020 to January, 2021.

3.2 Sampling technique and Sample collection

Pure (unadulterated) honey samples were collected from selected carefully (having quick test) from honey producer farmers in Jimma city and zonal districts depending on their colors and production area. Honey samples (1–2 kg) of each selected supplier were collected by using purposive sampling technique and transported to Jimma University analytical chemistry laboratory for further experiment.

3.3 Quick test for adulteration

The entire collected representative honey samples were evaluated at regular intervals before intentional addition of adulterant. Preliminary assessment (physical observation) was made to identify whether the honey was adulterated or not. The following observations of physical tests were used to identify pure and adulterated honey samples [4].

Flame test: The samples were ignited using candle flame to identify whether the flame is smokeless or not.

Heating effect: The honey samples were heated gently until it was dissolved and whether a clear transparent viscous solution (while wax materials floating on top) was observed or non-transparent, dispersed.

3.4 Instruments and apparatus

Instruments used in this study were thermo stated water bath supplied from (Grant Instruments (Cambridge) Ltd, England) was used for sample homogenizing, portable pH/conductivity multi-parameter (Bante902P, USA) used to measure pH value and electrical conductivity, Atago Abbe Refractometer (supplied by Bellinghant Stanley Ltd, England) used to measure the refractive index of honey samples, Karl Kolb Muffle Furnace (Nbertherm, Germany) used to determine ash

content, UV-VIS Double beam Spectrophotometers (SPECORD®200/PLUS, Germany) used to determine the absorbance of (HMF, TPC, DPPH), vortex mixer model FB15024 obtained from Fisher scientific (Merelbeke, Belgium) used to mix sample solutions, Microwave oven model (OV150SS, England), Heating mantle model CM2000/CE (Branstead/Electrothermal, UK) used to carbonize sample, magnetic stirrer, Whatman filter paper, desiccators, Pore size crucible, and ashing crucible.

3.5 Chemicals and reagents

Chemicals used were; Sodium hydroxide (NaOH) used for titration, Hydrochloric acid (HCl, 37%) used for inversion of sugar, deionized water, carrez solution I (potassium ferrocyanide $[K_4(Fe(CN)_6)].3H_2O$) was obtained from RIEDEL-DE HAEN AG SEELZE-HANNOVER (Rupert-Mayer-Str. Munich, Germany)) and carrez solution II (zinc acetate $(Zn(CH_3COO)_2.2H_2O)$ was obtained from Merck laboratory chemicals (Nagpur, Maharashtra, India) used in HMF analysis, methanol (CH_3OH) used as solvent, ascorbic acid used as positive control, methylene blue 0.2%, standardized Fehling solutions A & B ((Copper sulfate, $CuSO_4).5H_2O$, and potassium sodium tartrate $KNaC_4H_4O_6.4H_2O$) used in sugar analysis, 0.5 McFarland standard, methanolic solution containing DPPH radicals (0.024 mg/mL), Folin-Ciocalteu reagent used in TPC analysis, Na_2CO_3, gallic acid solutions used for calibration, buffer solutions from Blulux Laboratory reagent Ltd. (Faridabad, Haryana, India).

3.6 Sample preparation and pretreatment

Before the analysis for each parameter, samples were arrived laboratory; foreign matters such as wax, bees and particles of comb were separated manually according to international guideline. The resulting samples were mixed thoroughly by stirring before heating to 60 – 65 ^{o}C in water bath until it was liquefied. The liquefied honey then filtered by using 0.5 mm sieve mesh before cooling down. Then, it was kept in suitable condition in laboratory until further analysis.

3.6.1 Intentional adulteration of honey with sugar

Method used by Lewoyehu and coworkers, was modified for intentional adulteration. Before intentional adulteration; pure honey and pure sugar were analyzed for the selected parameters. Then the adulteration of honey, intentionally carried out by the proportion of honey to sugar ratio

as (8:2), (7:3) and (5:5) in grams at 45^{o}C for each composite light amber and amber honey mentioned above [5].

3.6.2 Color analysis

To determine color, honey sample was heated to 50 ^{o}c to dissolve sugar crystals, and the color was determined by measuring the absorbance of 50% honey solution (w/v) at 635nm. The honey samples were classified according to the Pfund scale after conversion of the absorbance value [19].

Intensity of honey color in the Pfund scale = (-38.70 + 371.39) x Abs ……1

3.6.3 Determination of honey moisture

The moisture content of honey was determined using refractometry at 20 ^{o}C. The refractive index of distilled water (1.33) was used as reference. The prism of the refractometry was calibrated by measuring refractive index of distilled water. Then the prism was covered by homogenized honey and the refractive index of each honey samples were measured in triplicate. The method was done by correlating refractive index of honey with its moisture content from table presented in standard method [31].

3.6.4 Determination of electrical conductivity

The electrical conductivity (EC) of a honey defined as that a 20% w/v of solution at 20^{o}c (20% dry matter basis) in CO_2-free deionized distilled water. Accordingly twenty gram of dry matter of honey was weighed and dissolved in 100 mL distilled water. The resulting solution was poured in a beaker and placed on hot plate at 20 ℃. Then the electrical conductance was measured in µS/cm using conductometer by immersing conductivity cell in to the sample solution (AOAC, 1990) [4, 13].

3.6.5 Determination of total ash content

Five gram of each honey sample was separately weighed out in to a porcelain crucible previously ignited and weighed. The organic matter was charred by igniting the sample on a hot plate in the fume cupboard. The crucible then placed in the muffle furnace and maintained at 600^{o}c for 6

hour. Then it was cooled in a desiccators and weighed immediately (AOAC, 1990) [12]. The percent ash was calculated as;

$$\text{Ash (\%)} = \frac{(weightofcrucible + ash) - (weightofemptycrucible) \times 100}{sampleweight} \quad \ldots\ldots\ldots 2$$

3.6.6 Determination of pH value and free acidity

The sample solution was prepared by dissolving 10 g sample in 75 mL CO_2-free water in a 250 mL beaker. Then, the solution stirred by magnetic stirrer, pH electrode was immersed in the solution and pH was measured and recorded. For acidity measurement the solution was titrated with 0.1M NaOH to pH 8.30(free acidity) for 2 minutes using 10 mL burette and the volume consumed was recorded. Then the free acidity was obtained by multiplying 10 times volume of 0.1M NaOH used for titration, and expressed in meq/kg [31].

3.6.7 Determination of reducing sugar and sucrose

For the determination of reducing sugar and sucrose modified Lane and Lynon procedure according to harmonized method international honey commission 2009 was used.

Preliminary titration was conducted in order to determine suitable amount of distilled water used. The total volume of the added reactants at the completion of the reduction titration must be 35 mL. Accordingly 5 mL Fehling's solution A and 5 mL Fehling's solution B was pipetted into a 250 mL Erlenmeyer flask. Then 7 mL distilled water added, followed by about 15 mL diluted honey solution from burette. After heating the cold mixture to boil over wire gauze, it was maintained to moderate ebullition for 2 minutes. 0.2 % aqueous methylene blue solution was added whilst still boiling and the titration completed within a total boiling time of 3 minutes, by repeated small additions of diluted honey solution used.

Titration

5 mL Fehling's solution A and 5 mL Fehling's solution B was Pipetted into 250 mL Erlenmeyer flask and add approximately. Eight mL distilled water was added, from a burette, 16.5 ml of the diluted honey solution volume determined in the preliminary titration (18 – 1.5) was added. The cold mixture was heated to boil over wire gauze and maintained at moderate ebullition for 2

minutes. One milliliter of methylene blue solution (0.2%) was added whilst still boiling and the titration completed within a total boiling time of 3 minutes by repeated small additions of diluted honey solution until the indicator was decolorized. Duplicate titration was done [31].

3.6.8 Determination of hydroxyl-methyl-furfural (HMF)

Determination of HMF was done according to harmonized methods of international honey commission 2009. The absorbance of clarified aqueous honey solution is determined against reference solution of the same honey. The chromophore of HMF at 284 nm was destroyed by addition of bisulphite. Thus; 5 g of honey was dissolved in 25 mL deionized water and the solution was transferred to 50 mL volumetric flask including washing the residue. Then, the solution was mixed with 0.5 mL carrez solution I and 0.5 mL carrez solution II. The flask was filled with deionized water. The first 10 mL of titrate was rejected by filtering through filter paper. Five milliliter of the titrate was added in to two test tubes each and 5 mL of deionized water was added in to one test tube and 5 mL 0.20 % bisulphite in to the other test tube. Each test tube was mixed using vortex mixer. At last the absorbance of the sample was measured against the reference at 284 nm and 336 nm [27, 31]. The HMF content of honey was calculated as;

$$\text{HMF (mg/kg honey)} = \frac{(A_{284} - A_{336}) \times 149.7 \times 5 \times D}{W} \quad \ldots\ldots\ldots\ldots \; 3$$

Where W = wt of sample (g), A_{284}, A_{336} = absorbance reading, Factor $= \frac{126 \times 1000 \times 1000}{16830 \times 10 \times 5} =$ 149.7, 126 = MW of HMF, 16830 = molar absorptivity of HMF at 284 nm,

D = (Final volume of solution/10) ………………………………………………………... 4

3.6.9 Antioxidant activity of honey

The antioxidant activity of honey was determined using DPPH method by using UV-vis spectrophotometer; thus 2 g of honey sample was dissolved in 20 mL methanol at concentration of 100mg/mL, vigorously shaken and filtered using filter paper. 0.5 mL of honey extract was mixed with 2.7 mL of methanolic solution containing DPPH radicals (0.024 mg/mL). Then, the mixture was left in the dark for 15 min and the absorbance was measured at 517 nm. Ascorbic acid is used as positive control [5]. Thus; DPPH scavenging activity was calculated as;

DPPH scavenging activity (%) = $\frac{A_{control} - A_{sample}}{A_{control}}$ x100% 5

Where $A_{control}$ is absorbance of control, A_{sample} is absorbance of sample

The percentage of scavenged DPPH was then plotted against the sample concentration to calculate graphically the amount of antioxidant required to decrease the initial DPPH concentration by 50% (IC_{50}), expressed in terms of (mg/mL) [32].

3.6.10 Determination of total phenolic content of honey

For the determination of total phenolic content method by Pauliuc and coworkers modified/optimized for these samples. Accordingly 5 g of honey sample was extracted with 50 mL of 70% methanol. The samples were stirred for 15 min with a magnetic stirrer. From the extract 1 mL was mixed with 5 mL of Folin-Ciocalteu reagent and 4 mL Na_2CO_3 10% (w/v). Then samples were kept in a dark place for 15 min and the absorbance was measured at 760 nm using UV-Vis spectrometer. A Gallic acid solution with concentrations 0, 5, 50, 100, 150 and 200 $mg.L^{-1}$ was used for calibration curve [14, 33].

3.7 Method Validation tools

The method of this study was validated through Accuracy, precision, detection limit and quantification limit.

3.8 Method of data analysis

Analysis was performed in triplicate, and the results are expressed as Mean±SD. the significance difference and correlation was tested by ANOVA at ($p \leq 0.05$) [11]

4. Results and discussions

4.1 Quick test for adulteration

The entire collected representative honey samples were evaluated at regular intervals by preliminary assessment (quick test) to identify the commonly used local adulterants (Table 2). The observations of physical tests were used to identify pure and adulterated honey samples. The flame test of the honey samples demonstrated smokeless flame and clear transparent viscous solution on heating. This result implies that the honey samples were pure (unadulterated) [4].

Table 2: Physical test observation

Test parameter	**Samples**	**Character observed**	**Suggestion**	
			Pure	**Adulterated**
Flame test	Pure LA	Gave smokeless flame	√	
	LA(8:2)	Gave smoky flame		√
	LA(7:3)	Gave smoky flame		√
	LA(5:5)	Gave smoky flame		√
Heating effect	Pure Am.	Clear transparent viscous solution	√	
	Am(8:2)	Dispersed and partially transparent liquid		√
	Am(7:3)	Dispersed and partially transparent liquid		√
	Am(5:5)	Dispersed and non-transparent liquid		√

4.2 Physico-chemical and biochemical properties of pure and adulterated honey

Results of the physico-chemical properties such as color, MC, EC, Ash content, pH values, FA, RS, SC, HMF, TPC and Antioxidant activity of honey samples are presented in Table 3.

Table 3: Physico-chemical and biochemical properties and antioxidant activity of honey samples

Honey Type	**L.A**	**L.A 8:2**	**L.A 7:3**	**L.A 5:5**	**Amber**	**Am 8:2**	**Am 7:3**	**Am 5:5**	**Sugar**
Color (Pfund , mm)	68.56 ± 0.12	47.90 ± 0.04	39.72 ± 0.12	28.77 ± 0.02	102.66 ± 0.11	79.20 ± 0.12	75.32 ± 0.03	55.43±0.03	12.12 ± 0.13
Moist. Cont (%)	19.62 ± 0.07	16.31 ± 0.10	13.42 ± 0.16	12.88 ± 0.00	19.44 ± 0.13	16.09 ± 0.10	13.38 ± 0.16	12.96 ± 0.01	ND
Elec. Cond (µS/cm)	180.94 ± 2.44	157.83 ± 0.29	147.83 ± 0.15	117.90±0.36	778.78 ± 1.62	763 ± 1.00	731.67 ± 0.58	667.00 ± 3.00	17.70 ± 0.12
Ash (%)	0.36 ± 0.00	0.31 ± 0.00	0.28 ± 0.01	0.2 ± 0.01	0.41 ± 0.00	0.37 ± 0.01	0.33 ± 0.01	0.25 ± 0.00	0.015±0.001
pH value	3.95 ± 0.01	3.96 ± 0.00	3.97 ± 0.00	4.00 ± 0.01	4.08 ± 0.08	4.12 ± 0.02	4.15 ± 0.01	4.19 ± 0.01	5.89 ± 0.16
Free acidity (meq/kg)	42.50 ± 0.50	33.17 ± 0.76	30.00 ± 1.00	18.50 ± 1.32	38.66 ± 0.58	31.17 ± 1.04	21.67 ± 0.76	12.83 ± 0.76	ND
Reducing sugar content (%)	61.41 ± 0.11	51.31 ± 1.32	45.63 ± 0.80	42.57 ± 0.90	62.83 ± 0.30	53.34 ± 0.71	48.82 ± 0.54	45.01 ± 0.52	ND
Sucrose content (%)	4.23 ± 0.02	13.82 ± 0.94	18.94 ± 0.56	22.55 ± 0.55	4.65 ± 0.04	14.10 ± 0.36	18.73 ± 0.23	25.47 ± 0.42	95.00 ± 0.15
HMF (mg/Kg)	42.33 ± 1.05	23.03 ± 1.50	4.39 ± 0.55	ND	34.73 ± 2.74	5.76 ± 1.78	ND	ND	ND
Total phenol content (mg. GAE/100g)	145.00 ± 01.40	169.06 ± 0.02	173.26 ± 0.00	178.40 ± 0.00	149.40 ± 1.54	162.53 ± 0.00	167.75 ± 0.00	178.30 ± 0.01	39.3 ± 1.13
Antioxidant Activity (IC_{50} mg/ml)	28.48	30.41	33.44	36.34	30.46	32.31	35.67	41.25	95.77

L.A: Light amber, A: Amber

4.2.1 Color analysis

The color of honey is a sensory parameter that varies between different types of honey and dependent on mineral content and polyphenols content.

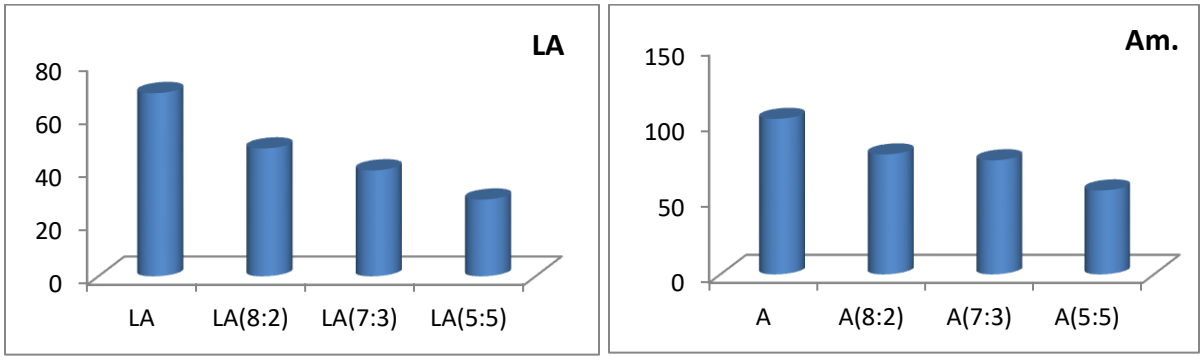

Figure 2: Color analysis for pure and adulterated light amber and amber honey samples

From Table 1; the Pfund scales in (mm) of the pure honey samples were identified as 68.56 ± 0.12 and 102.66 ± 0.11, implying light amber and amber color respectively. The color of both honey samples decreased its intensity as it was adulterated by sugar as 47.90 ± 0.04, 39.72 ± 0.12 and 28.77 ± 0.02 for light amber honey and 79.20 ± 0.12, 75.32 ± 0.03 and 55.43 ± 0.03 for amber per gram of the honey samples. When the honey samples were adulterated they gradually changed their color from light amber to white and amber to light amber. This adulteration trend was reported by Damto and coworkers; thus the color analysis reveals that the adulterated honey was brighter in color while the pure honey was more reddish [11].

4.2.2 Moisture content of honey

The moisture content is an important criterion to identify the shelf life of honey. High amount of water in honey can cause fermentation during storage. From, Table 3; the moisture content of the honey samples were 19.62 ± 0.07 for light amber honey and 19.44 ± 0.13 for amber. This result suggested to be good by honey standard according to codex Alimentarius, 2009, the moisture content shall not be more than 20%. This result shows some similarity for pure honey with 19.29 ± 1.62 reported by Gebremariam and coworkers. Also the result in line with samples of Yola (19.6%) and Ibadan (19.4%) reported in Lawal and coworkers; and 19.6% reported in Pauliuc and coworkers. But after adulteration water content per gram of the honey samples was decreased as 16.31± 0.105, 13.42 ± 0.156 and 12.88 ± 0.01; 16.09 ± 0.11, 13.38 ± 0.16 and

12.96 ± 0.01 for light amber and amber honey samples with significant difference at $p \leq 0.05$ [4, 14, 23].

4.2.3 Electrical conductivity of honey

The electrical conductivity of honey depend on ash content of the honey which intern varies depends on its botanical origin. The electrical conductivity of honey has close relationship with its mineral content.

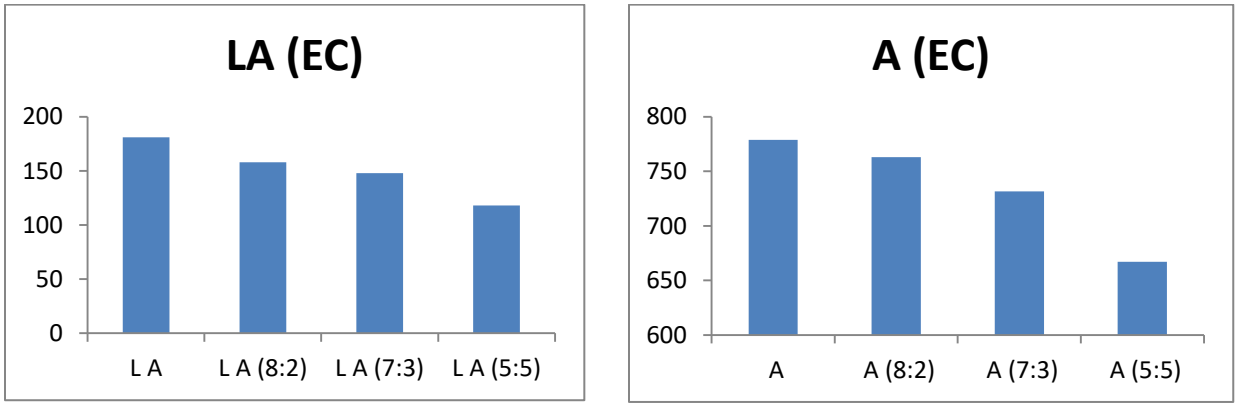

Figure 3: Electrical conductivity of light amber and amber honey samples

It is a parameter that shows great variability according to the floral origin and is considered one of the best parameters for differentiating between honeys with different floral origins, according to Lewoyehu and coworkers. The electrical conductivity results of the studied honey samples of the pure honey samples were 180.94 ± 2.44 for light amber honey and 778.78 ± 1.62 for amber honey. The pure honey samples showed similarity with result reported by Gebremariam and coworkers, which the honey samples had 130 µS/cm to 560 µS/cm electrical conductivity. Also this result satisfies codex Alimentarius for honey commission, 2009, since it recommends the electrical conductivity of honey shall be below 800 µS/cm. After adulteration the electrical conductivity was decreased to 157.83 ± 0.29, 147.83 ± 0.15 and 117.90 ± 0.36 for light amber honey, and 763.00 ± 1.00, 731.67 ± 0.58 and 667.00 ± 3.00 for amber honey, due to decrease in ionic species as a result of complex formation with large sugar and phenolic compounds. The adulteration trend of honey samples exhibited no significant difference at $p \leq 0.05$ for the electrical conductivity [4, 5, 23].

4.2.4 Ash content of the honey samples

Ash values depend on the mineral content of honey and directly measure inorganic residue after carbonization. The main minerals present in honey originate from soil and transported to trees by the roots.

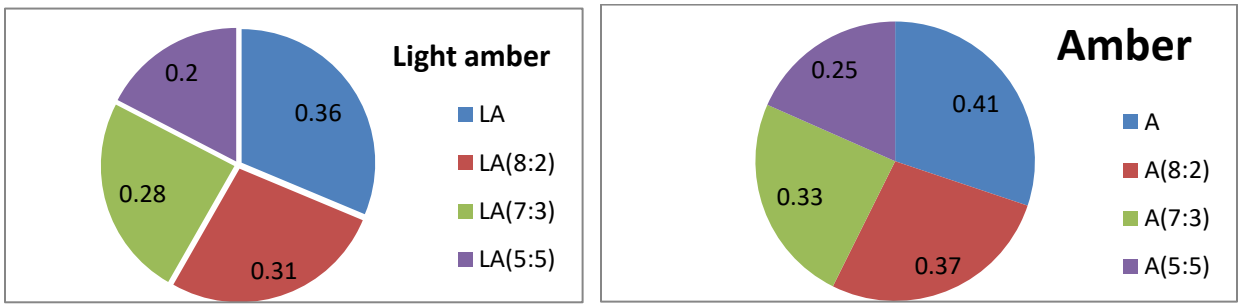

Figure 4: Ash content pure and adulterated honey of light amber and amber honey

The mean ash content of honey samples were 0.36 ± 0.00% for pure light amber honey and 0.41 ± 0.00% for pure amber honey. Thus it seems similar with report by Liberato and coworkers, which the ash content ranges from 0.01% to 0.71%. After adulteration the ash content of adulterated honey samples were decreased with increase in sugar ratios from 0.31 ± 0.00 – 0.2 ± 0.01% for light amber honey and 0.37 ± 0.01 – 0.25 ± 0.00% for amber honey with significant variation at $p < 0.05$ for different ratios of adulteration which might be the adulterant (sugar) organic molecule could evaporate at higher temperature (600 ^{o}C); this condition was also reported by Gebremariam and coworkers [4, 11, 24].

4.2.5 pH value and free acidity of the honey samples

One of an important thing for honey taste is stability and resistance to microorganisms is the acid content of honey which can be determined by pH meter.

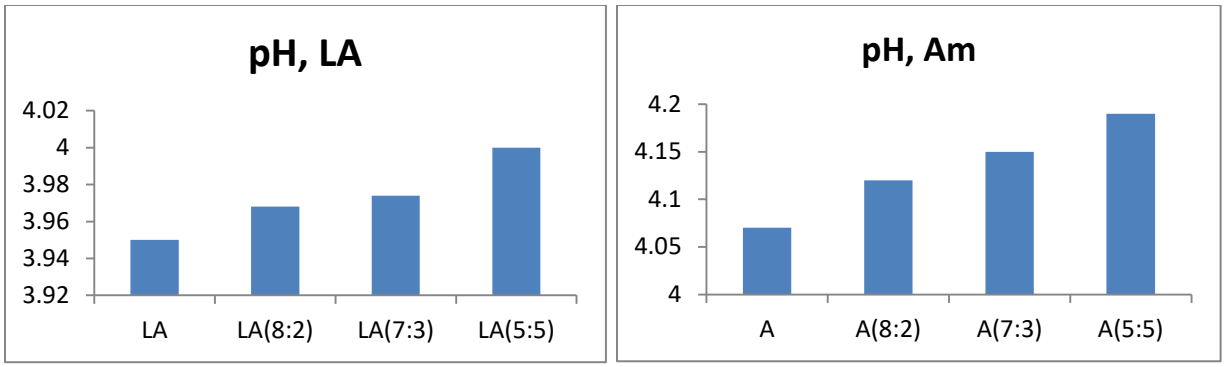

Figure 5: pH values pure and adulterated honey samples of light amber and amber honey

As reported by Damto and coworkers, pure honey normally contains relatively small amount of acid which is important for the honey taste. Thus, honey is mildly acidic and the average pH value for most honey sample was 3.9 which were closely similar with this result. The pH value of light amber honey was 3.96 ± 0.01 and 4.08 ± 0.08 for amber honey, and was similar with result 4.09 ± 0.36, and the pH values of the honey samples were increased as it was adulterated by sugar, decreasing the acidity of the honey according to Gebremariam and coworkers. The result was also similar and within the range of 3.58 ± 00 to 4.72 ± 0.01; which also in line with samples from Megusem (3.97 ± 0.01) and Tadjmount (4.08 ± 0.02) as reported by Zerrouk and coworkers. No significant differences were observed at $p \leq 0.05$ in pH value between the pure and adulated honey samples [4, 11, 13].

The acidity of honey is caused by organic acids and an important parameter for honey taste, stability and resistance to micro-organisms. The free acidity of honey samples in (meq/kg) were 42.5 ± 0.50 for light amber honey and 38.66 ± 0.58 for amber honey (Table 3), which shown similarity with the samples from Khat alouad and Ain Oussara 40.33 ± 0.28 and 40.08 ± 0.28 respectively as reported by Zerrouk and coworkers. Adulteration of the samples reduced free acidity as amount of sugar added increased from (8:2), (7:3) and (5:5) honey to sugar ratios and free acidity decreased as 33.17 ± 0.76, 30.00 ± 1.00 and 18.50 ± 1.32 for light amber honey; and 31.17 ± 1.04, 21.67 ± 0.76 and 12.83 ± 0.76 for amber honey (Table 3). The significant variation of free acidity at $p \leq 0.05$ will result in change in pH values to move to basic and neutral environment suitable for microbial growth [4, 13].

4.2. 6 Reducing sugar and sucrose content

Reducing sugar is the major component that governs honey property and its content is firmly related to the degree of maturity and botanical origin of honey. Honey storage and heating can affect its freshness. Figure 6 shows reducing sugar content of pure and adulterated honey.

The reducing sugar content (%) was 61.41 ± 0.11 for light amber honey 62.83 ± 0.30 for amber honey (Table 3). This content was decreased when the honey samples were adulterated by sugar as 51.31 ± 1.32, 45.63 ± 0.80 and 42.57 ± 0.90 for light amber honey samples and 53.34 ± 0.71, 48.82 ± 0.54 and 45.01 ± 0.52 for amber honey implying that, adulteration of honey with the sugar deteriorate the dominant and important type of monosaccharide sugars (glucose and fructose).

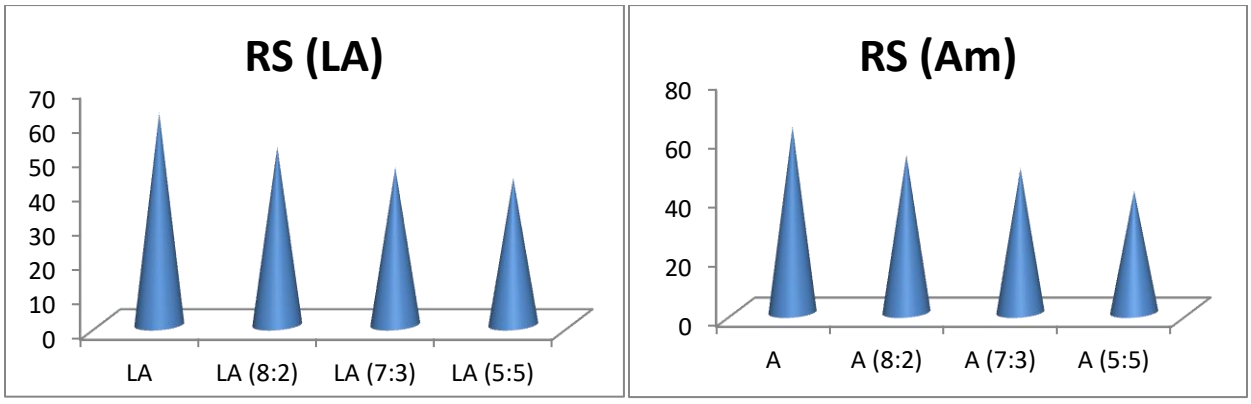

Figure 6: Reducing sugar content of pure and adulterated honey of light amber and amber honey

The sucrose content of pure and adulterated honey of light amber and amber honey are presented in Figure 7.

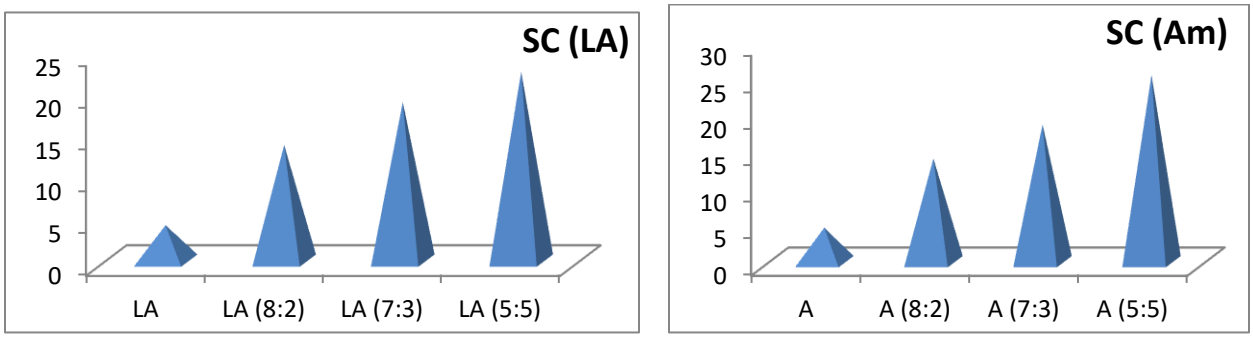

Figure 7: Sucrose content of pure and adulterated honey of light amber and amber honey

The sucrose content was 4.23 ± 0.02 and 4.65 ± 0.04 for light amber and amber honey samples respectively (Table 3). The sucrose content adulterated honey increased with the amount of sugar added. Thus, its content was 13.82 ± 0.94, 18.94 ± 0.56 and 22.55 ± 0.55 for light amber honey; and 14.10 ± 0.36, 18.73 ± 0.22 and 25.47 ± 0.42 for amber honey and their variation were significant at $p \leq 0.05$. The progress of sucrose content after adulteration was found to be significant and above the maximum limit (5 g/100 g honey) of codex alimentarius 2009, European union and Ethiopian standards. Inverse variation of reducing sugar and sucrose content seen in the result due to adulteration was also reported by Gebremariam and coworkers, in similar way [4, 23].

4.2.7 Hydroxyl-methyl-furfural (HMF) content

Hydroxyl-methyl-furfural (HMF) is a decomposition product of fructose. It is a major honey quality factor that indicates honey freshness and adulteration associated with overheating. The hydroxyl-methyl-furfural content (mg/Kg) of the honey samples were 42.33 ± 1.05 and 34.73 ± 2.74 for light amber and amber honey respectively (Table 3). As it was adulterated the content of HMF decreased. When light amber honey adulterated in (8:2) and (7:3) ratios as 23.03 ± 1.50 and 4.39 ± 0.55 respectively and for (5:5) ratios it did not detected; and the amber honey only detected for (8:2) ratio as 5.75 ± 1.78. Synthetic sugar mainly contains sucrose, as observed from the result in (Table 3) when adulterant concentration increase the amount of reducing sugar contributor for HMF formation significantly declined due to complex carbohydrates by linkage of monosaccharide unit; therefore this may result for decreased amount of HMF which is the byproduct of fructose. The Light amber honey which contains slightly higher than upper limit of codex alimmentarius commission 2009, ≤ 40 mg/kg [23] requires more sugar compared to the amber honey until the amount of HMF becomes not detected for both honey type at 5:5 equal ratio of honey and adulterant.

4.2.8 Total phenolic content

Phenolic compounds in foods are highly reactive. Depending on the geographical and climatic conditions and botanical origin, different types of honey contain a wide range of phytochemicals including polyphenols mainly flavonoids and phenolic acids [34].

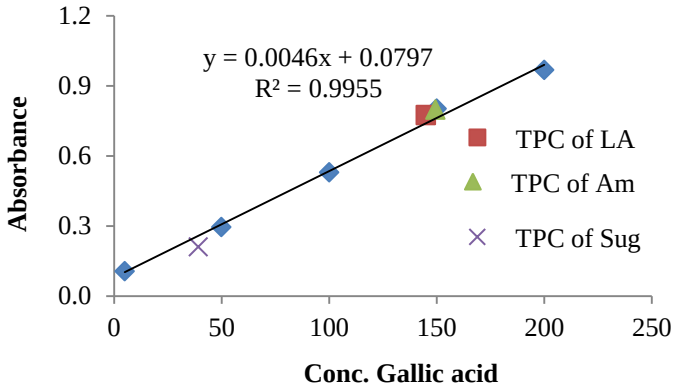

Figure. 8: Plot of calibration curve of Gallic acid equivalents

The total phenolic content in (mg.GAE/100g) was 145.00 ± 01.40 and 149.40 ± 1.54 for light amber honey and amber honey respectively (Table 3). When it was adulterated by sugar the total phenolic content increased to 169.06 ± 0.02, 173.26 ± 0.00 and 178.40 ± 0.00 for light amber honey and 162.53 ± 0.00, 167.75 ± 0.00 and 178.30 ± 0.01 for amber honey and this change might contributed from the phenolic content of adulterant sugar. The total phenolic content of sugar determined in this study was 39.25 ± 1.13, which is far different from the result (17.90 ± 0.30) reported by Kong and coworkers [20]. As a result pure sugar honey adulteration can contribute interims of increasing total phenol content of honey however; the declined vales of antioxidant activity of honey; which is considered as an implication of its medicinal value is quite questionable due to inverse correlation with increased amount of total phenol.

4.2.9 Radical scavenging activity by DPPH assay (% inhibition)

Antioxidant activity is a protection against oxidation free radicals. The lack of equilibrium between the production of free radicals and the antioxidant protective activity in human body is thought to be oxidative stress.

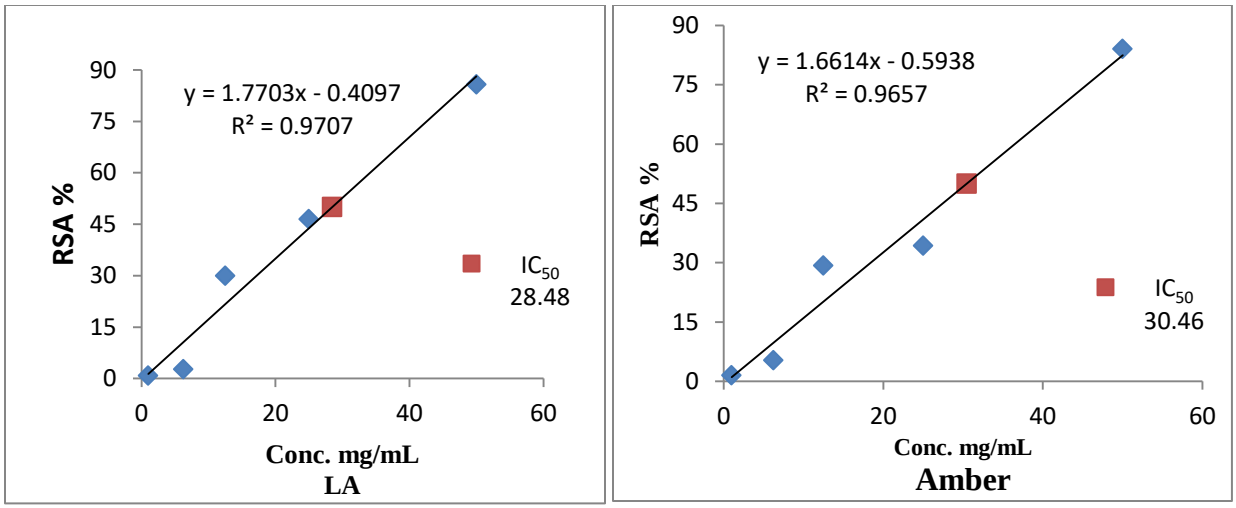

Figure 9: Plot of percentage scavenging activity of light amber and amber honey samples

The higher the consumption of DPPH by a given sample was the lower the IC_{50} value and the higher its antioxidant ability according to Vila and coworkers. As depicted above, the antioxidant activity of the honey samples were determined by 50% inhibition concentration of honey samples as, 28.48 and 30.46 for light amber and amber honey samples respectively. When adulterated the inhibition ability of the samples decreased to some extent, so that the IC_{50} of the adulterated samples increased accordingly (30.41, 33.44 and 36.34) for light amber honey and (32.31, 35.67 and 41.25) for amber honey. According to Vila and coworkers sugar cane has low antioxidant activity with IC_{50} = 100.2 showing slight similarity with this result (95.77) [32]. Therefore; adulteration of honey by cane sugar absolutely decreased the antioxidant activity of honey samples in this result. Recent studies on honeys indicated that the biological actions of honey can be ascribed to its polyphenol contents, which are elucidated by its antioxidant, anti-inflammatory and antimicrobial actions [29], however; as observed from this result, the contradiction between increased total phenolic content with radical scavenging activity is that only phenolic compounds with a certain structure and particularly hydroxyl position in the molecule can act as proton donating and show radical scavenging activity [34]. As also witnessed by [35] that, the health-promoting properties of Phenolic compounds are influenced by their structure, solubility, conjugation with other compounds and absorption and consequently on their metabolism.

5. Conclusion and Recommendation

5.1 Conclusion

The obtained results showed that adulteration of honey by synthetic cane sugar significantly affect the physicochemical and biochemical properties of honey such as color intensity, moisture, electrical conductivity, ash content, free acidity, reducing sugar, and antioxidant activities. As the amount of adulterant increase the content of monosaccharide sugars (glucose and fructose) exhibit significant decrease. The amount of reducing sugar (fructose), which is the main contributor for HMF formation significantly declined due up on the addition of sugar.

The finding also demonstrated that synthetic sugar contains some amount of phenolic contents. Thus, addition sugar increases the total phenol content of honey. However; the observed contradiction between increased total phenolic contents with decrease in the radical scavenging activity after sugar adulteration indicates that adulterated honey has less medicinal and therapeutic activities that the pure honey.

5.2. Recommendation

As a result the following conclusions are made by the researcher.

- ✓ It would be good to create awareness to the society about the impact of adulteration honey with sugar on the medicinal values of honey
- ✓ After addition of sugar, the contents of simple sugars in honey drastically decrease, this may require further investigation to check its impact on dietary value and human health.
- ✓ Physicochemical and biochemical properties of honey are significantly affected by intentional addition of sugar. Similarly, to know the impact the added sugar on microbial activities in vitro and in vivo tests are recommended.
- ✓ Quick tests shall be conducted by venders and individuals to check qualitative level of adulteration

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Appendix I

Tests for significance difference for adulteration trend for Physicochemical and biochemical parameters the present study

Color
ANOVA

Source of Variation	*Sum of Squares*	*df*	*Mean Square*	*F*	*P-value*	*F critical*
Between Groups	2037.134	1	2037.134	6.189706	0.047296	5.987378
Within Groups	1974.699	6	329.1165			
Total	4011.834	7				

Ash
ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.005513	1	0.005513	1.203822	0.314629	5.987378
Within Groups	0.027475	6	0.004579			
Total	0.032988	7				

Moisture
ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.0162	1	0.0162	0.001748	0.968012	5.987378
Within Groups	55.62155	6	9.270258			
Total	55.63775	7				

EC
ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	682082.8	1	682082.8	436.0969	7.85E-07	5.987378
Within Groups	9384.376	6	1564.063			
Total	691467.2	7				

pH value
ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.05445	1	0.05445	41.35443	0.000668	5.987378
Within Groups	0.0079	6	0.001317			
Total	0.06235	7				

Free acidity
ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	49.2032	1	49.2032	0.438416	0.53248	5.987378
Within Groups	673.3768	6	112.2295			
Total	722.58	7				

Redu. Sugar
ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	10.3058	1	10.3058	0.161427	0.701766	5.987378
Within Groups	383.0514	6	63.8419			
Total	393.3572	7				

Sucrose
ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1.453512	1	1.453512	0.020807	0.89003	5.987378
Within Groups	419.1492	6	69.8582			
Total	420.6027	7				

HMF
ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	107.0185	1	107.0185	0.328495	0.587368	5.987378
Within Groups	1954.707	6	325.7845			
Total	2061.725	7				

Phenolic C.
ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	7.48845	1	7.48845	0.041259	0.845752	5.987378
Within Groups	1088.985	6	181.4975			
Total	1096.473	7				

IC_{50}
ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	15.18005	1	15.18005	0.883841	0.383442	5.987378
Within Groups	103.0506	6	17.17509			
Total	118.2306	7				