PHYSICOCHEMICAL PROPERTIES, MICROBIAL QUALITIES AND ANTIMICROBIAL ACTIVITIES OF HONEYS FROM KELLEM AND WEST WOLLEGA ZONES, WESTERN ETHIOPIA

A THESIS SUBMITTED TO THE COLLAGE OF GRADUATE STUDIES, DEPARTMENT OF MICROBIAL, CELLULAR AND MOLECULAR BIOLOGY

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE

DEGREE OF MASTERS OF SCIENCE IN BIOLOGY (APPLIED MICROBIOLOGY)

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Declaration

I declare that this thesis is submitted to the School of Graduate Studies of Addis Ababa University for the Master's Degree in Biology (Applied Microbiology). I would like to prove through my signature below that it is my own independent work and has not earlier been submitted elsewhere by me or anybody else. All authors of the references cited in the current study were duly acknowledged.

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COLLAGE OF NATURAL AND COMPUTATIONAL SCIENCES

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This is to certify that the thesis prepared by Ofijan Tesfaye , entitled:“ Physicochemical properties, microbial qualities and antimicrobial activities of Honeys from Kellem and West Wollega Zones, Southwestern Ethiopia” and submitted in partial fulfillment of the requirements for the Degree of Master of Science in Applied Microbiology fulfills with the regulations of the University and meets the accepted standards with respect to originality and quality.

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Abstract

Honey is a natural substance synthesized by honey bees. Its physicochemical properties and biological composition differs among honey types. The aim of this study is, therefore, to determine the physicochemical and antioxidant properties, antimicrobial activities, microbial qualities and floral source of honeybee and stingless bee honeys collected from Kellem and West wollega zones of western Ethiopia. The physicochemical, antioxidant and pollen grain analysis of honey were determined using standard methods. Agar disc and well diffusion were used for antimicrobial susceptibility test while agar dilution was used to determine minimum inhibitory concentration (MIC). A microbial quality was carried out by pour plate technique. Honey from *Meliponulla beccarii* L. (MBH) was found to be more acidic (pH 3.00 ±0.11) with high level of free acidity (113.1±18.43 meq/kg) and moisture (33.55±2.82%) content, but with lower concentration of reducing sugar (37.84±4.04%) compared to honey from *Apis mellifera* L. samples. There was no statistically significant ($p > 0.05$) difference in the total phenol, flavonoid and antioxidant content of honey obtained from *Meliponulla beccarii* L (MBH), *Apis mellifera* market honey (AMMH) and *Apis mellifera* L fresh honey (AMFH). The overall average and the highest diameter of inhibition zone (mm) against clinical and standard isolates of *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus* and *Salmonella typhi* was recorded for MBH (15.71±2.78 mm) followed by AMFH (14.83±2.45 mm) and AMMH (10.51± 2.63 mm), while 25.16±2.50 mm for the positive control (chloramphenicol) by well diffusion method. On the other hand, the overall average inhibition zone (mm) recorded by disc diffusion from MBH, AMFH and AMMH were 10±1.21 mm, 9.04±1.67 mm and 7.62±1.32 mm, respectively against the tested bacterial pathogens. However, none of the honey samples inhibited *Candida albicans.* The overall mean of minimum inhibitory concentration (%) and minimum bactericidal concentration (%) against all the tested bacteria were 9.92 and 53.57 by MBH, 11.00 and 56.24 by AMFH, and 15.40 and 64.28 by AMMH, respectively. The highest *Staphylococci* (2.1 x 10^3cfu/g) and least spore forming bacteria (1.17 x 10^2 cfu/g) were counted from AMMH sample. MBH sample had the highest mean of 3.16 x 102 cfu/g by yeast and lowest mean of 1.0 x 10^2 cfu/g by mould, while AMFH sample became free of any microbial contamination. All types of honey samples were safe from Enterobacteriaceae and coliforms. *Coffee arabica* L., *Plantago lanceolate* L. and *Guizotia scabra* L. were the major floral sources of the tested honeys. Thus, Honey from the study area had good antioxidant, antibacterial and nutritional values.

Keywords: - *Apis mellifera L., Meliponula beccarii L.,* Microbiological qualities.

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Abbreviations and Acronyms

AEAC Ascorbic Acid Equivalent of Antioxidant content

AMFH *Apis mellifera* L. Fresh Honey

AMMH *Apis mellifera* L. Market Honey

ANOVA Analysis of Variance

CLSI Clinical and Laboratory Standard Institute

CSA Central Statistical Authority

EIAR Ethiopian Institute of Agricultural Research

EC Electrical Conductivity

EPHI Ethiopian Public Health Institute

ESA Ethiopian Standards Agency

EU European Union

GAE Gallic acid Equivalent

HMF Hydroxy Methyl Furfural

HSARC Haro Sabu Agricultural Research Center

HARC Holota Apiculture Research Center

IHC Harmonized methods of the International Honey Commission

QE Quercetin Equivalent

OARI Oromia Agricultural Research Institute

MBH *Meliponulla beccarii* L. Honey

M.a.s.l. Meter above sea level

NCCLS National Committee for Clinical Laboratory Standards

TAEAC Total Ascorbic Acid Equivalent of Antioxidant content

TFC Total Flavonoid Content

TPC Total Phenol Content

1. Introduction

1.1. Background and justification

Honey is a natural substance and sweet foods which is synthesized from flowers of bee flora by honeybees and has therapeutic activity and high nutritional values (Alvarez-Saurez *et al*., 2009).The presence of different floral source, entomological and geographical origin, climate, harvesting, processes and storage mechanisms of honey plays an important role on its flavor and biological properties (EI-Metwally, 2015; Faustino and Pinheiro, 2015) which are responsible for its the antimicrobial activities against pathogens. Bees (honey bees and stingless bees) are found under the family *Apidae* but they are separated under subfamilies *Apinae* and *Meliponiae*, respectively (Culliney, 1983). The most extensive genera under *Meliponiae* are *Trigona* and *Meliponula* (in Africa) while *Melipona* (in America) (Souza *et al*., 2006; Jalil *et al*., 2016). They are wild stingless bees (has no sting) unlike domesticated honey bees (*Apis mellifera L.*) in the hive (Souza *et al*., 2006). In Ethiopia, *Trigona* species make their honey in tree trunks while *Meliponula beccarii L.* make their honey in the underground (Pauly and Zewdu Ararso, 2013) and their honey has more medicinal value, locally expensive, acidic, high water content and less sugar content than the honey from honey bee (*Apis mellifera L.*).

Antimicrobial potency of honey depends on the peroxide and non-peroxide factors found in the honey sample. The peroxide (Hydrogen peroxide) which is produced by oxidation of Glucose by Glucose oxidase plays a key role in the antimicrobial activity of honey (Kacaniova *et al*., 2011). The non- peroxide factors include high osmotic effect (low water activity), acidic environment (low pH), phenolic acids and flavonoids, low protein content, high carbon to nitrogen ratio and low redox potential due to the high content of reducing sugars play antibacterial activity of honey (Ramos *et al*., 2018). The therapeutic potential of honey is associated with antioxidant capacity against reactive oxygen species (Ferreira *et al*., 2009). Components in honey reported to be responsible for its antioxidant effects are flavonoids, phenolic acids, ascorbic acid, enzymes (catalase and peroxidase), and carotenoids (Mohammed *et al*., 2014).

Honey is supersaturated sugar solution with approximately 17.1 % water. Fructose and glucose are the predominant sugar at 38.5% and 31% respectively. Disaccharides, trisaccharides and oligosaccharides were present in much smaller quantities (Saranraj *et al*., 2016). Other than

carbohydrates, honey contains numerous compounds like vitamins, organic acids, minerals, amino acids, proteins and aroma compounds (Alvarez-Suarez *et al*., 2018). Proline and lysine are the first and second most prevalent amino acids found in honey respectively whereas phenylalanine, tyrosine, glutamic and aspartic acids are also found in honey though their concentrations vary based on floral sources and bee types (Bogdanov *et al*., 2008). Glucose oxidase, invertase and diastase (amylase), which breaks down starch into dextrin and maltose are available in honey sample which all vary depending on floral source from which the honey is prepared (Alvarez- Suarez *et al*., 2010). Storing honey for long time as exposing to high temperatures inactivates their activity.

The quality of honey harvested directly from the hives and purchased from local markets may vary due to different factors. Among the factors, processing starting from harvesting to consumption and storage conditions such as materials used and length of storage can change its flavor, darken it and facilitate microbial growth (Ordó *et al*., 2005). Microbes of concern in post-harvest handling commonly found in honey are yeasts and spore-forming bacteria (Snowdon and Cliver, 1996). Therefore, comparing the honey quality between the fresh honeys directly from the hive with local market honey is important to know the effect of post-harvest handling. Antimicrobial agents are manufactured from microorganisms, plants and animal products and used to treat microbial diseases (Alkhyat and Al-Maqtari, 2014). However, through time, pathogenic microbes evolve multidrug and extended resistance mechanisms against antibiotics due to their massive and irrational usage (Levy and Marshall, 2004). Mulatu Getamesay *et al.*, 2014 and Feleke Moges *et al*., 2014, have reported the extensive development of resistance pathogenic bacteria against commonly available drugs due to misused and underused in developing countries like Ethiopia. Furthermore, the new generation antibiotics are less available and are expensive for resource poor societies (Yitbarek Habtamu *et al*., 2010). Thus, the resistance or slow killing of antibiotics against pathogenic microbes calls urgent search for more effective sources of natural products which has a multipurpose value like honey in order to tackle problems due to drugs failure to treat infectious diseases.

Yalemwork Ewnetu *et al*., (2013) have indicated that honey samples harvested from stingless bees caused maximum bacterial inhibition against clinical drug resistant pathogens (*Klebsiella pneumoniae*, Methicillin resistant *Staphylococcus aureus* and *Escherichia coli*) and standard

(*Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922) strains followed by honeys obtained from *A. mellifera* (white honey) and *A. mellifera* (yellow honey) from Gondar, Ethiopia. Similarly, Araya Gebereyesus and Berhe Gebreslassie, (2014) have showed better antibacterial activity of Tigray red honeys than the white honeys. In addition, honey from Samre area showed more inhibition than honey from Temben and Atsbi districts against multidrug resistant clinical isolates of *S. aureus*, *E. coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, Coagulase negative *Staphylococcus*, *Streptococcus pyogenes* and *Klebsiella pneumonia*

The sources of natural honey in Ethiopia vary from area to area depending on the agro-ecology, soil types, bee forages and bee species. For instance, the results reported by Berhanu Andualem, (2014) on stingless bee (*Trigona* spp.) from Gondar may be different with this work which was done on stingless bee (*M. beccarii*) found in the study area. Besides, there is limited information on scientific literature about the antagonistic effect of honey against human pathogens, physicochemical and antioxidant properties of stingless bee (*M. beccarii*) in Ethiopia in general and the study area in particular in comparison with honey produced by (*A. mellifera*). The previous investigations show that there is a difference on the efficacy of honey against human bacterial pathogens which depends on the color and site of honey collected as well as insects that produce honey.

Therefore, if different honey samples produced by different bee species have variations in their antibacterial activity, it is quite fundamental to elucidate honeys with its floral sources, physicochemical properties, antioxidant activities and biological composition (Muli *et al*., 2007; Alvarez-Suarez *et al*., 2010). This helps to identify and document the contents of honey from stingless bee (*M. beccarii*) in comparison with honey from honeybee (*A. mellifera*) that makes distinction between them in inhibiting human pathogens and knowing their components especially *M. beccarii* honey which add value for Ethiopian honey quality development. Moreover, the specified bee plant which contributes more forage sources for honeybees and at the same time enhances the medicinal value of honeys was identified. Finally, Ministry of agriculture, NGOs, Local community and, other stakeholders' become more beneficial from the result by cultivating those important major honey forages around their apiary site for surplus honey production which could be used in drug manufacturing and treating various diseases.

2. Objectives

2.1. General objective

- The general objective of this study was to determine the physico-chemical properties, antimicrobial activities, microbial qualities, and floral source of honey samples collected from the study sites.

2.2. Specific objectives:

The specific objectives of the current study were to:

- Analyze the physicochemical and antioxidant properties of honey samples harvested from *Apis mellifera* and *Meliponula beccarii.*
- Evaluate the antimicrobial efficacy of *Apis mellifera* and *Meliponula beccarii* honey samples against clinical isolates and standard strains.
- Analyze the plant source of each honey types.
- Determine microbial loads between different honey types.

3. Literature Review

3.1. Honey producing species of bees and their taxonomical position

Stingless bee (*Meliponini*) and honey bee (*Apini*) are highly eusocial corbiculate bees and common honey producers from the same bee family of Apidae (Eardley, 2004; Kek *et al*., 2017). Stingless bees belong to the subfamilies *Meliponinae* and keeping these bees is known as meliponiculture, while honey bees belonging to the subfamilies *Apinae*, and keeping of these bees is called Apiculture (Eardley, 2004). Honey bees consist of 11 species, genus *Apis* with *Apis mellifera* as the most extensive species in the world. There are more than 500 species (about 60 genera) of stingless bees: *Trigona,* and *Meliponula* (in Africa) while *Melipona* (in America) are the two extensive genera found in the world (Souza *et al*., 2006; Jalil *et al*., (016). According to Pauly and Zewdu Ararso, (2013), in Ethiopia: *Meliponula beccarii*, *Liotrigona bottegoi, Liotrigona baleensis, Hypotrigona gribodoi, Hypotrigona ruspolii* and *Plebeina armata* which are wild bees were collected as compared with the domesticated one (*Apis mellifera*).

The stingless bees construct horizontal pots made of cerumen for their nests to store honey while honey bees (*Apis mellifera*) build hexagonal-shaped combs with wax in the nests and the honey produced is known as comb honey. In Ethiopia, *M. beccarii* builds their nests in cavities in the ground while other stingless bees species make their honey by building their nest in tree trunks mostly of an *Acacia* tree (Pauly and Zewdu Ararso, 2013), and honey produced is known as pot honey and less yielder than *Apis mellifera* (Vit *et al*., 2004 ; Jalil *et al.*, 2016).

As opposed to honey bees, the stingless bees do not sting; thus, it is easier to extract their honey frequently. Stingless bee honeys are often not sweet, taste acidic, less viscous and have high water content (Torres *et al*., 2004; Bijlsma *et al*., 2006). Although the stingless bees produce less amount of honey, their honey fetches higher price than those from *Apis* honey bees. The price of stingless bee honey reaches up to ten times of the price of honey from *Apis mellifera* in tropical Africa due to beliefs of stingless bee honey has higher medicinal values and healing qualities (Vit *et al*., 2013) which makes the honey highly appreciated locally and traditionally than honey of *Apis mellifera* (Bijlsma *et al*., 2006). From *Meliponula* species, a range of 2-4 litre honey/ nest can be harvested and during harvesting the hunters dig in to the ground up to 10 to 15 cm below the nest (Awraris Getachew *et al*., 2012; Teklu Gebretsadik and Dinku Negash, 2016).

However, one cup of honey from *Trigona* species can be obtained per colony (Awraris Getachew *et al*., 2012). Compared to stingless bee, *Apis mellifera* bees have better productivity and the average annual yield of honeys in traditional, intermediate and modern hives is 5-10, 20-40 and 40-60 kg/hive/year (Admasu Adi *et al*., 2014), respectively.

3.2. Bee flora of honey and their sources

The floral source and geographical location from which bees harvest raw materials can greatly influence the composition and other characteristics of the honey (Baltrušaitytė *et al*., 2007). Just as some fruits and vegetables contain higher levels of beneficial phenolic compounds, different honeys can have varying levels of these beneficial compounds. Honey always includes numerous pollen grains mainly from the plant species when they forage (Abera Belay *et al*., 2015). Melisopallynology is the identification and quantification of pollen grains in honey sediment and it is the most important method for determining the botanical origin of honey (Anklam, 1998).

Honeys that are made from nectar belonging to a single plant comprising pollen count percentage to an extent of at least 45% are called monofloral. The honey that contains nectar and honeydew collected from several honeybees' forages is known as multifloral or polyfloral honey. In addition to predominant frequency, different levels of abundance of given pollen type in nectar such as secondary, tertiary and quaternary enrichment are required for botanical description of honey (Abera Belay *et al*., 2015). One can infer the source of the nectar used to produce the honey by determining the source of the pollen it contains (Nuru Adgaba, 2007). Production of honey and of other products depends on availability of floral resources. Bee floras do not contribute equally for honey bees because there is a preference between different vegetation by bee forager depending on the quality and contents of the resources released by plants. In the beekeeping point of view, bee plants are divided in to major and minor plants. Major bee plants are those plants which are visited by honey bees throughout their flowering season, but minor bee plants are those plants that are visited less often by bees (Nuru Adgaba, 2007). The common honey bee forages of Ethiopia are listed in Table 1.

Table 1: Common honeybee forages of Ethiopia

Family	Scientific name and authority	Source	Flowering period	Afan oromo	Amharic
Araliaceae	*Schefflera abyssinica*	Pollen & nectar	March to May	Gatama	Geteme, gustiye
Arecaceae	*Borassus aethiopum* Mart.	Pollen & nectar	September- October		Zembaba
	Phoenix reclinata Jacq.	Pollen & nectar	September- October	Meexii	Zembaba
Asteraceae	*Bidens macroptera* (Sch.Bip. ex Chiov.) Mesfin	Pollen	September to December	Kello	Adey Abeba, Meskel Abeba
	Bidens pachyloma (Oliv. & Hiern) Cufod.	Pollen			
	Bidens pilosa L.	Pollen & nectar	September and October	Maxxannee	Chegogit, Yesetan Merfe
	Bothriocline schimperi (Oliv. & Hiern ex) Benth.	Pollen & nectar	September to May		
	Guizotia scabra (Vis.) Chiov.	Pollen & nectar	all year round	Hadaa, Tuufoo	Adey Abeba Mech
	Vernonia rueppellii Sch. Bip. ex Walp.	Pollen & nectar	November to May	Redjii, Reejjii	Gujo
	Vernonia amygdalina Del.	Pollen & nectar	January to February	Ebicha	Grawa
Boraginaceae	*Cordia africana* Lam			Wodessa	Wanza
Combretaceae	*Combretum molle* R. Br. ex G. Don	Pollen & nectar	December and January	Daannisa Dandamsa	Abalo, Avalo
Euphorbiaceae	*Croton macrostachyus* Del.	Pollen & nectar	April to July	Cacaniraa, Makkanissa	Bisana
Fabaceae-Mimosoideae	*Acacia abyssinica* Hochst. ex Benth.	Pollen & nectar	September and October	Lafto, Garbi	Bazra-Grar
	Acacia etbaica Schweinf.	Pollen & nectar	August to October	Bate, Dodota	Derie, Doret
	Albizia schimperiana Oliv.	Pollen & nectar	January to May	Ambabessa,Mukarbaa	Sesa
Fabaceae-Papilionoideae	*Trifolium ruppellianum* Fresen.	Pollen & nectar	September October	Amaqeta, Sidisa	Amaget
Myrtaceae	*Callistemon citrinus* (Curtis) Skeels	Pollen & nectar	all year round		
	Eucalyptus camaldulensis Dehnh	Pollen & nectar		Bargamoo Dimma	Key Bahir Zaf
	Eucalyptus globulus Labill.	Pollen & nectar		Bargamoo Adii	Nech Bahirzaf
	Syzygium guineense (Willd.) DC. subsp. Afromontanum	Pollen & nectar	March and April	Aacha, Baddessa	Anqa, Dgta, Dokma
	Syzygium guineense subsp. Macrocarpa	Pollen & nectar	March and April	Gossuu	
Plantaginaceae	*Plantago lanceolata* L.	Pollen & nectar	whenever it rains	Qortobi	Gurteb, Gortem
Poaceae	*Zea mays* L.	Pollen		Boqolo	Beqolo
Rubiaceae	*Coffea arabica* L.	Pollen & nectar	after rains	Buna	Buna

Source: Admassu Adi *et al*., (2014): Honeybee Forages of Ethiopia.

3.3. Beekeeping and its importance

Beekeeping is a valuable small-scale activity and it plays a significant role in providing nutrition, cash income, pollination service and social benefits by generating work employment. Honey is an excellent energy source because it contains simple sugars that are ready for assimilation immediately on reaching the intestine. Beekeeping work doesn't require huge land, needs low initial investment to start, has a positive relationship with its environment, and doesn't differentiate sex of the individual and can be done as additional work. In Ethiopia, the selling price of single established colony from traditional hive ranged from 300 to 800 Ethiopian birr (Nuru Adgaba, 2007). The annual honey production of Ethiopia is estimated to be 45,300 metric tons which makes the country first honey producing in Africa and ninth in the world (FAO, 2010).

In addition bees are used for pollinating especially agricultural crops which increases food security and improving livelihoods. According to Ethiopian Institute of Agricultural Research EIAR, (2000), the value obtained from pollination service by bees is estimated to be over 15 times more than the value of all hive products together. Hackett, (2004) estimated the worth of honeybee agricultural pollination to be 14 billion United States Dollars in United States annually. Reduced agricultural yields and deformed fruit often result from insufficient pollination (Hepburn and Radloff, 2011). In Ethiopia, the seed yield of Niger (*Guizotia abyssinica*) was increased by about 43% (Admasu Adi and Nuru Adgaba, 2002) and Onion (*Allium cepa*) by two fold (Admasu Adi *et al.,* 2004) after pollinated by bees.

3.4. History of honey as traditional and modern medicine

Honey was used by ancient Greeks, Chinese, and Assyrian and Indians peoples for thousands of years to treat wound and diseases of the gut (Al-Jabri, 2005; Satarupa and Subha, 2014). Moreover, honey has been used for the treatment of irritating cough, keeping the teeth and gums healthy, insomnia/unable to sleep due to its hypnotic action, skin disorders (such as wounds and burns), chest (cardiac pain), all imbalances of the lungs and anemia, eye illnesses and preserving the dead, baldness, cleansing, sore throat, topical antisepsis (skin disinfectant), treatment of tuberculosis (Asadi-Pooya *et al*., 2003; Eteraf-Oskouei and Najafi, 2012). Furthermore, it was used as modern medicine in laboratory and clinical investigations (Dustmann, 1979).

In Ethiopia, *Meliponula beccarii* (Stingless bee) honey is traditionally used for treating respiratory ailments and surface infections (Mogessie Ashennafi, 1994 ; Berhanu Andualem, 2014) in line with treatments conducted using honeybee honey, whereas the society of this study area used this honey for treatment of cold, cough, asthma, diarrhea and respiratory infections (personnel communication).

3.5. Physicochemical properties of honey

The physicochemical properties for a given honey is influenced by the nectar types that the honeybee used, bee species, geographical ecology (climatic and soil) and post-harvest honey handling practices. Honey sold shall not have added to it any foreign food ingredient, especially during processing and storage shall not have any objectionable matter, flavor, aroma absorbed from foreign matter (Codex Alimentarius Commission, 2001; ESA, 2013). Therefore, analysis of the physicochemical properties of honey is used to verify the genuineness of the product and to tell the possible presence of artificial components or adulterants (Meda *et al.*, 2005; Abera Belay *et al.*, 2013). Physicochemical parameters including moisture, reducing sugar, sucrose, water insoluble, ash, free acidity, hydroxymethylfurfural contents, pH, diastase activity and electrical conductivity (Khalil and Suhaliman, 2010). Some of the physicochemical properties of honey are indicated in Table 2.

Table 2: Physicochemical properties of honey

Bee species	Physicochemical parameters						Country	Reference
	pH	Free acidity(meq/kg)	Ash (g/100 g)	EC(ms/cm)	HMF(mg/kg)	MC(g/100g)		
Melipona (Stingless Bee)	3.27	41.6	ND	ND	2.4	29.5	Brazil	Souza *et al*., 2004
	3.27	43.5	ND	3.52	5.8	28.8	Brazil	Alves *et al*., 2005
	4.66	28.3	0.17	ND	18.9	25.3	Brazil	Evangelista-Rodrigues *et al*., 2005
	3.2	41.52	0.46	0.5	9.2	28.62	Cuba	Alvarez-Suarez *et al*., 2018
	4.18	5.9	ND	0.66	5.4	27.0	Mexico	Santiesteban-Hernández *et al*., 2003
	3.53	79.0	0.15	ND	0.8	26.0	Brazil	Villas-Bôas and Malaspina, 2004
	4.21	11.23	ND	0.238	5.50	13.8	Nigeria	Nweze *et al*.,2017
Trigona (Stingless	2.87	36	1.8	3.27	4.3-39.1	25	Ethiopia	Berhanu Andualem, 2014

Bee)	3.75	30.69	ND	0.303	16.58	17.50	Nigeria	Nweze *et al*.,2017
	3.69	26.0	0.32	ND	8.1	25.5	Brazil	Almeida and Marchini, 2004
	3.88	7.7	ND	0.78	4.3	26.7	Mexico	Santiesteban-Hernández *et al*., 2003
	3.26	136.8	ND	1.08	ND	33.24	Malaysia	Kek *et al*.,2017
Apis mellifera	3.8	6.25	0.2	0.488	ND	18.5	Ethiopia	Berhanu Andualem, 2014
	4.76	32.65	0.18	0.33	16.54	16.74	Cuba	Alvarez-Suarez *et al*., 2018
	3.75	32.43	0.21	0.69	36.35	18.80	Ethiopia	Bekele Tesfaye *et al*.,2016
	4.24	18.67	ND	0.207	13.75	11.74	Nigeria	Nweze *et al*.,2017
	3.33-5.54	16.05-34.1	ND	0.143-2.006	0.58-4.25	15.56-18.15	Turk	Kivrak *et al*.,2017

EC (ms/cm): electrical conductivity in millisiemen per centimeter; HMF (mg/kg): Hydroxymethyl furfural in milligram per kilogram of honey; MC (g/100g): moisture content in gram per 100 gram of honey, Free acidity (meq/kg): mill equivalent of free acidity per kilogram of honey, Ash (g/100g): Ash contents in gram per 100g of honey sample, ND: not done.

3.6. Nutritional composition of honey.

On account of high energy value (303 kcal per 100 g) honey and fast absorption of its carbohydrate, honey is a food suitable for humans of every age (Blasa *et al*., 2006). When orally consumed, its carbohydrates are easily digested and quickly transported into the blood and can be utilized for energy requirements by the human body. It is for this reason that honey is particularly recommended for children and sportsmen because it can help to improve on the efficiency of the system of the elderly and invalids (Alvarez-Suarez *et al*., 2010). Honey is supersaturated sugar solution with approximately 17.1 % water. Fructose and glucose are the predominant sugar at 38.5% and 31% respectively and they depend on the invertase enzyme present in honey which decomposes sucrose in to fructose and glucose. In the presence of oxygen, glucose is decomposed to gluconic acid and hydrogen peroxide by glucose oxidase enzyme found in honey. The other enzyme found in honey is diastase (amylase), which breaks down starch into dextrin and maltose (Alvarez- Suarez *et al*., 2010). These enzymes vary depending on floral source from which the honey is prepared. Storing honey for long time as exposing to high temperatures inactivates their activity. Disaccharides, trisaccharides and oligosaccharides were present in much smaller quantities (Saranraj *et al*., 2016).

Besides carbohydrates, honey contains numerous compounds like vitamins, organic acids, minerals, amino acids, proteins and aroma compounds (Alvarez-Suarez *et al*., 2018). Proline and lysine are the first and second most prevalent amino acids found in honey respectively whereas phenylalanine, tyrosine, glutamic and aspartic acids are also found in honey though their concentrations vary based on floral sources and bee types (Bogdanov *et al*., 2008). The amount of sugar found in honeys from different honey bees are presented in Table 3.

Table 3.Sugar analysis of honey

Bee spp.	Sugars					Countries	References
	RS (g/100g	Sucrose(g/100 g)	Glucose g/100 g	Fructose g/100 g	Total sugar		
Melipona honey	75.64	5.06	ND	ND	80.71	Nigeria	Nweze *et al*., 2017
	68.9	4.7	ND	ND	ND	Brazil	Souza *et al*., 2004
	74.8	2.9	ND	ND	ND	Brazil	Alves *et al*., 2005
	68.0	1.6	ND	ND	ND	Mexico	Santiesteban-Hernández *et al*., 2003
Trigona honey	60.49	1.83	ND	ND	62.32	Nigeria	Nweze *et al*., 2017
	70.0	2.4	ND	ND	ND	Mexico	Santiesteban-Hernández *et al*., 2003
Apis mellifera honey	66.41	4.48	ND	ND	ND	Ethiopia	Bekele Tesfaye *et al*.,2016
	63.3.	6.1	ND	DN	69.3	Bangladesh	Islam *et al*.,2017
	70.34	2.36	ND	ND	72.70	Nigeria	Nweze *et a*l., 2017
	15.11-2.3	1.34-3.59	10.63-6.5	4.48-0.7	ND	Egypt	Sohaimy *et al*., 2015
	67.7-80	1.27-4.24	ND	ND	70.19-81.61	Ethiopia	Gebreegziabher Gebremedhin *et al*.,2013
	62.51	3.02	ND	ND	65.53	Malaysia	Moniruzzaman *et al*..,2013
	610.79	2.76 -28.60	246.68	357.03	ND	Portugal	Aazza *et al*.,2013

RS (g/100g): reducing sugar in gram per 100 gram of honey sample, Sucrose (g/100g): sucrose in gram per 100 gram of honey sample, Glucose g/100g: Glucose content in gram per 100 gram of honey sample, Fructose g/100g: Fructose content in gram per 100 gram of honey sample, ND: not done.

3.7. Antimicrobial activity of honey and its mechanisms of action

The production of hydrogen peroxide and gluconic acid which are the major antimicrobial components present in honey is the result of action of the glucose-oxidase activity in presence of oxygen, glucose will be converted in to gluconic acid and Hydrogen peroxide with the following reaction : glucose + H_2O + O_2 ------ gluconic acid + H_2O_2 (Molan, 1999; Cooper, 2014). Intrinsic physicochemical properties like high sugar content represents greater than 80% of honey's dry weight. This means, there is a low water activity (a_w) or high osmotic effect in a solution of honey, which makes little or no water to support the growth of microorganisms (Molan, 1992**;** Bogdanov, 1997). Many species of bacteria can grow if the a_w is between 0.94 - 0.99 values. The a_w of ripened honey (< 0.6) and its low pH of 3 to 4.5, do not support the growth of microorganisms (Cooper, 2014).

In addition, the presence of polyphenol in honey have antibacterial activity (Cabrera *et al.*, 2006). These compounds vary depending on the plant species from which the bees gather their nectar and seem to remain unchanged even after long periods of storage (Cooper, 2007**;** Viuda-Martos *et al*., 2008). Studies on antimicrobial activities of honey indicated that *Eucalyptus* honey from the Andean region of Ecuador (Valdes-Silverio *et al*., 2018), processed and raw honeys from Mauritius (Kinoo *et al*., 2012) and Polish market (Makarewicz *et al*., 2017) inhibited the growth of *Staphylococcus aureus*, *Klebsiella pneumoniae, Streptococcus pyogenes, Proteus mirabilis, Pseudomonas aeruginosa* and *Escherichia coli*, but with less or no effect against *Candida albicans* and *Aspergillus niger*.

At national level, there is shortage of study on antifungal activity of honey. However, previous studies have reported potent antibacterial activities of honey against drug resistance microbes such as *Pseudomonas aeruginosa, Salmonella typhi, Shigella flexneri, Escherichia coli, Klebsiella pneumoniae* and *Staphylococcus aureus* (Berhanu Andualem 2013; Yalemwork Ewnetu *et al*., 2013; Araya Gebereyesus and Berhe Gebreslassie, 2016) from Ethiopia (Gojjam and Tigray) honey, and stingless bee (*Trigona* spp) was more potent than *Apis mellifera* honey.

3.8. Multiple drug resistance mechanisms

Development of multidrug-resistant organisms is documented as a major public health problem affecting humans worldwide in high illness and death as well as increasing economic problem (Feleke Moges *et al*., 2014; Munita and Arias, 2016). Antibiotic resistance pathogens arise from mutations occurred at the gene level through horizontal gene transfer between microorganisms. Furthermore, after a bacterium gains resistance genes to protect itself from numerous antimicrobial agents, the bacteria uses different self-protective mechanisms (Mims *et al*., 2004 ; Giedraitienė *et al*., 2011; Munita and Arias, 2016) such as: (1) Antibiotic inactivation by enzymatic modification and degradation (β-lactamases, aminoglycoside-modifying enzymes, and chloramphenicol acetyl transferases) and inhibit on cell wall synthesis; (2) Target modification- an interaction between an antibiotic and a target molecule is very specific. For instance, macrolides and tetracycline are used for inhibition of protein synthesis while fluoroquinolones and rifampin acts against nucleic acid synthesis; (3) Efflux pumps and outer membrane permeability- efflux pumps are membrane proteins that pass on antibiotics from the cell and maintain their low intracellular concentrations. Most of efflux pumps are multidrug transporters that pump a wide range of dissimilar antibiotics such as macrolides; tetracycline, fluoroquinolones and significantly contribute to multidrug resistance (Džidic *et al*., 2008). For instance, *Pseudomonas aeruginosa* uses more than four powerful multidrug resistance efflux pumps (Mex) to eliminate toxic compounds from the cytoplasm and periplasm (Strateva and Yordanov, 2009)

3.9. Antioxidant activity of honey

Phytochemicals are substances that are found in plant and are recognized in food including honey for health encouraging actions (Saranraj *et al*., 2016). Antioxidant, which is a major group of phytochemicals, decreases oxidative stress within the human body and has a capacity to scavenge free radicals. According to (Meda *et al.*, 2005; Khalil *et al.*, 2010), honey has been found to contain significant antioxidant activity which includes both enzymatic (catalase and glucose oxidase) and non-enzymatic substances (organic acids, amino acids, proteins, α-tocopherol, flavonols, catechins and carotenoids). Many flavonoides (such as apigenin, pinocembrin, kaempferol, quercetin, galangin, chrysin and hesperetin), phenolic acids (such as ellagic, caffeic, p-coumaric, ferulic acids and ascorbic acid) have synergistic antioxidant effect

(Turkmen *et al*., 2006; Rakha *et al*., 2008). The effectiveness of honey's antioxidant role depends on honeybee species producing the honey, botanical origin, processing, handling and storage.

Honey has been used in ethno-medicine since the early humans history and in more recent times its role in the treatment of burns, gastrointestinal disorders, asthma, infected and chronic wounds, skin ulcers, cataracts and other eye diseases has been rediscovered (Ferreira *et al.*, 2009). However, since some of these diseases are a result of oxidative damage, it seems that part of the therapeutic properties of honey is due to its antioxidant capacity. Antioxidants can slow down biologically destructive chemical reactions in living organisms. These compounds are considered to defend humans from disease, in part, through their ability to scavenge oxidants and free radicals, absorbing molecular damage that might otherwise compromise the function of essential lipids, proteins, and nucleic acid (Schramm *et al.*, 2003).

In Ethiopia, there is limited information on comparison of bioactive compounds and antioxidant capacity between and within bee species. However, studies at elsewhere indicated the presence of bioactive compounds (total phenol, total flavonoid, total carotenoids, vitamin C, total free amino acids, total protein and folic acid content) and antioxidant capacity of honey from stingless bee (Alvarez-Suarez *et al*., 2010 ; Alvarez-Suarez *et al*., 2012; Alvarez-Suarez *et al*., 2018). The total antioxidant activity in the honey from *Tetragonula carbonaria* (stingless bee) was proven to be higher than that of the European honeybee honey (Oddo *et al*., 2008). These findings are supported by another study on the *Melipona fasciculate* (another stingless bee species), where the polyphenol content of honey was the highest in comparison with other South American honeybees (Isla *et al*., 2013). Some of the antioxidant components of honey from different areas of the world are well addressed (Table 4).

Table 4: Antioxidant properties of honey

Bee species	Parameters			Country	Reference
	TPC	TFC	AEAC		
Melipona	94.39±14.55 mgGAE/100 g	4.19±0.37 mgQE/100g	42.23±1.66 mg AEAC/100g	Cuba	Alvarez-Suarez *et al*., 2018
	371.98±14.18 mgGAE/kg	86.39 ± 4.69 mg CE/kg	296.99±3.40mg AEAC/kg	Nigeria	Nweze *et al*., 2017
Trigona	527.41 ± 3.60 mgGAE/kg	41.37±10.65 mg CE/kg	342.33±0.78 mgAEAC/kg	Nigeria	Nweze *et al*., 2017
Apis mellifera	110.39- 196.50	18.51- 32.88	9.650- 50.169	Malaysia	Chua *et al*.,2013
	54.30 ± 7.19 mgGAE/100 g	2.68 ± 0.38 mgQE/100g	31.06±2.19 mg AEAC/100g	Cuba	Alvarez-Suarez *et al*., 2018
	459.83 ± 1.92 mgGAE/kg	54.23 ± 0.62 mg CE/kg	236.8-315.9 mgAEAC/kg	Algeria	Khalil *et al*., 2012
	757.2±30.8 mgGAE/kg	43.1±2.1(mg CE/kg)	-	Banglade sh	Islam *et al*.,2017
	243.01 ± 74.91 mgGAE/kg	37.70±19.75 mg CE/kg	276.96-324.47 mg AEAC/kg	Malaysia	Moniruzzaman *et al*., 2013

TPC: total phenol content, mgGAE/100g: milligram of gallic acid equivalent per 100 gram or kilogram of honey, TFC: total flavonoid content, mgQE/100g: milligram of Quercetin equivalent per 100 gram or kilogram of honey,mg CE/kg) : milligram of Catchein equivalent per 100 gram or kilogram of honey, mg AEAC/g: milligram of Ascorbic acid equivalent of antioxidant content per 100 gram or kilogram honey

3.10. Microbiology of honey

If matured honey is not harvested and handled properly as other ready to eat food, it can be contaminated by microorganisms. These microorganisms are those that can withstand the concentrated sugar and acidity of honey. There are two sources of honey contamination by microbes; these are primary and secondary sources (Snowdon and Cliver, 1996)

3.10.1. Primary sources

Primary sources of microbial contamination include pollen, the digestive tracts of honey bees, dust, air, nectar and flowers, sources which are very difficult to control (Troller, 1979). Bacteria introduced to honey by the bees are probably members of the genus *Bacillus.* Sackett, (1919) observed that *Bacillus, Micrococcus* and *Saccharomyces* species could be readily isolated from honeycombs.

3.10.2. Secondary sources

Possible ways of microbial transmission into honey are humans or food handlers (from skin infections, sneezing or frank fecal contamination), equipment during harvesting, containers, wind, dust, animals and water. Yeasts and spore-forming bacteria are very common microbes found in honey during a contamination of post-harvest handling (Gadisa Natea and Delelegn Woyessa, 2014) and microorganisms like fecal and total coliforms can be supported and grows in honey and those that under certain conditions could cause human illness. According to research done by Tysset *et al*., (1970), from 12 honey samples analyzed, more microbial contamination occurred during post-harvest handling than in the hive.

Even though honey has low water activity which prevents multiplication and survival of bacteria, few pathogens have been found in honey (Snowdon and Cliver, 1996; Snowdon, 1999). *Bacillus* sp. and *Clostridium* sp. were described in honey. From *Clostridium* spp, only *Clostridium botulinum* was found in honey (Snowdon, 1999) which enters the beehive through the contaminated water or even by contact of the product with soil. *Clostridium botulinum* does not cause damage to the honeybees, but it is responsible for the development of botulism in humans, especially in children or people with weakened immune systems (Poormontaseri *et al*., 2014). *Bacillus cereus* is an important pathogen that can be found in honey; it is an enterotoxin producer in pH 6.0-8.0 and temperature ranging from 6°C to 21°C, but it is necessary to ingest 10^7 cells/mL to reach toxic effect (Jay *et al*., 2005). In terms of microbial loads, a market honey may contain much contaminated microbial loads than fresh honey in the hive. This may be due to a market honey may be adulterated and has hygroscopic characteristics especially when the

container is not sealed well or harvesting unripe honey changes honey quality and more water content leads to fermentation.

3.11. Challenges and opportunities of beekeeping in Ethiopia

Ethiopia has immense untapped potential like natural resources for beekeeping activity; the sector has been seriously devastated by complicated constraints while it varies depending on the agro-ecology of the country. According to national investigators (Kerealem Ejjigu, 2005; Awraris Getachew *et al*., 2012 ; Teklu Gebretsadik and Dinku Negash, 2016), lack of beekeeping knowledge, shortage of skilled manpower, shortage of bee equipment, honeybee diseases and pests, chemical poisoning, poor infrastructure development and shortage of bee forage are the major constraints that affect beekeeping sub-sector in Ethiopia.

In addition to the direct effect of chemicals on bees, herbicides may create a shortage of bee forages by eradicating some of the important bee forage weeds (Teklu Gebretsadik and Dinku Negash, 2016). Weeds like *Guizotia scabra* and *Bidens* species are among the most important honeybee forages and are sources of honey in the country (Admasu Adi *et al*., 2014). The availability of different agro-ecology which contains a variety of bee plant diversity counts over 7000 species of flowering plants which has supported the existence of large number of local bee colonies in Ethiopia (Nuru Adgaba,2002). The country has the largest bee population in Africa and about 10 million bee colonies are found, out of which about 5 to 7.5 million are estimated to be hived (MoARD, 2007; CSA, 2009).

4. Materials and Methods

4.1. Description of the study area

The study was conducted in West and Kellam Wollega Zones of Western Oromia, Ethiopia. The study Zones are naturally gifted with suitable climate and natural resources which provide favorable environment for beekeeping. The area is covered with virgin forests like Didessa, Abba Sena, Dalati Park and Garjeda that harbor different bee forage diversity including herbs, shrubs and woody tree plants. Moreover, the sites also comprise cultivated crops like cereals, pulses, oils and horticultural crops that are suitable for honey production. The study area has long rainy season which begins in April and ends in October with major in middle of June to the end of August that helps for availability of bee forages. The study area is also characterized by two dry seasons or dearth periods from beekeeping point of view. The main dearth period starts from the middle of February to end of March where flowering plants scarce and dry and the second starts dearth period from July to August due to long rainy, which is not conducive for honeybees to forage.

Ghimbi is the capital town of West Wollega Zone and is located at a distance of 474 km away from Addis Ababa City. The agro- climatic zone of the West Wollega including Gimbi is classified as lowland (28.7% of the total area), Midland (60. 9%) and Highland (10. 4%) with altitudinal ranges from 645 to 2935 m.a.s.l. The mean annual minimum and maximum temperature of West Wollega Zone is 11.2 and 28.1°C, respectively, with the average annual temperature of 19.65°C. It has also the highest and lowest percentage relative humidity of 84.05 in August and 56.8 in February, respectively. The mean annual rainfall ranges from 650 to 2,700 mm with an average of about 1,675 mm.

Dembidollo is the capital town of Kellem Wollega Zone and is located at a distance of 654 km away from Addis Ababa city. The agro- climatic zone of the Kellem Wollega is classified as lowland (38.4% of the total area), Midland (47.7%) and Highland (13.9%) with altitudinal ranges from 500 to 3335 m.a.s.l. The annual temperature and rainfall varies between 12°C to 36°C and 700 to 2800 mm, respectively. The highest percentage relative humidity is 92 in July whereas the lowest is 58.6 in February.

Honey is harvested two or three times annually in all the study areas depending on the availability of bee forages. In the late September or early November, herbaceous bee plants such as *Bidens* spp (Meskel flower or Adey Abeba in Amharic), *Trifolium* spp and *Plantago lanceolate L.* (Yebeg lat in Amharic whereas literally Qorxobbii in Afaan oromoo) are the dominant species which release both pollen and nectar sources (Nuru Adgaba,2007). This makes the main honey harvesting season to be practiced at the first of October and honey type is said to be Meskel flower honey which is Monofloral (Literally DammaTuufoo in Afaan oromoo) at all studies areas. In addition to this, other honey harvesting season is practiced in February from *Vernonia amaygdalina* L. and *Coffee arabica L.*, in all the study areas. In some places where *Eucalyptus* spp, *Syzygium guinensee* L. and *Croton macrostacyus L.* plants are available, there is a possibility of harvesting multifloral honey at the end of May and this might be realized due to the mixture of nectars that is collected from different plants as per communication with Zonal Livestock and Fisheries office of the study sites. In all the study areas, honey production system is mostly traditional, while in a very rare case by transitional and improved hives.

4.2. Honey sample collection

Three sources of treatments were used and for each treatments thirteen (13) honey samples, a total of 39 kg both honey samples of about 1kg per each sample were collected randomly. The first source of the treatment was fresh honey directly harvested from the modern hives. For this, ripened honey from *Apis mellifera* was collected during honey harvesting period (from 11-26, February, 2018 G.C) from apiary sites of Haro Sabu Agricultural Research Center (HSARC). For this, from Haro Sabu district (Hawetu Gandaso and Hawetu birbir kebele) and Chanka Sedi district (Kombo kebele) of Kellem Wollega Zone was used. From West Wollega Zone: Nedjo district (Lalisa Qambi kebele) and Gulliso district (Moga Kobore and Kurfessa birbir kebele) was included. The second source of treatment was *Apis mellifera* honey samples purchased from local market of each woreda. The third source of the treatment was honey samples from stingless bees (*Meliponulla beccarii)*. For this treatment, honey of stingless bee was purchased from the farmers of the study area. The collected honey samples were brought to Addis Ababa University, Microbiology, using sterile glass cup honey containers until further analysis. During harvesting, visual observation of apiary environment was carried out in addition to personal communication with very experienced beekeepers of each study area to get the botanical origin of the honey samples and bee forages growing in the area.

Table 5.Description of study area for geographical locations

	Kellem wollega zone			West wollega zone		
Parameter	Haro sebu district		Chanka sedi district	Nedjo district	Gulliso district	
	H. Gandaso Kebele	H. Birbir Kebele	Kombo kebele	L. qambi Kebele	M. Kobore Kebele	K. Birbir kebele
Latitude	08°52'51"N	08°53'52"N	08°56'53"N	08° 37'40"N	08° 38' 40"N	08° 30'40" N
Longitude	35°13'18"E	35°14'18"E	35°15'19"E	34° 42'41"E	34° 42'41"E	34° 42'41"E
Altitude(m.a.s.l.)	1515	1563	1654	2050	1805	1716

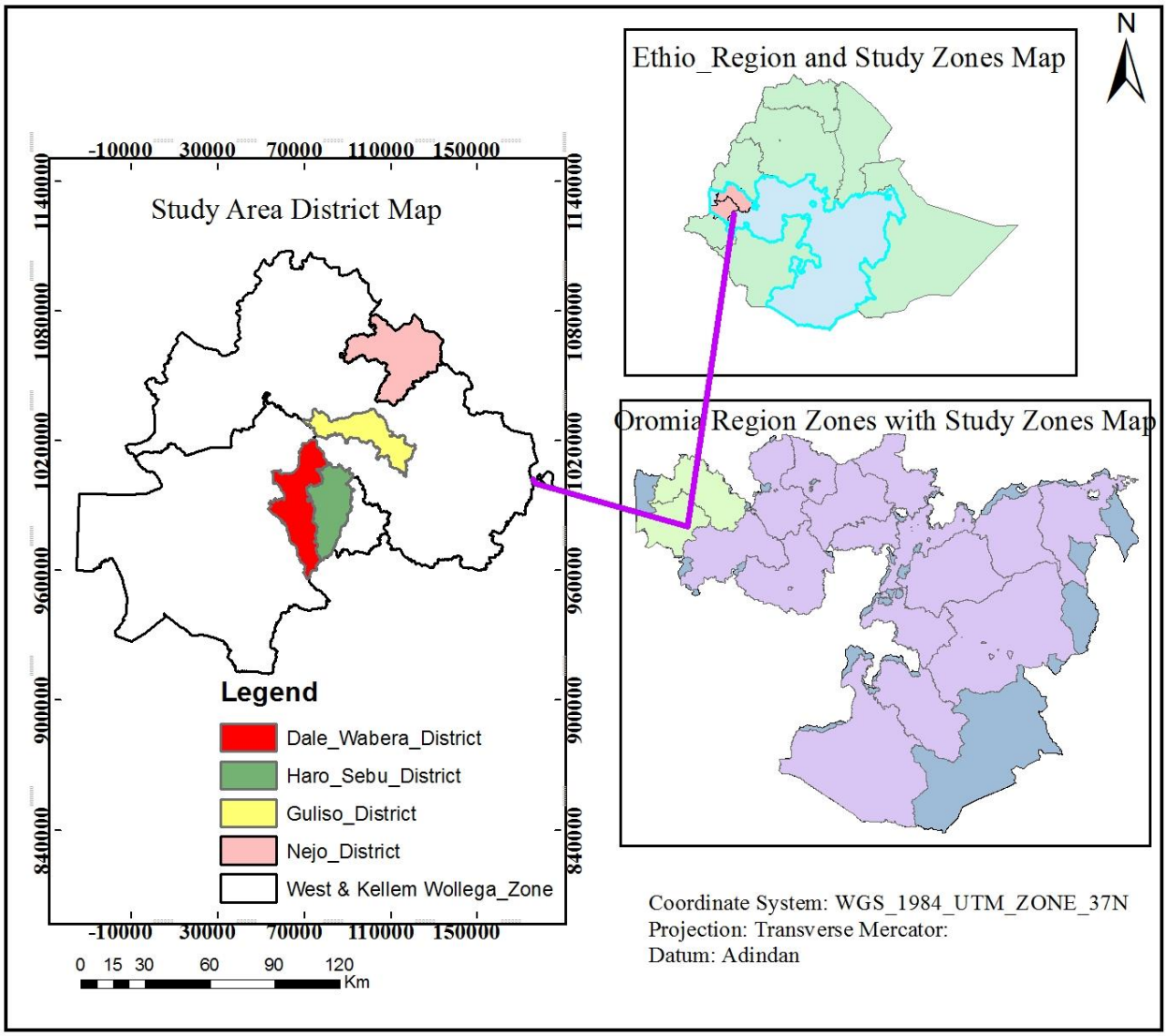

Figure 1.Map showing area of honey sample collected.

4.3. Analyzing botanical origin of honey

Pollen analysis of honey was carried out using the method of Louveaux *et al.*, (1978). For this, ten gram of honey was dissolved in 20 ml of warm distilled water in centrifuge tube at temperatures that ranged from 20-40°C. The solution was centrifuged at 3800 rpm for 10 minutes and the supernatant was decanted. Distilled water of 20 ml again was added to completely dissolve the remaining sugar crystals and centrifuged at 3800 rpm again for 5 minutes and supernatant was removed completely. The sediment was spread evenly using a sterile micro spatula on microscope slide and the sample was dried for a while. Thereafter, one drop of glycerin jelly was added to the cover slip and the pollen grains were identified using pollen atlas (Nuru Adgaba, 2002).The percentage of pollen types in each honey samples was calculated based on the total number of different types of pollen grains counted in each sample. If pollen grains counted was greater than 45 %, used as predominant pollen (unifloral honey), while a honey with no predominant pollen was used as mixed honey (Louveaux *et al*, 1978). The pollen count was done under light microscope (Swift instrument international, serial number 8750038, Japan, high power 400x) linked to a computer. Physicochemical and pollen grain analysis of honey samples were done at Laboratory of Holota Apiculture Research Center, while antimicrobial effects, antioxidant activity and microbial quality analysis were carried out at Addis Ababa University department of MCMB (Applied Microbiology), and Food Science and nutrition Laboratories.

4.4. Physicochemical analysis of honey

4.4.1. Moisture content

The moisture content of honey samples was determined using an Abbé refractometer (ABBE- 5 Bellingham Stanley. Ltd, United Kingdom) that can be thermo stated at 20°C and regularly calibrated with distilled water. Honey samples were homogenized and placed in a water bath until all the sugar crystals were dissolved. After homogenization, the surface of the prism of the refractometer was covered with honey and after 2 minutes refractive index for moisture was determined. The value of the refractive index of the honey sample was determined using standard table designed for this purpose (Bogdanov, 2009).

4.4.2. pH and free acidity

From each honey sample, ten gram of honey was dissolved in 75 ml of distilled water in 250 ml beaker and stirred using magnetic stirrer. The electrode of pH meter (METTLER TOLEDO, CHINA) was immersed in the solution and the pH of honey was recorded. For measurement of free acidity, the solution was further titrated with 0.1 M sodium hydroxide (NaOH) solution to pH 8.30. For precision, the reading to the nearest 0.2 ml was recorded using a 10 ml burette. Free acidity is expressed as mill equivalents or a mill mole of acid/kg honey and is equal to ml of 0.1M NaOH x 10. The result is expressed to one decimal place following the procedure of Bogdanov (2009).

Acidity =10 V, Where: V = the volume of 0.1N NaOH in 10 g of honey.

4.4.3. Determination of total ash content

Determination of ash content was carried out by incinerating honey samples at 600°C in a muffle furnace (BioBase JKKZ.5.12GJ, Shandong, China) to constant mass (Bogdanov, 2009). First, the ash dish was heated in an electrical muffle furnace at ashing temperature and subsequently cooled in a desiccator to room temperature and weighted to 0.001 g (M_2). Then 5 g ($M_{0)}$ of each honey sample was weighed to the nearest 0.001 g and taken into a platinum dish and two drops of olive oil were added to prevent foaming. Water was removed and started ashing without loss at a low heat rising to 350 - 400°C using electrical devices. After the preliminary ashing, the dish

was placed in the preheated furnace and heated for at least 1 h. The ash dish was cooled in the desiccators and weighed. The ashing procedure was continued until constant weight was reached (M_1). Lastly, % of weight of ash in g/100 g honey was calculated using the following formula: -

$$WA = \frac{M1 - M2}{M0}$$

Where, M0= Weight of honey taken.

M1= Weight of ash + dish.

M2= Weight of dish.

4.4.4. Determination of sugars

Honey sugars were determined using high performance liquid chromatography (HPLC- 1260 Infinity Series Agilent Technologies, Germany). Five gram of honey was dissolved in 40 ml distilled water. A 25 ml of acetonitrile was pipetted into a 100 ml volumetric flask and the honey solution was transferred to a flask and filled to the mark with distilled and the solution of each honey sample was filtered using syringe filter (0.45 µm) before chromatographic analysis. The HPLC separation system was composed of analytical stainless steel column, 4.6 mm in diameter, 250 mm length, containing amine modified silica gel with 5-7 µm particle size. Flow rate 1.3 ml/min, mobile phase Acetonitrile: water (80:20, v/v) and sample injection volume 10 µl. The sugars were detected by a Refractive Index Detector thermo stated at 30^oC temperature regulated column oven at 30^0C. The identification of honey sugars was obtained by comparison of their retention times with those of the standard sugars (Bogdanov, 2009). Standard sugars with their purity level in percent used were glucose (>99.5%), sucrose ((>90 %), maltose ((>90%) and fructose (>99.5%) made in Germany, sigma Aldrich.

4.4.5. Electrical conductivity

The electrical conductivity of a solution of 20 g dry matter of honey in 100 milliliter distilled water was measured using an electrical conductivity cell (BANTE Instrument- 520 conductive and temperature meter, China). A 0.745 g of potassium chloride (KCl), was dried at 130°C, dissolved in freshly distilled water in a 100 ml flask and filled to volume with distilled water.

Forty milliliter of the potassium chloride solution was transferred to a beaker and the conductivity cell connected to the conductivity meter, the cell rinsed thoroughly with potassium chloride solution and immerse the cell in the solution, together with a thermometer and reading of the electrical conductance of the solution in mill siemen after the temperature has equilibrated to 20^0C was taken as described in harmonized IHC (Bogdanov, 2009).

The cell constant K, was calculated using the following formula:

K=11.691x1/G

Where:

K= the cell constant in cm^{-1}

G= the electrical conductance in mS, measured with the conductivity cell.

11.691= the sum of the mean value of the electrical conductivity of freshly distilled water in $mS.cm^{-1}$ and the electrical conductivity of a 0.1M potassium chloride solution, at 20°C.

4.4.6. Determination of hydroxyl methyl furfural (HMF)

HMF was determined using 6800 UV– Vis spectrophotometer (JENWAY, United Kingdom). A 5 g honey sample was weighed in small beaker and mixed in 25 ml distilled water and transferred into 50 ml volumetric flask (Bogdanov, 2009). A 0.5 ml carrezz solution I (15 g $K_4Fe(CN)_6.3H_20$ /100 ml distilled water) was added and mixed with 0.5 ml carrezz solution II (30 g Zn acitate /100 ml distilled water). The solution was mixed with the honey solution. A droplet of alcohol was added into the solution. The solution was filtered through a filter paper and the filtrate (10 ml) was discarded. A 5 ml filtrate was added into each of two test tubes and 5 ml distilled water was added into the first test tube (sample solution), while 5 ml sodium bisulfite solution (0.20% of 0.20 g $NaHSO_3$/100 ml distilled water) was added into the other test tube (reference). The contents of both test tubes were well mixed by vortex mixer and their absorbance was recorded spectrophotometric ally by subtracting the absorbance measured at 284 nm for HMF in the honey sample solution against the absorbance of reference (the same honey solution treated with sodium bisulfite, 0.2%) at 336 nm and the result was calculated and expressed according to international honey commission (Bogdanov, 2009). Hydroxyl methyl furfural (HMF)/100 g honey= $(A_{284} - A_{336})$ x 14.97 X 5/g sample,

Where A_{284}= absorbance at 284, A_{336} = absorbance at 336, 14.97= constant, 5= theoretical nominal sample weight and g = mass of honey sample.

4.5. Antioxidant properties of honey

4.5.1. Total phenolic contents

To analyze and compare the total phenol content between honey samples, Folin-Ciocalteu method was used (Chua *et al*., 2013). Honey stock solution was prepared by mixing 5 g of honey sample in 50 ml of distilled water and filtered through Whatman no.1 filter paper. From this stock solution, 0.5 mL aliquot was mixed with 2.5 ml of 0.2N Folin- Ciocalteu reagent and incubated for 5 min. A 2 ml of 75 g/l sodium carbonate solution was added into the solution. Finally, after the solution was incubated for 2 h at 25°C, the absorbance of the reaction mixture was measured at 765 nm using UV (PerkinElmer Lambda 950 UV /VIS/NIR Spectrophotometer). Gallic acid (0-200 mg/L) was used as a standard chemical to produce calibration curve and finally, the total phenol content was expressed as milligram of gallic acid equivalent (GE) in 100 gram of honey from the mean value of triplicate data using the calibration equation ($y=0.0084x + 0.2822$; $R^2 = 0.9883$), derived from calibration curve.

4.5.2. Total flavonoid content

The total flavonoid content of each botanical sources of honey samples were determined (Chua *et al.*, 2013). The stock solution was prepared by diluting five gram of honey sample in fifty milliliters of distilled water and filtered through Whatman no.1 paper. Five milliliters from honey stock solution was pipetted and mixed in five milliliters of 2% aluminum chloride ($AlCl_3$) solution. After incubation for 10 min, the absorbance of the reaction mixture was measured at 415 nm by using Spectrophotometer (Perkin Elmer Lambda 950 UV/VIS/NIR Spectro photometer). Quercetin (0-200 mg/L) was used as a standard chemical to produce calibration curve and finally, the total flavonoid content was reported as mean value of triplicate assays and expressed as milligram of Quercetin equivalent (QE) per100 gram of honey from the mean value of triplicate data using the calibration equation ($y = 0.002x + 0.0623$; $R^2 = 0.9907$), derived from calibration curve.

4.5.3. Antioxidant content of honey

Antioxidant compounds in honey samples were evaluated by measuring the ascorbic acid equivalent antioxidant capacity (AAEAC) following standard methods (Islam *et al*., 2012). The

DPPH (2, 2- diphenyl-1-picrylhydrazyl) solution (20 mg/l) was prepared by dissolving 0.5 mg of DPPH in 25 ml of methanol. Honey solution was prepared by mixing 30 mg honey in 1 ml methanol and 0.75 mL of methanolic honey solution was added to 1.5 ml of DPPH solution. The absorbance was measured at 517 nm after 15 min of incubation at room temperature. The blank was composed of 0.75 ml of a methanolic honey solution mixed with 1.5 ml of methanol. Ascorbic acid (0-200 mg/l) was used as a standard chemical to produce calibration curve. Finally, the measurements were replicated three times and the mean value was expressed as milligram of ascorbic acid equivalent antioxidant content per 100 g of honey from the mean value of triplicate data using the calibration equation ($y= -0.002x + 0.4951$; $R^2 =0.9911$), derived from calibration curve.

4.6. Preparation of honey samples

Each of the honey samples was filtered using sterile gauze to remove impurities or debris in the crude honey such as bee wax, brood and dead bees. From pure honey, 75% w/v honey solution was prepared to test the susceptibility of honey. Three millilitre of pure honey was transferred into 1ml of sterile distilled water during the preparation of honey samples for antimicrobial test.

4.7. Test microorganisms used for the current study

For this work, the standard bacterial strains *Staphylococcus aureus* (ATCC-25923), *Escherichia coli* (ATCC-25922) and *Pseudomonas aeruginosa* (ATCC-27853) and clinical microbes (*Staphylococcus aureus* from pus, *Escherichia coli* from urine, *Pseudomonas aeruginosa* from fluid, *Salmonella typhi* from blood and *Candida albicans* from urine) were obtained from Ethiopian Public Health Institute (EPHI).

4.8. Inoculums preparation

Standard 0.5 McFarland was prepared by mixing 0.5 ml of 0.048M $BaCl_2$(1.175%W/V $BaCl_2.2H_2O$) with 99.5 ml of 0.18 M H_2SO_4 (1% V/V) and thoroughly mixed to ensure that it is evenly suspended (Andrews, 2006).Then, the standard was distributed in to a capped test tube of the same size and volume as those used to prepare the test inoculums. Inoculums was prepared by picking 2-3 colony from 24-hour old culture that was grown on their selective media (*Eschericia coli* on eosin methyl blue, *Staphylococcus aureus* on mannitol salt agar, *Salmonella*

typhi on selenite agar, *Pseudomonas aeruginosa* on pseudomonas agar and *Candida albican* on Potato dextrose agar) and suspended in 5 ml saline solution (0.85% saline). The suspended inoculums were vortexed for 15 seconds and their turbidity was adjusted by adding colony or saline solution into microbial stock solution and compared visually with standardized 0.5 McFarland (turbidity adjusted spectrophotometric ally to 0.08-0.12 at OD_{625} nm) against a white paper background and contrasting black lines.

4.9. Determination of antimicrobial activity of honey

4.9.1. Agar well diffusion assay

Agar well diffusion test was carried out according to (Moussa *et al.*, 2011). Muller Hinton Agar (Meat infusion, 2.00 g; Casein(protein and aas source breakdown by acid(HCL)) acid hydrolysate, 17.50 g; Starch, 1.50 g; Agar, 17.00 g; distilled water, 1000 ml; pH, 7.3±0.1 at 25ºC) and Potato dextrose agar (potatoes infusion, 200.00 g; Dextrose, 20.00 g; Agar, 15.00 g; distilled water, 1000ml; pH, 5.6±0.2 at 25ºC) plates were uniformly seeded by means of sterile cotton swab from inoculated saline solution containing bacteria and fungus strains, respectively. The plates were left on the bench for excess fluid to be absorbed. Using a sterile cork borer, 6 mm diameter and 4 mm deep wells were made in the seeded agar medium. Using a micropipette, 60 µL of honey sample with the concentration of 75% each sample was added into the wells in the plates. Positive controls (chloramphenicol (30µg/60µL) and griseofulvin (25 µg/60 µL) as well as sterile distilled water negative control was equally filled in the well. The plates were incubated at 37°C for 24 h. The diameters of inhibition zones of each sample including the wells were measured using caliper in mm and the results were recorded accordingly. All the assays were done in triplicate.

4.9.2. Agar disc diffusion assay

Agar disc diffusion test was carried out according to (Moussa *et al.*, 2011). The solidified Muller Hinton agar plate for bacteria and Potato Dextrose Agar plate for fungus were uniformly seeded using sterile cotton swab from inoculated saline solution containing the respective test strains. The plates were left on the bench for excess fluid to be absorbed. Using a puncher, 6 mm discs were made from Whatman no 1 and sterilized to be placed on the seeded plates. A 25 µL of the

prepared honey solution was pipetted on the surface of the disc. Chloramphenicol (30 µg was dissolved in 25 µL sterile distilled water per a disc) and griseofulvin (25 µg was dissolved in 25 µL sterile distilled water for a single disc) were used as a positive control for bacterial and fungal strains, respectively, while 25 µL sterile distilled water was used as a negative control. All the assays were replicated three times.

4. 9. 3. Minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) was determined by broth dilution method (Boateng and Diunase, 2015). To determine the lowest concentration of antimicrobial activity, the stock honey solution (75%) which was prepared by diluting 3 g/ml of Muller Hinton Broth that showed high inhibition zone against the test microbes was used. A two-fold serial dilution was prepared from the stock solution. A 18-24 h bacterial cultures were used and standardized by comparing its turbidity with McFarland 0.5 standard. Nine sterile capped test tubes were prepared and labeled. Then 4 ml (75%), 2 ml (37.5%), 1 ml (18.75%), 0.5 ml (9.375%), 0.25 ml (4.68%), 0.125 ml (2.35%) and 0.0625 ml (1.17%) were pipetted from stock honey solution (75%) with in a series of seven tubes. Then to each test tube, 1 ml of the standardized inoculum suspensions were pipetted while negative control tube (eighth test tube) contained only broth and the positive control tube (ninth test tube) contained one milliliter tested bacteria plus broth for comparison. The tubes were vortexed and incubated for 24 h at 37°C. Finally, by comparing with control tube which showed clear tubes was used as the least honey concentration that inhibits bacterial growth.

4. 9. 4. Minimum bactericidal concentration (MBC)

A dilution showing no visible growth during the MIC test was sub cultured onto a fresh Muller Hinton Agar plates by streaking using sterile inoculating loop and incubated at 37°C for 24 h. The lowest concentration of the extracts showing no growth on the Muller Hinton Agar plate was recorded as MBC (Yalemwork Ewnetu *et al*., 2013).

4. 10. Microbiological quality of honey samples

A 20 g of each honey sample was mixed with 180 ml sterilized distilled water and homogenized using orbital shaker (110 rpm) for five to ten minutes and used as stock solution for further serial dilution. A 0.1 ml from 10^{-1} serial dilution was pipetted on to the center of sterilized respective plates. Accordingly, 20-25 ml sterilized respective media was poured on to the plate containing the aliquots and well mixed. The inoculated plates were incubated at appropriate temperature for 24-96 h. The colonies were enumerated from countable plates and expressed as colony forming units per gram or ml.

4.10.1. Aerobic mesophilic counts

From 10^{-1} serial dilution, a 0.1 ml of aliquot was poured in duplicate onto plates of Nutrient Agar (Oxoid) and the colonies were counted after the plates were incubated under aerobic condition at 30-32°C for 24-48 hours (Deriba Muleta and Mogessie Ashenafi, 2002).

4.10.2. Staphylococci counts

From appropriate serial dilution, 0.1 ml of aliquot was poured in duplicate onto plates of Mannitol Salt Agar (Oxoid). The plates were incubated under aerobic condition at 30-32°C for 36 h. After incubation, yellow colonies with yellow zones and colorless or red colonies with red zones was counted as staphylococci (Deriba Muleta and Mogessie Ashenafi, 2002).

4.10.3. Enterobacteriaceae counts

From appropriate serial dilution, 0.1 ml of aliquot was poured onto plates of violet red bile glucose agar (Oxoid) (g/l: peptone-source of nitrogen, 7.00 g; yeast extract, 3.00 g; agar, 12.00 g; glucose, 10.0g; sodium chloride, 5.00 g; bile salts, 1.50 g; neutral red-as dye, 0.03 g; Crystal Violet, 0.002 g; Distilled water, 1000 ml with final pH of 7.4±0.2. Colonies were counted after the plates incubated under aerobic condition at 30-37°C for 18-24 h and after which, pink to red purple colonies were considered as member of the family Enterobacteriaceae (Deriba Muleta and Mogessie Ashenafi, 2002).

4.10.4. Coliforms

From a ten-fold appropriate serial dilution, 0.1 ml of aliquot was poured onto plates of violet red bile agar (Oxoid) (lactose, 10.0 g; peptic digest of animal tissue, 7.0 g; sodium chloride, 5.0 g; yeast extract, 3.0 g; bile salts mixture, 1.5 g; neutral red, 0.03 g, crystal violet, 0.002 g; agar, 15.0 g; distilled water, 1000 ml with pH of 7.4±0.2. Colonies were counted after the plates incubated under aerobic condition at 30-32°C for 20- 24 h (Deriba Muleta and Mogessie Ashenafi, 2002).

4.10.5. Yeasts and moulds

From appropriate serial dilution, 0.1 ml of aliquot was poured onto plates of Potato Dextrose Agar (Oxoid) containing (g/l) potatoes infusion, 200.00 g; dextrose, 20.00 g; agar, 15.00 g; chloramphenicol , 0.1 g; distilled water, 1000 ml with pH value of 5.6±0.2 and incubated at 25-28 °C for three to five days. Smooth (non-hairy) colonies without extension and hairy colonies with extension at periphery were counted as yeasts and molds, respectively (Deriba Muleta and Mogessie Ashenafi, 2002).

4.10.6. Aerobic bacterial spore formers

For aerobic bacterial spore counts, 10^{-1} ml of the serial dilutions was heated in a water bath at 80°C for 10 min to kill vegetative cells and cooled rapidly in tap water. A 0.1 ml of aliquot was poured onto plates of Nutrient Agar (Oxoid). The grown colonies were counted as aerobic spore former bacteria after incubation at 30-32^{o}C for 48 h. nutrient agar (Deriba Muleta and Mogessie Ashenafi, 2002)

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5. Data analysis

SAS version 9.1.3 (SAS Institute, 2003) computer package was used for analyzing all the data. Means and standard deviations of the recorded data were calculated using SAS Software (SAS Inc., 2003). Determination of the significant differences between honey samples was done using one-way ANOVA. Inhibition zone, minimum inhibitory concentration, minimum bactericidal concentration, physicochemical and antioxidant parameters of the tested honey samples were used for mean separation.

6. Results and discussions

6.1. Botanical origin

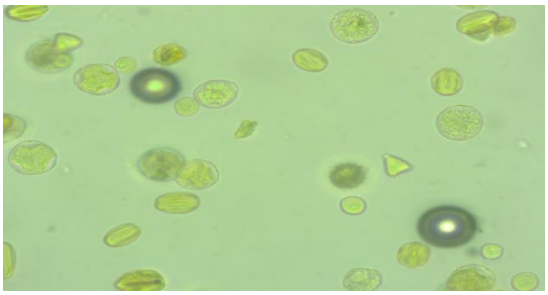
From this study (Table 6), AMMH sample was considered as multifloral (mixed) honey since each plant species found in honey samples were below 45% pollen count. The plant species found in multifloral honey were *Guizotia scabra*, *Coffee arabica*, *Vernonia amygdalina*, *Eucalyptus* spp, *Combretum molle*, *Trifolium ruppelianum* and *Syszgium guineense*. This might be because of the honey found at commercial level is the mixture of different botanical and geographical origin of honey or it blended with honeys harvested from different seasons by honey traders. On the other hand, AMFH sample was dominated by *Coffee arabica* which could be found as monofloral honey since its pollen count was 68 % (Table 6). *Coffee arabica* is common cash crop in western Oromia, particularly in the study area and contributed much to monofloral nature of the harvested honey samples in February. This is supported with field observation and personal communication with local experienced beekeepers who stated that *Coffee arabica* flowers provide abundant pollen and nectar in January for honeybees (Admasu Adi *et al*., 2014).

The dominant pollen grains counted in MBH samples were *Guizotia scabra* and *Plantago lanceolate* accounting 50.3 and 44.5% (Table 6), respectively. Honey from stingless bee (*Meliponula beccarii*) is harvested once annually in the month of October unlike honeybee honey (*Apis mellifera* L.). From the pollen analysis, *Meliponula beccarii* visits herbaceous plants compared to *Apis mellifera* which forages all herb, shrubs and tree plants. (Alvareez-suarez *et al*., 2018) have demonstrated the presence of difference between stingless and *Apis mellifera* on foraging flowers due to the short flight rate of stingless bees or the difficulty of access to flowers used by *Apis mellifera L*. Most of the stingless bees nest on cultivated field and fallow land that favor the growth of herbaceous flora. On the other hand, stingless bees are unable to compete with *Apis mellifera* (Cairns *et al*., 2005). Moreover, the specialty of the stingless bees is the ability to pollinate small-sized flowers due to their diminutive figure which cannot be achieved by the relatively big honey bee and besides stingless bees are not selective in building a colony hive (Jalil *et al*., 2017).

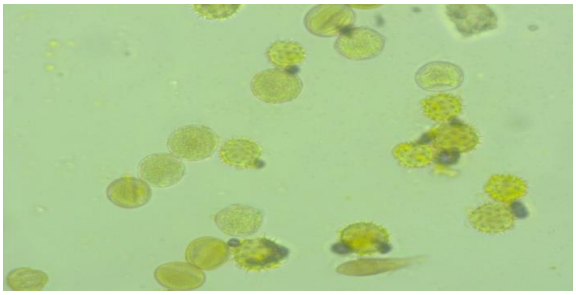
Table 6: Relative frequency of enriching nectariferous species from sources of honey treatments (% distribution)

Honey type	Plant species (%)							
	Guizotia scabra	*Plantago lanceolate*	*Coffee arabica*	*Vernonia amygdalina*	*Eucalyptus* spp	*Combretum molle*	*Trifolium ruppellianum*	*Syzgium guineense*
AMMH	13.7	ND	11.2	17.2	21.5	12.9	8.6	14.6
AMFH	16.5	ND	67.9	2.9	5.8	ND	5.8	ND
MBH	50.3	44.5	ND	ND	ND	ND	5.2	ND

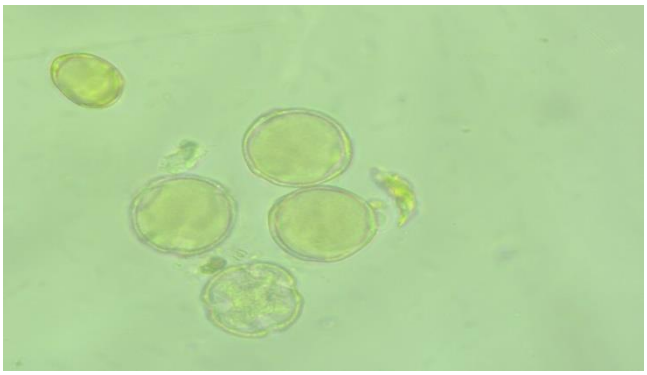
Notice: AMMH-*Apis mellifera* market honey; AMFH- *Apis mellifera* fresh honey; MBH-*Meliponulla beccarii* honey and ND – not detected.



A)



C)



B)

Figure 2: **Pollen grain morphology identified from honey samples**. A) *Apis mellifera* market honey sample (Multifloral honey pollen), B) *Apis mellifera* fresh honey sample (*Coffee Arabica*, Monofloral honey*)* and C) *Meliponula beccarii* honey sample (*Plantago lanceolate* and *Guizotia scabra)*

6.2. Physicochemical properties of different sources of honey

6.2.1. Moisture content

Different honey types were analyzed for their moisture content (Table 7). Statistically ($p< 0.0001$) highly significant difference was observed among the three honey types (AMMH, MBH and AMFH) analyzed. The highest moisture content was recorded for MBH (33.55 ± 2.82 %) and the lowest was recorded for AMFH (18.64 ± 1.25%). The moisture content of AMFH of this study was comparable with (Sisay Gobessa *et al*., 2012), 17.2 ± 0.86%, (Bekele Tesfaye *et al*., 2016) , 18.80±0.36%, and (Gebreegziabher Gebremedhin *et al*., 2013) , 18.4±0.8% but higher than (Tewodros Alemu *et al*., 2013) with 16.0 ± 1.25% from Ethiopian *Apis mellifera* honey that was directly harvested from the hive. Likewise, the moisture content of AMMH (21.71 ± 2.90) in present study is higher (18.60 ± 0.17) than commercial honey (Kinoo *et al*., 2012).

The mean moisture content of AMFH was in agreement with National standard (ESA, 2013) and International (Codex, 2001 and EU, 2002) parameters recommended for *Apis mellifera* honey which should be a maximum of 20%, while the moisture content of AMMH was found to be slightly above the national and international standards. The differences in the moisture content of AMMH (21.71±2.90%) and AMFH (18.64±1.25%) might be due to harvesting procedures. AMMH were purchased from market sites and they were initially harvested by farmer beekeepers mostly from traditional hive without following the standard procedures and waiting for the proper level of maturity. Generally, uncapped honey that contains more water is not recommended for harvesting. The season of honey production also affects the moisture content. For instance, the honey produced during rainy season contains more moisture content compared to those produced during dry season (Ordó *et al*., 2005).

In Ethiopia, information on physicochemical properties of MBH is not available. As a result, the moisture content of MBH from the present study was compared with investigators from other countries. The moisture content of (33.55 ± 2.82 %) was slightly higher than the previous researchers (Vit *et al*. 2004; Alvarez-Suarez *et al*.,2018) and honey from other stingless bees (*Trigona* species) (Berhanu Andualem, 2014) . Such differences were also reported by Kek *et al*. (2017), where Kelulut honey harvested from stingless bees (*Heterotrigona itama*) showed significantly higher moisture content compared to honey produced from *Apis* species. Factors such as bee species, floral source, honey harvesting time, the degree of maturity achieved in the hive and climatic factors affect the moisture content of honey (Bogdanov *et al*., 1999; Kamal *et al*., 2002; Sisay Gobessa *et al*., 2012).

Table 7: Physicochemical composition of honey from different sources

Parameter	Sources of honey samples (Mean ±SD)			X	LSD	P-value	Standard			
	AMMH	AMFH	MBH				Codex Alimentarius,2001* *Apis mellifera*	EU council,2002** Apis mellifera	Ethiopia*** *Apis mellifera*	Vit *et al*,.2004**** *Meliponiae*
MC (%)	21.71^{b}±2.90	18.64^{c}±1.25	33.55^{a}± 2.82	24.63	2.24	<.0001	≤20	≤20	≤21	≤ 30.0
FA (meq/kg)	25.30^{b}±8.81	32.80^{b}±5.77	113.1^{a}±18.43	57.87	12.76	<.0001	≤50	≤50	≤40	≤ 70.0
pH	3.82^{a}± 0.14	3.60^{b}±0.101	3.00^{c} ±0.11	3.48	0.10	<.0001	-	-	-	-
HMF (mg/kg)	15.56^{a}±8.11	5.58^{b} ±3.33	4.40^{b}±2.06	8.51	5.36	0.0003	≤40	≤40	≤40	≤ 40.0
ASH(g/100g)	0.33^{a}± 0.15	0.40^{a}± 0.67	0.26^{a} ±0.10	0.33	0.37	0.7498	≤0.6	≤ 0.6	-	≤ 0.5
EC (mS/cm)	0.26^{b} ±0.08	0.38^{a}± 0.01	0.25^{b}± 0.07	0.29	0.06	0.0003	≤ 0.8	-	≤ 0.8	-

Means with different superscript (a, b, c) within the rows are statistically different at $p \leq 0.05$. Note: SD= Standard deviation, LSD= Least Significant Difference at alpha=0.05, X= overall mean, AMMH= *Apis mellifera L*. honey sample from market, AMFH= *Apis mellifera L*. fresh honey directly from hive and MBH= *Meliponula beccarii L*. honey from underground, MC (%)= Moisture content of honey in percent, FA (meq/kg)= Free Acidity in mill equivalents per kilogram of honey, HMF (mg/kg) = Hydroxymethyl Furfural in mg/kg of honey, ASH(g/100g) = Ash content of honey in g/100g, EC (ms/cm) = Electrical conductivity in milli Siemen per centimeter,*= Codex Alimentarius Commission(2001), **=The Council of the European union(2001),***=Ethiopian Standards Agency(2013) and ****=Guatemala, Mexico and Venezuela(2004)

6.2.2. Free acidity, pH and HMF

The free acidity of MBH (113.1 ± 18.43 meq/kg) significantly ($p < 0.0001$) differed from AMMH (25.30±8.81meq/kg) and AMFH (32.80±5.77 meq/kg) (Table 7). However, statistically ($p>0.05$) significant difference was not observed in the free acidity between AMMH and AMFH. The highest mean free acidity value of 113.1 ± 18.43 meq/kg was recorded for MBH. Free acidity indicates one of the quality parameters of honey samples and it reveals whether the honey is fermented or not (Silvano *et al*., 2014) and corresponds to the presence or absence of organic acids in the product.

With the exception of MBH, the mean free acidity of other honey samples (AMMH and AMFH) fits to the national (≤ 40 meq/kg) (ESA, 2013) and international (Codex, 2001 and EU, 2002) quality standards which should be a maximum of 50 meq/kg from *Apis mellifera* honey. None of the honey samples from *Apis mellifera* exceeded the acceptable limit. Free acidity values of honey close to the result obtained in the current study were reported from Tigray (29.89±5 meq/kg; Gebreegziabher Gebremedhin *et al*., 2013) and from Amhara (27.34 ± 5.06 meq/kg; Tewodros Alemu *et al*., 2013). However, the mean free acidity of honey (*Apis mellifera*) from this study was higher than the free acidity of honey (*Apis mellifera*) reported from Nigeria (18.67 ± 0.64 meq/kg; Nweze *et al*., 2017) and from Polish market (14.40 ± 0.58 meq/kg; Makarewicz *et al*., 2017).

MBH sample from the *Meliponula* species in this study had free acidity value (113.1±18.43 meq/kg) which was higher than the previous reports (Vit *et al.,* 2004; Berhanu Andualem, 2014; Nweze *et al*., 2017; Alvarez-Suarez *et al*., 2018). The mean free acidity of MBH (113.1±18.43meq/kg) was 3.7 times higher than *Apis mellifera* honeys (32.80±5.77meq/kg) and this result is in agreement with other studies (Vit *et al*., 2004; Kek *et al*., 2017), who reported that free acidity of stingless bee is higher compared to *Apis mellifera* honey. The free acidity of MBH from this study was comparable with Malaysia stingless bee (*Heterotrigona itama*) (136.8±7.6 meq/kg) (Kek *et al*., 2017), Australia stingless bee (*Tetragonula carbonaria)* 124.2 ± 22.9 meq/kg (Oddo *et al*., 2008), *Melipona quadrifasciata* and *Tetragonisca angustula* from Brazil (Souza *et al*., 2006) with average 103.3 meq/kg and 109.0 meq/kg, respectively. The

difference in the free acidity of honey samples produced by different bee species might be due to fermentation of sugar in to organic acids (Silvano *et al*., 2014).

The MBH sample had more acidity with mean pH of 3.00 ± 0.11 which is less than the value (3.2 ± 0.21) reported by (Alvarez-Suarez *et al*., 2018) from Cuba stingless bees (*Meliponula species*) but higher than the value from Ethiopia (Berhanu Andualem, 2014) from different species of *Trigona*. Statistically ($p<0.0001$) highly significant differences were observed between all sources of honey types.

The mean pH result of honey (*Apis mellifera*) observed from the present investigation was within the pH range of international standard (3.2-4.5). The mean pH value from AMMH (3.82± 0.14) of this study was equal with Malaysian honeys (pH=3.78 ± 0.21; Moniruzzaman *et al*., 2013) but lower than pH value from Istanbul market (pH=4.32; Uran *et al*., 2017). The differences in the pH values of the samples might be due to the differences in the source of honey such as entomological, botanical and processing methods **(**Alvarez-Suarez *et al*., 2018**).**

The highest concentration of HMF was recorded for AMMH (15.56 ± 8.11 mg/kg). The amount of HMF of AMMH significantly ($p < 0.01$) differed from AMFH (5.58 ± 3.33 mg/kg) and MBH (4.40±2.06). However, significant difference was not ($p>0.05$) observed in the HMF concentration of AMFH and MBH (Table 7). The amount of hydroxymethyl furfural (HMF) and enzymatic activity in honey is one of the important indicators of honey's quality (freshness) indicating whether the honey is aged or over-heated (Mairaj *et al*., 2008; Subramanian *et al*., 2007). None of the investigated samples exceeded the allowed limit of National (ESA, 2013) and International (Codex, 2001 and EU, 2002) quality standards (Table 7). Hydroxymethyl furfural should be a maximum of 40 mg/kg from *Apis mellifera* L., whereas (Vit *et al.*, 2004) have reported value <40 meq/kg) from *Melipona* species honey.

From the present study, honey samples from market had low free acidity, high pH and high hydroxymethyl furfural (HMF) compared to fresh honey directly harvested from modern hive and underground nests of stingless bees. The differences observed might be associated with beekeepers or honey retailers, processing of the honey samples through straining and filtering though exposure to heating or sunlight which deactivate glucose oxidase activity which limits the production of acids. The differences in the amount of HMF was also reported by other

researchers (Bogdanov *et al*., 2002 ; Chua *et al*., 2014). According to these authors, the HMF content of honey increases through over-heating, ageing and poor storage conditions. After a year storage of honey produced in Greek (Thrasyvoulou, 1986) reported an average HMF increase from an initial value of zero to 8.8 mg/kg.

6.2.3. Ash content and electrical conductivity

The average ash contents of different honey samples are indicated in Table 7. There was no statistically significant ($p > 0.05$) variation between ash contents of both sources of honey samples observed. Mean ash content (g/100 g) of AMMH, AMFH and MBH were 0.33±0.15, 0.40± 0.67 and 0.26 ± 0.10, respectively. The ash contents of AMFH and AMMH samples fell within the concentration in the norm of International Honey Commission (Codex, 2001; EU, 2002) which is not more than 0.6% for *Apis mellifera*. Similarly, the ash content of MBH was found within the concentration proposed by (Vit *et al*., 2004) who suggested that the ash content of honey from stingless bee (*Melipona beechei*) should be not more than 0.5% (Table 7).

The ash content MBH of this study was lower than the result (0.46 ± 0.03) reported by (Alvarez-Suarez *et al*., 2018) from stingless bee (*Melipona beechei*). The difference in the ash content might be due to floral origin and soil features, and honeys with ash content of $\leq$ 0.6% have the nectar source (Andrade *et al*., 1999). The blossom honeys (nectar of plants) have lower ash content than the honeydew (secretions of living parts of plants or excretions of plant-sucking insects on plants`) (Bogdanov, 2009)

Electrical conductivity (mS/cm) of AMFH (0.38 ± 0.01) significantly ($p < 0.01$) differed from the EC values of AMMH (0.26±0.08) and MBH (0.25±0.07) (Table 7). Electrical conductivity is determined by the ability of ions present in a sample to conduct electrons and it has been found to assist in the determination of the botanical origin of honey samples. The electrical conductivity of honey from the three sources fell within the value recommended by International Honey Commission (Codex, 2001) and Ethiopian standard (ESA, 2013) with the maximum EC value of 0.8% for honey from *Apis mellifera L*. (Table 7). The electrical conductivity of MBH (0.25 ± 0.07) in this study was less than the value (0.58 ± 0.14) reported by Alvarez-Suarez *et al*., (2018) for *Melipona beecheii* honey and (Berhanu Andualem, 2014) (3.27±0.01) for *Trigona* species honey.

Generally, from the present investigation, it can be observed that as the value of electrical conductivity increased, the value of ash content was also increased between each source of honey treatments which is in agreement with the result reported by Kek *et al*., (2017). In this report, as the ash content increased the electrical conductivity also increased. Such direct proportionality between electrical conductivity and ash content of honey samples were also reported by Acquarone *et al*., (2007). According to Biluca *et al*., (2016), variations in electrical conductivity of honey samples were also linked to variations in the geographical, botanical and entomological origins of the honey samples.

6.2.4. Reducing sugar (glucose, fructose and maltose) and non-reducing sugar

The average concentration of reducing sugars (sum of glucose, fructose and maltose) of honey samples analyzed were 69.85±3.35% for AMMH, 74.73±2.54% for AMFH and 40.88±5.01% for MBH and statistically very highly significant difference ($p< 0.0001$) was observed between all sources of honey samples (Table 8). Except for honey samples from stingless bee (*Meliponula beccarrii*) with the lowest concentration of reducing sugar, all the honey samples from *Apis mellifera* exceeded the national (ESA, 2013) and international quality requirements(Codex alimentarius, 2001; EU, 2002) which should be a minimum of 65% and 60%, respectively.

The concentration of reducing sugar from AMFH of this study result was higher than the report from Tigray, Ethiopia (71.2±2.5%; Gebreegziabher Gebremedhin *et al*., 2013) and Amhara, Ethiopia from *Apis mellifera* that was directly harvested (67.3 ± 2.42%; Tewodros Alemu *et al*., 2013). Similarly, a very close concentration of reducing sugar (70.34 ± 7.49%) to the concentration in the current study (69.12 ± 3.21%) was also reported for honey samples from Nigeria (Nweze *et al*., 2017) , but the concentration of reducing sugar from AMFH and AMMH of this study was higher than the concentration of reducing sugar (62.51 ± 1.20%) reported for Malaysian honey (Moniruzzaman *et al*., 2013) and Bangladesh (63.3±1.5%; Islam *et al*., 2017).

The principal carbohydrate constituents of honey are fructose (32.56 to 38.2%) and glucose (28.54 to 31.3 %), which represents 85–95% of total sugars that are readily absorbed in the gastrointestinal tract (El-Arab *et al*., 2006; Saranraj *et al*.,2016). The concentration of glucose very highly significantly ($p<0.0001$) differed among the honey samples analyzed from the three sources. Highly significant differences were also observed ($p < 0.0001$) in the concentration of

fructose between honey samples from different honey bee types, but there was no significant difference in the concentration of fructose for honey samples from the same honey bee types (Table 8). Generally, from this study, fructose and glucose sugars are the dominant sugars found in all sources of honey samples, while fructose was more abundant than glucose. This is also supported by research results from Nigeria (Adenekan *et al*., 2012), Turkey (KIVRAK *et al*., 2017) and Poland (Makarewicz *et al*.,2017) for *Apis mellifera* honey samples.

The presence of enzyme in bees and nectar as well as the presence of sugar in nectar of plant are the main factor for sugar production of any honey (Maurizio, 1959 ; Cavian, 2002) and this is why statistically great variation was observed between the species (*Apis mellifera* and *Meliponula beccarri*) that produce honey. The reducing sugar produced from MBH of the present study was much less and did not fit the standard proposed by several investigators, (Vit *et al*., 2004) (>50%), 63% by (Fonte *et al*., 2013), and 75.64±1.99% by (Nweze *et al*., 2017) from the same species of stingless bee. These results indicate that the sample of MBH might not be harvested during its appropriate time or not matured, or adulterated with the addition of water, which can alter the normal values of carbohydrate composition in the honey. Furthermore, from (Table 7), the moisture content of MBH sample which records 33.55% indicated the external addition of water. Moreover, the amount of rainfall affects the concentration of sugars in honey with more sugars being available in the plant in the dry season than in rainy season (Azonwade *et al*., 2018).

Statistically no significant difference ($p>0.05$) was recorded on a disaccharide maltose (glucose + glucose) between AMFH and MBH, but the concentration of maltose from AMMH significantly ($p<0.0001$) differed from the other two honey types (AMFH and MBH) (Table 8). The maximum and minimum average of maltose was recorded from MBH (3.04 ± 0.97) and AMMH (0.73±0.14), respectively. From these result except the honey sample from AMMH, other honey samples were found within the range 1.88±0.11 to 6.64±0.15 reported by (Makarewicz *et al*., 2017) from Polish market. Diastase activity in honey significantly affected by heating, heat exposure and storage time. This will directly affect the concentration of maltose in the sample especially at market honey.

From the present analysis (Table 8), statistically ($p<0.05$) significant difference in the sucrose concentration was recorded between AMMH and MBH and the highest (1.03±1.50%) was produced by AMMH sample which followed by AMFH (0.26±0.32%) and MBH (0.18 ± 0.13%). The level of sucrose present in honey varies and it depends on its maturity (ripeness) and sources of nectar compounds from which honey produced. All of the honey samples fit norm of national (ESA, 2013) and international (Codex Alimentarius, 2001 and EU, 2002) quality requirements with a maximum of 10% and 5% sucrose content from *Apis mellifera,* respectively. The present study values were less than previously reported for honey samples from Malaysia (3.02±1.33; Moniruzzaman *et al.*, 2013), from Bangladesh honey (6.1±0.1; Isalm *et al*., 2017) and from Nigeria (2.36 ± 0.05; Nweze *et al*., 2017). The sucrose content in mature honeys could be low, due to the invertase enzyme, which degrades the disaccharide(sucrose) into two simple sugars (glucose and fructose) (Fonte *et al*.,2013). However, high temperature (overheat) and long storage of honey deactivates the enzymatic activity. Sucrose content of honey is used to detect adulteration of honey by addition of cane or beet sugars. From the present study, honey collected and analyzed from different sources showed the absence of adulteration with external sugar. Quality standard of stingless bee honey was not legislated and comparison was carried out with the norm by (Vit *et al*., 2004), who proposed a maximum of 6% for the *Melipona* honey; which agrees with the sucrose content of the finding obtained in this study.

Table 8: Sugar contents of honey samples from different sources

Sugar	Sources of honey samples (mean ± SD)			X	LSD	P-value	Standard			
	AMMH	AMFH	MBH				Codex Alimentarius,2001* *Apis mellifera*	EU council, 2002 ** *Apis mellifera*	Ethiopia*** *Apis mellifera*	Vit *et al.*, 2004**** *Meliponiae*
Fructose (%)	38.84^{a} ± 2.65	38.59^{a} ± 0.51	19.61^{b} ± 2.45	32.35	1.93	<.0001	-	-	-	-
Glucose (%)	30.84^{b} ± 3.37	33.56^{a} ± 1.54	18.23^{c} ± 1.62	27.54	2.14	<.0001	-	-	-	-
Maltose (%)	0.73$^{\mathbf{b}}$ ± 0.14	2.57 $^{\mathbf{a}}$ ± 0.70	3.04$^{\mathbf{a}}$ ± 0.97	2.11	0.64	<.0001	-	-	-	-
RS (Fru + Glu+Malt) (%)	69.85$^{\mathbf{b}}$ ± 3.35	74.73$^{\mathbf{a}}$ ± 2.54	40.88$^{\mathbf{c}}$ ± 5.01	61.82	2.90	<.0001	≥60	≥60	≥65	≥50
Sucrose (%)	1.03$^{\mathbf{a}}$± 1.50	0.26$^{\mathbf{ab}}$± 0.32	0.18^{b}± 0.13	0.49	0.81	0.08	≤5	≤5	≤10	≤ 6

Means with different superscript (a, b, c) within the rows are statistically different at p≤0.05.

Note : SD = Standard deviation, X = overall mean, LSD = Least Significant Difference at alpha = 0.05, AMMH = *Apis mellifera L*. honey sample from market, AMFH = *Apis mellifera L*. fresh honey directly from hive and MBH = *Meliponula beccarii L*. honey from underground, RS (%) = percent of Seducing Sugar in honey , Fru = Fructose, Glu = Glucose, Mal = Maltose, * = Codex Alimentarius Commission (2001), ** = The Council of the European Union (2001),*** = Ethiopian Standards Agency (2013) and **** = Guatemala, Mexico and Venezuela (2004)

6.3. Antioxidant activity of honey

6.3.1. Total phenolic content (TPC)

The total phenolic content between different sources of honey samples did not show statistically ($p>0.05$) significant difference (Table 9). Numerically high TPC was recorded from MBH sample with mean value of 273.2 ± 9.2 mg GAE/100 g followed by AMMH (149±22.3 mg GAE/100 g) and AMFH (137.8±7 mg GAE/100 g). Compared to the TPC values from the result of other countries , the mean values of TPC for *Apis mellifera* honey of this study was higher than that of Malaysian samples (186.70±0.84 mg GAE/kg from Acacia forage) and (226.29±1.18mg GAE/kg from pineapple forage) (Moniruzzaman *et al*., 2013). In addition, quite variable and lower TPC values were measured from Poland (Kaškonienė *et al*., 2009), Burkina Faso (Meda *et al*., 2005) and Cuba (Alvarez-Suarez *et al*., 2018). However, the TPC of honey from *Apis mellifera* of this study result was less than Sudanese *Acacia nilotica* honey sample (201.08±2.49 mg GAE /100 g honey) (Idris *et al*., 2011) and market honey from Egypt 5,500-14,120 mg GAE/kg (EI-Borai *et al*., 2018). This is in agreement with the finding of Alvarez-Suarez *et al*., (2010) who noted on factors such as bee species, bee forage and geographical origin of honey samples that represent its chemical composition and phenolic content.

From the present study, MBH contained more total phenol compared to other sources of the tested honey samples. This is in agreement with the investigation by Alvarez-Suarez *et al*., (2018) who proved that both *Apis mellifera* and *Melipona beecheii* are important sources of bioactive compounds even though *Melipona beecheii* showed the highest values in the concentration of the compounds (total phenol) , and the biological properties and composition of honey samples depend on bee species. Furthermore, Kek *et al*., (2016) have suggested that Malaysian TPC by stingless bees (*Heterotrigona itama*) honey (80 mgGAE/100 g) was higher than *Apis* species honey (51.04 to 78.43 mg GAE/100 g) and TPC of Plebeia stingless bees (106.01 ± 9.85 mg GAE/100 g) of honey was slightly higher than those from *Apis mellifera* (92.34 ± 13.55 mg GAE/100g) as reported by Duarte *et al*., (2012).

The total phenol content of MBH from this study had higher values compared to other countries such as polyfloral honey from Cuba by *Meliponula beecheii* L. and Nigerian Nsukka sample (Nweze *et al*., 2017) from stingless bee (*Hypotrigona* spp.). The total polyphenol content

investigated in current study is found in similar range in most scientific literature. The total phenolic compounds are sensitive to phenol and polyphenol units and other electron-donating antioxidants such as ascorbic acid and vitamin E. Phenolic acid such as 4-Dimethylamino benzoic acid, caffeic acid, p-Coumaric acid, gallic acid, vallinic acid, syringic acid and chlorogenic acid are the major identified phenolics in honey which all varies between honey samples and depends on bee species, floral and geographical origin (Alvarez-Suarez *et al*., 2010).

6.3.2. Total flavonoid content (TFC)

The highest TFC was recorded from MBH with mean of (41.0±21.0 mg QE /100 g) followed by AMMH (30.7±15.2 mg QE /100 g) and AMFH (22.2±10.4 mg QE /100 g (Table 9). The TFC from this study was higher than Turkish honey (1.12-9.24 mg QE /100 g) from *Apis mellifera* (Kivrak *et al*., 2017) and commercial Portuguese honeys (1.73 ± 0.80 mg QE /100 g in citrus sample) but slightly similar (21.16 ± 0.80 mg QE /100 g in heather sample) from *Apis mellifera* of Portuguese country (Aazza *et al*., 2013). From this study, as TPC increased, the TFC also increased which showed high polyphenol composition of honey sample. Phenolic acid, flavonoid and tannins are the common classes found under polyphenol (Kečkeš *et al*., 2013) and show wide structural differences.

MBH had the highest TFC value compared to other sources of honey samples and also higher than Cuban stingless bee (*Melipona beecheii* L.) 4.19 ± 0.37mgCE/100g (Alvarez-Suarez *et al*., 2018). Honey from *Meliponula beccarri* L. was from herbaceous plants such as *Guizotia scabra* and *Plantago lanceolate*. These plant species in addition to bee species might be the source of high concentration of bioactive compounds of the samples. It was investigated that honey samples from the monoflora (*Guizotia abyssinica)* had high antioxidant properties from other samples of Bangladesh honey (Islam *et al*., 2012). The deviation occurred between this study and somewhere might be due to source of honey and methods used.

High TFC and TPC were obtained from AMMH compared to AMFH. The aggregation of compounds that are released from different plant diversity such as herbaceous, shrubs and tree types might bring the *Apis mellifera* market honey (multifloral honey) to have more TFC and TPC than honey collected directly from hives which was monofloral honey (*Coffee Arabica* L.).

Similar study has been reported that multifloral honeys have higher antioxidant properties based on their high levels of phenolics, flavonoids, Ascorbic Acid equivalent of Antioxidant content (AEAO) and DPPH (2,2-diphenyl-1-picrylhydrazyl) compared to monofloral honeys (Islam *et al*., 2012).

6.3.3. Antioxidant content

Statistically no variation ($p>0.05$) was observed between all sources of honey samples with regard to antioxidant content. However, the highest concentration of antioxidant (104 ± 22.6 mg AAE /100 g) was recorded for MBH samples while the lowest (42.2±5.7 mg AAE /100 g) by AMFH (Table 9). The mean values of antioxidants from AMFH was within the range reported from Burkina Faso honey sample 11.27 ± 0.02 mg AAE /100g (*Lannea* floral origin) to 65.86 ± 0.1 mg AAE /100g (*Vitellaria* floral origin) from *Apis mellifera* (Meda *et al*., 2005), while, higher than Bangladesh honeys ranging from 18.4 ± 0.7 mg AAE /100g to 34.1±1.4 mg AAE /100g by *Apis mellifera* L. (Islam *et al*., 2012), and Indian honey samples ranging from 15 to 30 mg AAE /100g (Saxena *et al*., 2010).

The present study result is also in agreement with (Kek *et al*., 2016) who verified that Malaysian stingless bees (*Heterotrigona itama*) had higher total ascorbic acid equivalent antioxidant content than *Apis* spp foraged on pineapple honey. Likewise (Meda *et al*., 2004) have reported that honey with the highest Ascorbic acid equivalent antioxidant content (AEAC) value had the highest antioxidant activity. From this study, it can be forwarded that the honey produced by *Melipona* stingless bees (*Meliponula beccarii*) exhibited significantly higher values in TPC, TFC, AOC and showed better antioxidant properties compared to honey produced by *Apis mellifera*. Similar supportive investigation has been demonstrated that the Kelulut honey produced by *Trigona* stingless bees, *Heterotrigona itama* showed significantly higher values in moisture content, water activity and free acidity with better antioxidant properties (high TPC and TAEAC) compared to honey produced by *Apis* spp (Kek *et al*., 2016). AMMH of this study records high phenol, flavonoid and antioxidant content as compared with AMFH. The cumulative of compounds from different plants by AMMH showed variation than monofloral honey (*Coffee arabica*) from AMFH. However, the high value by MBH sample might be due to the difference in plant (*Guizotia scabra* and *Plantago lanceolate*) and bee species.

Table 9: Antioxidant properties of honey

Parameters	Sources of honey sample (mean ± SD)			X	LSD	P-value
	AMMH	AMFH	MBH			
TPC (mgGAE/100g of honey)	149^{a} ±22.3	137.8^{a} ±7	273.2^{a} ±9.2	183.3	165.9	0.1507
TFC (mgQE/100g of honey)	30.7^{a} ±15.2	22.2^{a} ±10.4	41.0^{a} ±21.0	31.3	32.3	0.4145
AOC (mgAAE/100g of honey)	93.5^{a} ±16.0	42.2^{a} ±5.7	104^{a} ±22.6	83.2	62.1	0.5538

Means with the same letter (^{a}) within the row are not statistically significant ($p \leq 0.05$).

Notice: - AMMH: *Apis mellifera* L. market honey sample, AMFH: *Apis mellifera* L. fresh honey sample, MBH: *Meliponula beccarii* L. honey sample, SD: Standard Deviation, X: overall mean, LSD: least significant difference at alpha =0.05, TPC (mgGAE/100g of honey): Total phenolic content in milligram of gallic acid equivalent per one hundred gram of honey sample, TFC (mgQE/100g of honey) : Total flavonoid content in milligram of Quercetin equivalent per one hundred gram of honey sample, AOC (mgAAE/100g of honey): Antioxidant content in mg of Ascorbic acid equivalent in one hundred of honey sample.

6.4. Antimicrobial activity of honeys

6.4.1. Inhibition zone by agar well diffusion

The measured diameter of inhibition zone (mm) that was performed by well diffusion at 75% w/v concentration of honey samples against various pathogenic microorganisms are shown in Table 10 and all the honey samples showed inhibition against all the tested microorganisms except *Candida albicans*. Relatively, the smallest diameter (mm) of zones of inhibition was observed against clinical *Escherichia coli* (CEC) and standard *Escherichia coli* (SEC; ATCC 25923) with a mean of 9.56±2.04 mm and 9.93±2.31 mm by AMMH, 13.46±2.37 mm and 13.36±1.69 mm by AMFH, 13.86±3.24mm and 13.96±3.39mm by MBH, respectively. The honey samples showed highly significant ($p<0.0001$) difference with positive control (Chloramphenicol) which showed strong inhibition (20.40±1.22 - 23.06±1.67 mm) against CEC and SEC respectively (Table 10).

From this study, the largest mean inhibition zones were recorded against *Staphylococcus aureus*. The mean of inhibition zones against clinical *Staphylococcus aureus* and standard *Staphylococcus aureus* (ATCC 25923) were 12.76 ± 4.09 mm and 11.73 ± 3.64 mm by AMMH, 17.80 ± 2.34 mm and 16.00 ± 4.67 mm by AMFH, and 18.26 ± 3.24 mm and 15.73 ± 3.18 mm by MBH respectively, whilst chloramphenicol inhibited a diameter from 26.20 ± 2.28 to 28.26 ± 4.18 on both types of *Staphylococcus aureus* and very highly significance difference ($p<0.0001$) was observed with honey samples. However, clinical *Candida albicans* showed strong resistance against all the honey samples tested while weak inhibition zone (11 ± 2.12 mm) diameter was measured by griseofulvin.

The mean diameter of zone of inhibition (mm) in the current study ranged from 9.56±2.04 (CEC) to 12.76±4.09 (CSA) for AMMH, 13.36±1.69 (SEC) to 17.80±2.34 (CSA) for AMFH and 13.86± 3.24 (CEC) to 18.26±3.24 (CSA) for MBH compared to 20.40±1.22 (CEC) to 28.26±4.18 (CSA) for Chloramphenicol (Table 10). The overall mean diameter of inhibition recorded against all the tested pathogens was 10.80±2.63 by AMMH, 14.83±2.45 by AMFH, 15.71±2.78 by MBH and 25.16 ±2.50 by Chloramphenicol (Table 10).

The mean inhibition zone from all types of honey sample in this study was less than the result by Yalemwork Ewnetu *et al*., (2013) who recorded 22.27±3.79 mm by stingless bee honey,

21.0±2.7 mm by *Apis mellifera* L. (white honey), 18.0±2.3 mm from *Apis mellifera* L. (yellow honey) at 50%(v/v) honey concentration by using agar well diffusion method. However, from their results, honey from stingless bee showed stronger antibacterial activity than honey from *Apis mellifera* L. against the tested pathogenic microbes which supports our results. Another study measured mean diameter zone of inhibition (mm) of 20±1 against *Staphylococcus aureus* at 50 % (v/v) stingless honey concentration by using agar well diffusion method (Berhanu Andualem, 2013), which is slightly similar with our result (Table 10) even if at high concentration (75% v/v).

The mean diameter of inhibition zone from this study was lower than the one reported by Boateng and Diunase, (2015), who found out that honey from Cameroonian market exhibited 37.0±0.0 mm, 30.0±0.0 mm and 29.3±0.6 mm against *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Escherichia coli,* respectively at 75% (w/v) concentration of honey from *Apis mellifera* using agar well diffusion method. The variation with the previous investigators might be due to the methods used like inoculum density, Incubation time, size of plate, depth of agar medium and composition of the medium (Coyle, 2005), in addition to honey components.

Clinical *Staphylococcus aureus* (CSA) was more susceptible to both sources of honey treatments compared with clinical *Escherichia coli* (CEC) which is less susceptible. In general, it was noticed that gram-positive bacteria were more susceptible to honey samples compared with gram-negative bacteria. This might be due to the differences in bacterial cell walls, in gram-negative organisms the complex cell wall with additional outer membrane protects the bacteria from the environment by excluding toxic molecules and provides an additional stabilizing layer around the cell (Silhavy *et al*., 2010).

In addition, gram- positive bacteria were more sensitive to stingless bee honeys compared to gram-negative using agar well diffusion assay method (Nishio *et al*., 2016). None of the honey samples from this study showed clear zone against *Candida albicans* as noted by other investigators (Nishio *et al*., 2016; Assagid Garedew *et al*., 2003). For instance, Moussa *et al.,* (201l) have reported that *Candida albicans* was resistant to all Algerian honey concentrations by using agar and disc diffusion assays. In another study, *Candida albicans* was found to be resistant against honey samples collected from peasant farmers of Igara in Edao state, Nigeria

(Omoya, 2012). Similarly, the report by Wahdan, (2008); Xesús and Estevinho, (2011) demonstrated that inhibition of fungal growth by honey is not related to its osmotic effect.

Overall, the higher and lower antimicrobial activities were exhibited by MBH and AMMH samples respectively which might due to high free acidity, low pH and high polyphenol content of MBH sample. This result is in agreement with several findings (Chan-Rodríguez *et al*., 2012 ; Alvarez- Suarez *et al*., 2018), who verified more efficiency of stingless bee (*Melipona beecheii* L.) honey samples against human pathogens compared to honey from *Apis mellifera* L. due to lower pH, high acidity, high polyphenol and hydrogen peroxide.

The main compound responsible for the antibacterial activity of honey are H_2O_2 and acids which are toxic to bacteria, and their amount depends on the presence of glucose oxidase during glucose oxidation (Weston, 2000; Brudzynski, 2006; Al-Naama, 2009). If a honey contains high concentration of this enzyme, bacteria cannot respond normally to proliferative signals, and their growth remains arrested even when the honey is used in diluted forms (Brudzynski *et al*., 2011). Drastic variations in cytoplasmic pH can harm bacteria by disrupting the plasma membrane or inhibiting the activity of enzymes.

The peroxide group like H_2O_2, which is composed of two oxygen ions which has an unpaired electron, reacts with bacterial cell walls and disrupts their chemical structure by oxidizing their cell walls, stealing electrons from them (Brudzynski *et al*., 2012). A honey H_2O_2 exert bacteriostatic and DNA degrading effects on bacterial cells (Alzahrani *et al*., 2012), which is powerfully influenced by the bacterial sensitivity to oxidative stress (Brudzynski *et al*., 2011). On the other hand, the enzymatic activity of honey is deactivated when honey sample is taken in to light or heating during processing and purification. AMMH sample accounts less effective against the tested pathogenic microbes compared to other sources and recorded high hydroxymethylfurfural which is a sign of honey heating.

Table 10: Mean Inhibition zone of the tested samples against pathogenic microorganisms by agar well diffusion method including the well (6mm diameter) at 75% honey concentration.

Microbes	Inhibition zone in mm (Mean ± SD)			CONT	LSD	p-value
	AMMH	AMFH	MBH			
CEC	9.56^{c}±2.04	13.46^{b}±2.37	13.86^{b}±3.24	20.40^{a}±1.22	1.19	<.0001
SEC (ATCC 25922)	9.93^{c}±2.31	13.36^{b}±1.69	13.96^{b}±3.39	23.06^{a}±1.67	1.21	<.0001
CPA	10.13^{d}±1.87	14.70^{c}±1.39	16.66^{b}±2.33	25.26^{a}±2.93	1.73	<.0001
SPA (ATCC 27853)	11.06^{c}±2.49	14.70^{b}±2.56	15.70^{b}±2.38	25.60^{a}±2.43	1.26	<.0001
CSA	12.76^{c}±4.09	17.80^{b}±2.34	18.26^{b}±3.24	28.26^{a}±4.18	1.81	<.0001
SSA (ATCC 25923)	11.73^{c}±3.64	16.00^{b}±4.67	15.73^{b}±3.18	26.20^{a}±2.28	1.82	<.0001
CST	10.46^{d}±2.01	13.83^{c}±2.13	15.80^{b}±1.74	27.36^{a}±2.82	1.13	<.0001
X	10.80±2.63	14.83±2.45	15.71±2.78	25.16±2.50		
CCA	ND	ND	ND	11 ± 2.1		

Note: Means followed by different letters (a, b, c, d) within the row are statistically significant difference at p≤ 0.05. AMMH: *Apis mellifera* L. honey purchased from market, AMFH: *Apis mellifera* L. honey harvested directly from hive, MBH: Honey sample from *Meliponula beccarii* L. CONT: Positive control, LSD: Least Significant Difference at alpha=0.05, SD: Standard Deviation, X: Overall mean, CEC: Clinical *Escherichia coli*, SEC (ATCC 25922): Standard *Escherichia coli* American Type Culture Collection number 25922, CPA: Clinical *Pseudomonas aeruginosa*, SPA (ATCC 27853): Standard *Pseudomonas aeruginosa* American Type Culture Collection number 27853, CSA: Clinical *Staphylococcus aureus*, SSA (ATCC 25923): Standard *Staphylococcus aureus* American Type Culture Collection number 25923, CST: Clinical *Salmonella typhi*, CCA: Clinical *Candida albicans* and ND: not detected.

6.4.2. Inhibition zone by agar disc diffusion

As with well diffusion, both sources of honey samples were less effective by disc diffusion against both types of *Escherichia coli* (Table 11). The mean inhibition zone of clinical *Escherichia coli* and standard *Escherichia coli* was 6.0±0.94 and 6.8±1.87 by AMMH**,** 6.5±0.70 and 7.5±1.50 by AMFH, and 7.2±1.03 and 8.4±1.07 by MBH, respectively. On the other hand,

the highest efficacy of honey samples were observed against clinical *Staphylococcus aureus* and standard *Staphylococcus aureus* with the mean inhibition (mm) of 9.2±1.03 and 8.8±1.31 by AMMH, 11.4±2.06 and 10.1±1.96 by AMFH, and 10.9±1.59 and 12.4±1.64 by MBH, respectively. Standard antibiotic (chloramphenicol) showed very highly significant different ($p<0.0001$) compared to other treatments and showed the lowest and highest inhibition zone with average values of 17.9±1.59 to 18.1±1.44 against both types of *Escherichia coli* and 23.3±1.41 to 24.0±2.75 against both types of *Staphylococcus aureus*, respectively (Table 11).

Generally, the diameter of inhibition zone (mm) by disc diffusion method ranged from 6.0 ± 0.94 (CEC) to 9.2 ± 1.03 (CSA) by AMMH, 6.5 ± 0.70 (CEC) to 11.4 ± 2.06 (CSA) by AMFH, 7.2 ± 1.03 (CEC) to 12.4 ± 1.64 (SSA) by MBH and 17.9±1.59 (SEC) to 24.0±2.75 (CSA) by chloramphenicol (Table 11). However, clinical *Candida albicans* was resistant to all the honey samples and weak inhibition zone (9 ± 1.2 mm) as observed by griseofulvin (Table 11). The overall mean against the tested microorganisms by disc diffusion assay was 7.62±1.32 by AMMH, 9.04±1.67 by AMFH, 10±1.21 by MBH and 20.92±2.04 by chloramphenicol (Table 11).

Al- Naama (2009) has studied the antimicrobial activity of honey from Basrah/Iraq region and found inhibition zone (diameter in mm) of 11, 12 and 10 against *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas* spp, respectively at 75%(w/v) concentration of honey from *Apis mellifera* L. using disc diffusion method which is slightly higher than the findings of the present study (Table 8). The disparity might be due to honey sources and methods of the study. In this study, high inhibition zone diameter was measured by agar well diffusion than agar disc diffusion method. This might be due to the difference in contenting the amount of antimicrobial substances by well and disc diffusion method in our study. In well diffusion assay, 60 µl honey concentration was used while 25 µl during disc diffusion. The well diffusion method is reported to be better than the disc diffusion method for producing zones of inhibition (Moumbe *et al*., 2013). Minutes particle in honey tend to migrate more easily in the well diffusion assay than the disc diffusion to influence the growth of pathogenic micro-organisms (kinoo *et al*., 2012). Consequently, agar well diffusion assay was considered as the best method and more accurate than disc diffusion method (Rahimifard *et al*., 2016), and is simple, easy to reproduce and inexpensive (Magaldi *et al*., 2004) in drug susceptibility testing.

Table 11: Mean inhibition zone of the tested samples against clinical and standard pathogens by disc diffusion method with the disc diameter (6 mm) at 75% (w/v or v/v) honey concentration.

Microbes	Zone of inhibition in mm (Mean ± SD)			CONT	LSD	p-value
	AMMH	AMFH	MBH			
CEC	6.0±0.94^{c}	6.5±0.70^{bc}	7.2±1.03^{b}	18.1±1.44^{a}	0.96	<.0001
SEC (ATCC 25922)	6.8±1.87^{c}	7.5±1.50^{bc}	8.4±1.07^{b}	17.9±1.59^{a}	1.39	<.0001
CPA	7.5±1.08^{d}	9.3± 1.63^{c}	10.9±1.19^{b}	19.4±1.42^{a}	1.22	<.0001
SPA (ATCC 27853)	7.9±1.66^{b}	8.9±1.79^{b}	8.4±1.17^{b}	20.6±1.42^{a}	1.39	<.0001
CSA	9.2±1.03^{b}	11.4±2.06^{b}	10.9±1.59^{b}	24.0±2.75^{a}	2.90	<.0001
SSA (ATCC 25923)	8.8±1.31^{c}	10.1± 1.96^{c}	12.4±1.64^{b}	23.3±1.41^{a}	1.45	<.0001
CST	7.2±1.39^{d}	9.6±2.06^{c}	11.0±0.81^{b}	23.2±1.31^{a}	1.33	<.0001
X	7.62±1.32	9.04±1.67	10±1.21	20.92±2.04		
CCA	ND	ND	ND	9 ± 1.2		

Note: different letters (a, b, c and d) on the superscripts within the row are statistically significant difference at $p \leq 0.05$.

AMMH: *Apis mellifera* L. honey purchased from market, AMFH: *Apis mellifera* L. honey harvested directly from hive, MBH: Honey sample from *Meliponula beccarii* L. species. CONT: Positive control, LSD: Least Significant Difference at alpha=0.05, SD: Standard Deviation, X: Overall mean, CEC: Clinical *Escherichia coli*, SEC (ATCC 25922): Standard *Escherichia coli* American Type Culture Collection number 25922, CPA: Clinical *Pseudomonas aeruginosa*, SPA (ATCC 27853): Standard *Pseudomonas aeruginosa* American Type Culture Collection number 27853, CSA: Clinical *Staphylococcus aureus*, SSA (ATCC 25923): Standard *Staphylococcus aureus* American Type Culture Collection number 25923, CST: Clinical *Salmonella typhi* , CCA: Clinical *Candida albicans* and ND: not detected.

6.4.3. Minimum inhibitory concentration (MIC) of honey

The least concentration of different sources of honey sample which inhibited microbial growth was shown in (Figure 3). The minimum MIC values for AMMH, AMFH and MBH samples were 9.4% (w/v), 7.02% (w/v) and 7.02% (v/v), respectively against both types of *Staphylococcus aureus*. The maximum MIC values for AMMH, AMFH and MBH samples were 18.6 % (w/v), 14.06 % (w/v) and 11.52 % (v/v), respectively against both types of *Escherichia coli*.

The mean MIC of the current study from MBH was higher than the study by Berhanu Andualem, (2014) who recorded 6.25% MIC from stingless bee (*Trigona* spp.) honey and 6.25-12.5% MIC from honeybee honey, and (Yalemwork Ewnetu *et al*., 2013) who investigated less MIC (6.25%) of honey from stingless bee against clinical and standard human pathogens (*Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae* and *Salmonella typhi)* compared to 12.5% from *Apis mellifera* yellow and white honey. Our study and others indicated above revealed more efficacy of honey from stingless bee against the test microbes than honey from *Apis mellifera*. However, the variation observed might be due to differences in the honey producing bee species, floral types and production process which all bring a disparity on the components of honey. Furthermore, the isolated pathogens of this study might be differed with the above tested pathogens which showed a difference in the result.

In this study, *Staphylococcus aureus* was more sensitive to all sources of honey samples as it had the greatest zone of inhibition and the reverse is true for *Escherichia coli*. Similar result was indicated by other researchers (Mundo, 2004; Agbaje, 2006; Alvarez-Suarez *et al*., 2010; Oses *et al*., 2016) who showed the most susceptibility of *Staphylococcus aureus* by different monofloral honey than other microorganisms. This observation is in agreement with *Eucalyptus* honey from the Andean region of Ecuador (Valdés-Silverio *et al*., 2018), to which *Staphylococcus aureus* was the most sensitive with a mean minimum active dilution of 6% v/v, followed by *Streptococcus pyogenes* (9% v/v), *Escherichia coli* (11% v/v) and lastly *Pseudomonas aeruginosa*, with the highest value (14% v/v) by *Apis mellifera* honey.

The overall mean of MIC produced by AMMH (15.4%) was high with low inhibition zone followed by AMFH (11%), and MBH (9.92%) (Figure 3). The less effectiveness of AMMH

samples against all the tested organisms might be associated with long storage and heating of honey at market level. During commercialization of honey which is mixed with impurities of hive product was heated or taken in to sunlight to change in to table honey (walela mar in Amharic) (personnel communication). According to Jahan *et al*., (2015), the MICs of the investigated honey samples ranged from 6.25% to 20.00% (w/v) for unheated and increased to 50% after honey was heated to 90°C against *Staphylococcus aureus, Escherichia coli, Salmonella typhi, Salmonella paratyphi* and *Chromobacterium violaceum*.

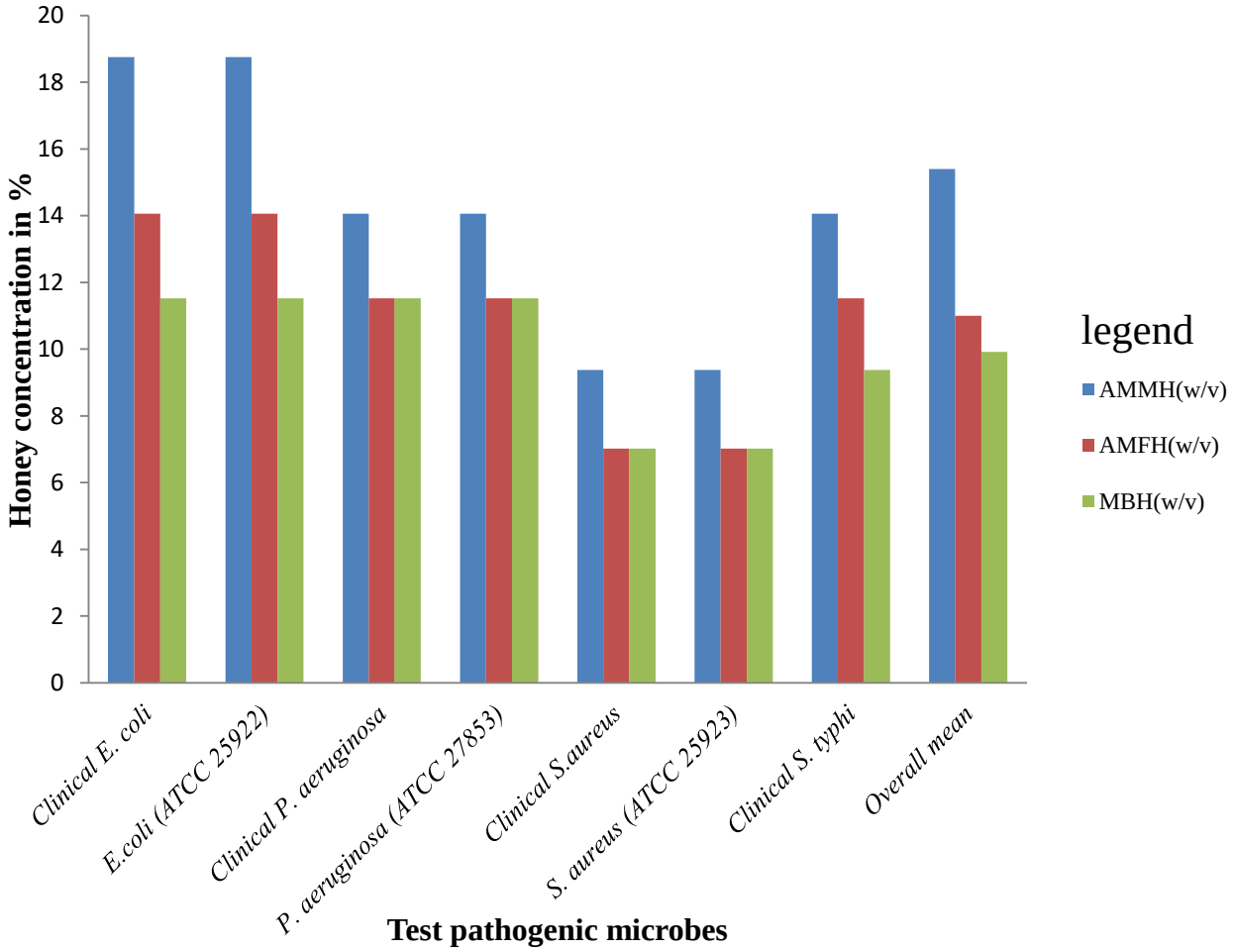


Figure 3: Mean minimum inhibitory concentration of honey samples at different concentrations (75, 37.5, 18.75, 9.375, 4.68 and 2.34%) by using broth dilution method. AMMH*: Apis mellifera L.* honey purchased from market, AMFH*: Apis mellifera L.* honey harvested directly from hive, MBH*:* Honey sample from *Meliponula beccarii L.* species.

6.4.4. Minimum bactericidal concentration (MBC) of honey

The mean MBC of honey from different sources against the tested microbes is indicated (Figure 4). No visible colony was observed against *Escherichia coli* and *Pseudomonas aeruginosa* at 75 % (w/v) and *Staphylococcus aureus* at 46.87 % (w/v) honey concentration by AMMH. AMFH also killed *Escherichia coli* and *Pseudomonas aeruginosa* at 75 % (w/v and *Staphylococcus aureus* at 28.12% (w/v) honey concentration by AMFH. The MBC of MBH against *Escherichia coli* and *Staphylococcus aureus* was 75 and 28.12 % (v/v), respectively.

The mean MBC of MBH from this study was higher than the result by (Berhanu Andualem, 2013) although difference exists with regards to species of bee (*Trigona* species). Cooper *et al*., (1999) have also used similar bacteria and methods and found percentage inhibition ranging from 6.25 to 12.50% (v/v) for honey from stingless bee. Our result is also found within the range of other studies (Araya Gebereyesus and Berhe Gebreslassie, 2016), who reported the overall mean MIC and MBC of 6.25–50 and 12.5–100 % (v/v), respectively against similar test pathogens from *Apis mellifera* (white and red) honey.

The overall mean MBC which killed all the examined microbes was 64.28% (w/v) by AMMH, 56.24% (w/v) by AMFH and 53.57% (v/v) by MBH. This indicates that although there is significant variation between the sources of honey samples, they all have potential bactericidal activities against both standard and clinical drug susceptible and resistance (Annex 20) pathogenic bacteria, but *Candida albican* was resistant to the tested honey concentrations. The small variation in their potency might be due to the source of flower, honey bees, harvesting and handling, besides differences in the season of honey collection that might contribute to differences in their components and antibiotic property of the honey samples. The low pH (3.00 ± 0.11), high free acidity (113.1 ± 18.43 meq /kg) and high antioxidant content (104 ± 22.6 mg AAE /100g) of MBH sample might kill by less concentration compared with others. The bacteriostatic and DNA damaging potency of honey is constituted with phenolic and H_2O_2-induced oxidative stress (Brudzynski *et al*., 2012).

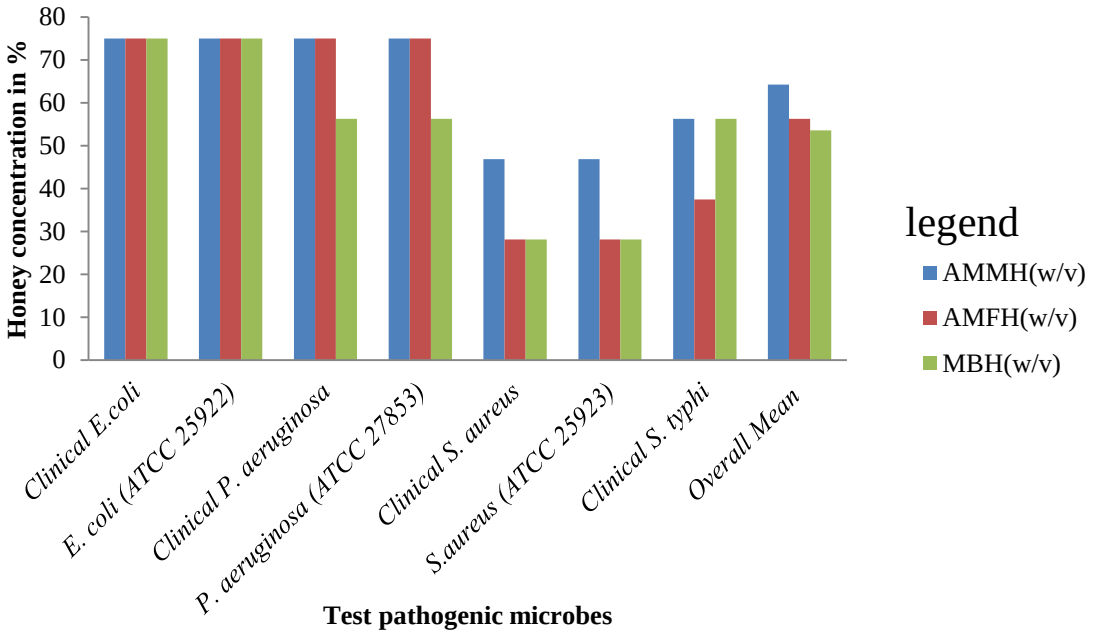


Figure 4: Mean minimum bactericidal concentration of honey samples at different concentrations (75, 37.5, 18.75, 9.375, 4.68 and 2.34%) on agar plate. AMMH: *Apis mellifera* L. honey purchased from market, AMFH: *Apis mellifera* L. honey harvested directly from hive, MBH: Honey sample from *Meliponula beccarii* L. species,

6.5. Microbiological profiles of honey samples

The microbial quality from different sources of honey treatments are summarized in Table 12. From the result, the highest and least mean counted was *Staphylococci* ($2.1x10^3$ cfu/g) and spore forming bacteria ($1.17x10^2$ cfu/g), respectively from AMMH sample. MBH had the highest mean count of $3.16x10^2$ cfu/g by yeast while the least mean count of $1.0x10^2$ cfu/g by mould. However, none of honey types were positive to Enterobacteriaceae and Coliforms indicating the absence of fecal contamination of the honey samples and the hygienic status of honey on sale by the retailers or there might be fecal contamination earlier but the organisms couldn't survive or die because of unsuitability of the honey samples. The acidic character honey sample made microbes not to survive. No microbial count was recorded for samples directly collected from the hive (AMFH). Less microbial load was recorded from MBH sample as compared with AMMH sample. This might be the acidic character (low pH and high free acidity) of MBH sample made microbes not to survive. The pH of honey is major influence on microbial growth in honey (Adadi and Obeng, 2017).

The microbial count from AMMH and MBH of the present study was less than commercial honey samples from Jimma town, Ethiopia (Gadisa Natea and Delelegn Woyessa, 2014) who recorded mean aerobic mesophilic count of $2.2x10^4$ cfu/g, *Staphylococci* ($4.11x10^3$cfu/g), aerobic bacterial spore count of $7.33x10^3$cfu/g, yeast ($4.65\ x10^2$cfu/g), moulds ($5.9x10^2$ cfu/g) but no coliforms. In commercial honey from Nigeria (Oshomah and Ummulkhair, 2014), bacteria count ranged from 1.0×10^4 to 2.0×10^4 Cfu/ml, yeast ranged from 1.0×10^4 to 1.2×10^5 cfu/ml and coliforms only detected in one sample (2.0×10^4). (Gomes *et al*., 2010) have also recorded the mean of aerobic mesophiles (<20 cfu/g), moulds and yeasts (< 22 cfu/g) and no fecal coliforms from commercial honeys of Portugal.

The current study is in agreement with commercial honey from Edo State, Nigeria (Oshomah and Ummulkhair, 2014) who reported the absence of microorganisms from freshly harvested honey samples compared to some level of contamination for samples obtained from market and other retail outlets. Similarly, from Transylvania honey (Popa *et al*., 2009), Romania country, micro-organisms were not detected in the samples collected from primary sources or directly from hives, whereas the bacterium (*Bacillus* spp) and eight types of fungi were detected from honey samples from local markets. This indicates that the sanitary conditions during hive inspection, extraction and handling in the apiaries were quite efficient, while the microbial contamination at market honey may originate from secondary sources during processing, packaging, storing or intentional adulteration.

AMFH samples were free from any microbial contamination. Matured AMFH samples for this study were harvested by bee technicians with proper care for storage materials used and honey combs to be harvested and extracted. Honeys that are well preserved provide unfavorable condition to microorganisms to survive and reproduce (Adenekan *et al*., 2012). The presence of certain amount of bacteria and fungi in the samples is considered to be associated with the contamination during straining, transportation, packaging, storage or marketing (Tchoumboue *et al*., 2007; Uran *et al*., 2017). Relatively as compared with other microbes, high population of *Staphylococci* was recorded from AMMH sample which could be an indicator of poor handling and contact with bare hands of honey handlers. It is normally part of the skin flora found in the nose and on skin and can be transmitted from person to product by unhygienic practices (DÜMEN *et al*., 2013).

Table 12: Microbial counts between sources of honey samples

Microbial groups	Counts (cfu/g)		
	AMMH	AMFH	MBH
Aerobic mesophilic	2.1×10^2	ND	2.5×10^2
Staphylococci	2.1×10^3	ND	1.5×10^2
Yeast	1.9×10^3	ND	3.16×10^2
Mould	3.0×10^2	ND	1.0×10^2
Spore forming bacteria	1.17×10^2	ND	1.5×10^2
Enterobacteriaceae	ND	ND	ND
Coliforms	ND	ND	ND

ND: Not detected (no microbes found).

Cfu/g: Colony forming unit per one gram of honey.

AMMH: *Apis mellifera* honey sample from market.

AMFH: *Apis mellifera* honey direct from hive.

MBH*: Meliponula beccarii* honey from market.

7. Conclusions and recommendations

From the present study, *Coffee arabica* L. is the major honey plants and its honey harvested with in the month of February, while *Guizotia scabra* and *Plantago lanceolate* are the two most attractive forages and their honey is harvested in the October. Furthermore, *Guizotia scabra*, *Coffee arabica*, *Vernonia amygdalina*, *Eucalyptus* spp, *Combretum molle*, *Trifolium ruppelianum* and *Syszgium guineense* were honey plants analyzed from polyfloral market honey even though, their pollen dominance varies.

Honey from *Apis mellifera* L. harvested directly from the hive and purchased from the local market of the study area showed excellent quality for their physicochemical properties based on recommendation by International (European Union Commission and Codex Alimentarius) and National (Ethiopian standard agency).

Regarding antioxidant activities, the higher antioxidant activity was showed by *Meliponulla beccarii* honey followed by *Apis mellifera* market honey and *Apis mellifera* fresh honey samples depending on their values of total phenol, flavonoid and antioxidant content.

All sources of honey treatments from the study area showed their antibacterial activities against selected drug resistance human pathogens although their diameter zone of inhibition varied. *Meliponulla beccarii* honey was more effective against the tested pathogens followed by *Apis mellifera* fresh honey and *Apis mellifera* market honey samples. A gram positive bacterium (*Staphylococcus aureu*s) was more susceptible compared to gram negative bacteria (*Escherichia coli, Salmonella typhi* and *Pseudomonas aeruginosa).* However, none of the sources of honey samples inhibited the tested fungi (*Candida albicans*).

The overall mean of minimum inhibitory concentration and minimum bactericidal concentration was 15.40 and 64.28% by *Apis mellifera* market honey, 11.00 and 56.24% by *Apis mellifera* fresh honey and 9.92 and 53.57% by *Meliponulla beccarii* honey samples respectively. From the result, the mean least honey concentration to inhibit and kill was recorded against *Staphylococcus aureus* compared to the tested gram negative bacteria. *Apis mellifera* market honey and *Meliponulla beccarii* honey samples showed microbial contamination even though below detectable count while *Apis mellifera* fresh honey were safe to microbial loads.

Based on the results of this study, the following recommendations are made for future consideration.

- Unlike honeybee (*Apis mellifera*) honeys, there is no national standard to regulate quality parameter of stingless bee honeys. Therefore, it is recommended that future work will be expected to standardize on the components of honey between the bee species.
- Stingless bee is not domesticated and there is limited information on its characteristics contrasting with *Apis mellifera*. This makes stingless bee honey more expensive and not available all the time disparating with *Apis mellifera* honey. Therefore, scientific work will be expected from the stakeholders on how to change the wild stingless bee in to hive in order to harvest high quality and quantity of its products.
- Since the composition and biological activities of honeys depends on its entomological and botanical origin, studies on different Ethiopian monofloral honeys with their respective bee species against human pathogenic microorganisms is expected. This helps to recognize the specific major honey plant which will be cultivated around apiary site of beekeepers to harvest multipurpose honey.

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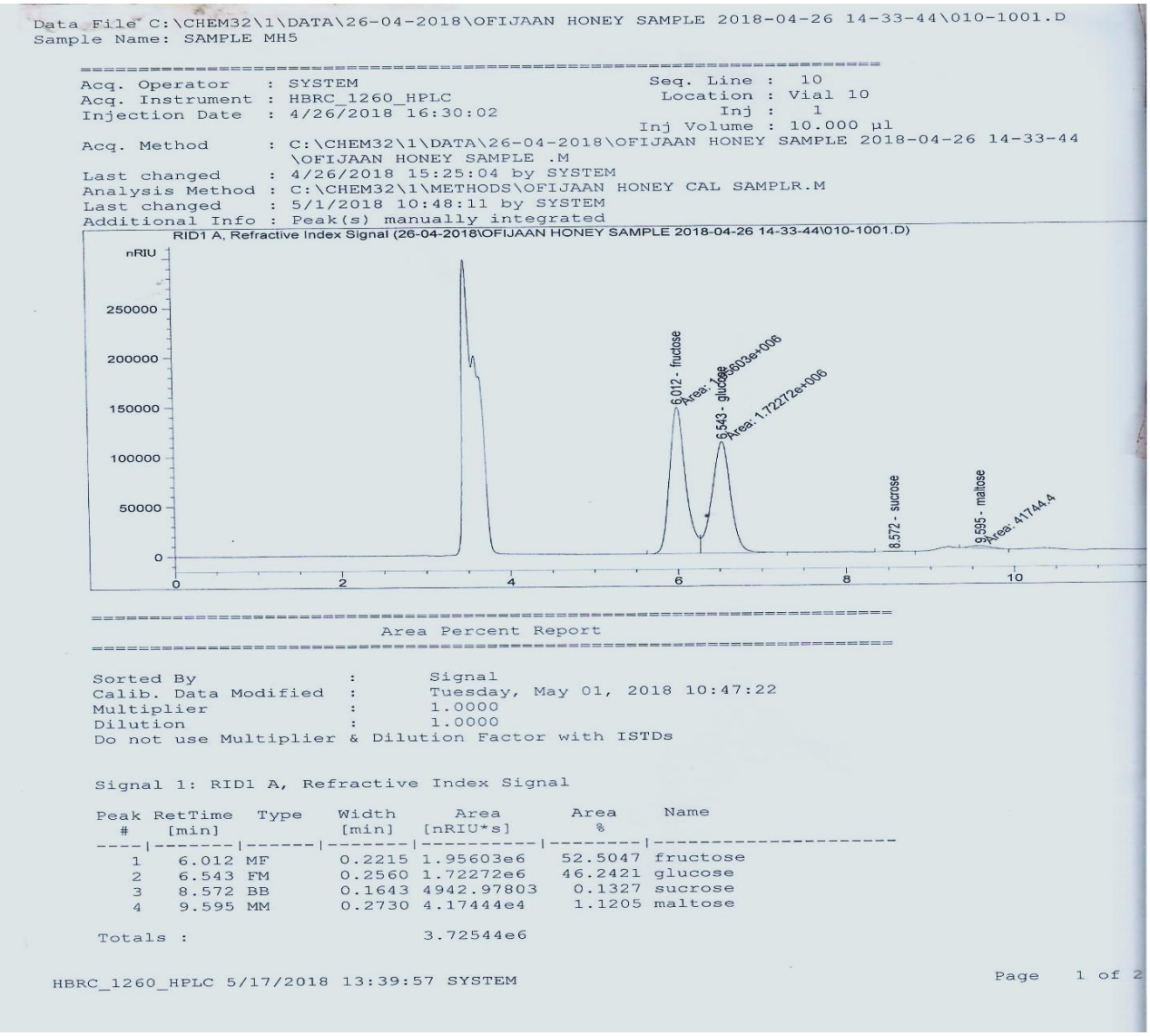
9. Appendixes

Annex 1: Honey sample collection

Honey harvesting and making table honey by using honey presser and filtration.

Annex 2: Sugar analysis using HPLC

Sample preparation, filtering and running HPLC



Data File C:\CHEM32\1\DATA\26-04-2018\OFIJAAN HONEY SAMPLE 2018-04-26 14-33-44\010-1001.D
Sample Name: SAMPLE MH5

Acq. Operator : SYSTEM
Seq. Line : 10
Acq. Instrument : HBRC_1260_HPLC
Location : Vial 10
Injection Date : 4/26/2018 16:30:02
Inj : 1
Inj Volume : 10.000 µl
Acq. Method : C:\CHEM32\1\DATA\26-04-2018\OFIJAAN HONEY SAMPLE 2018-04-26 14-33-44 \OFIJAAN HONEY SAMPLE .M
Last changed : 4/26/2018 15:25:04 by SYSTEM
Analysis Method : C:\CHEM32\1\METHODS\OFIJAAN HONEY CAL SAMPLR.M
Last changed : 5/1/2018 10:48:11 by SYSTEM
Additional Info : Peak(s) manually integrated

RID1 A, Refractive Index Signal (26-04-2018\OFIJAAN HONEY SAMPLE 2018-04-26 14-33-44\010-1001.D)

Area Percent Report

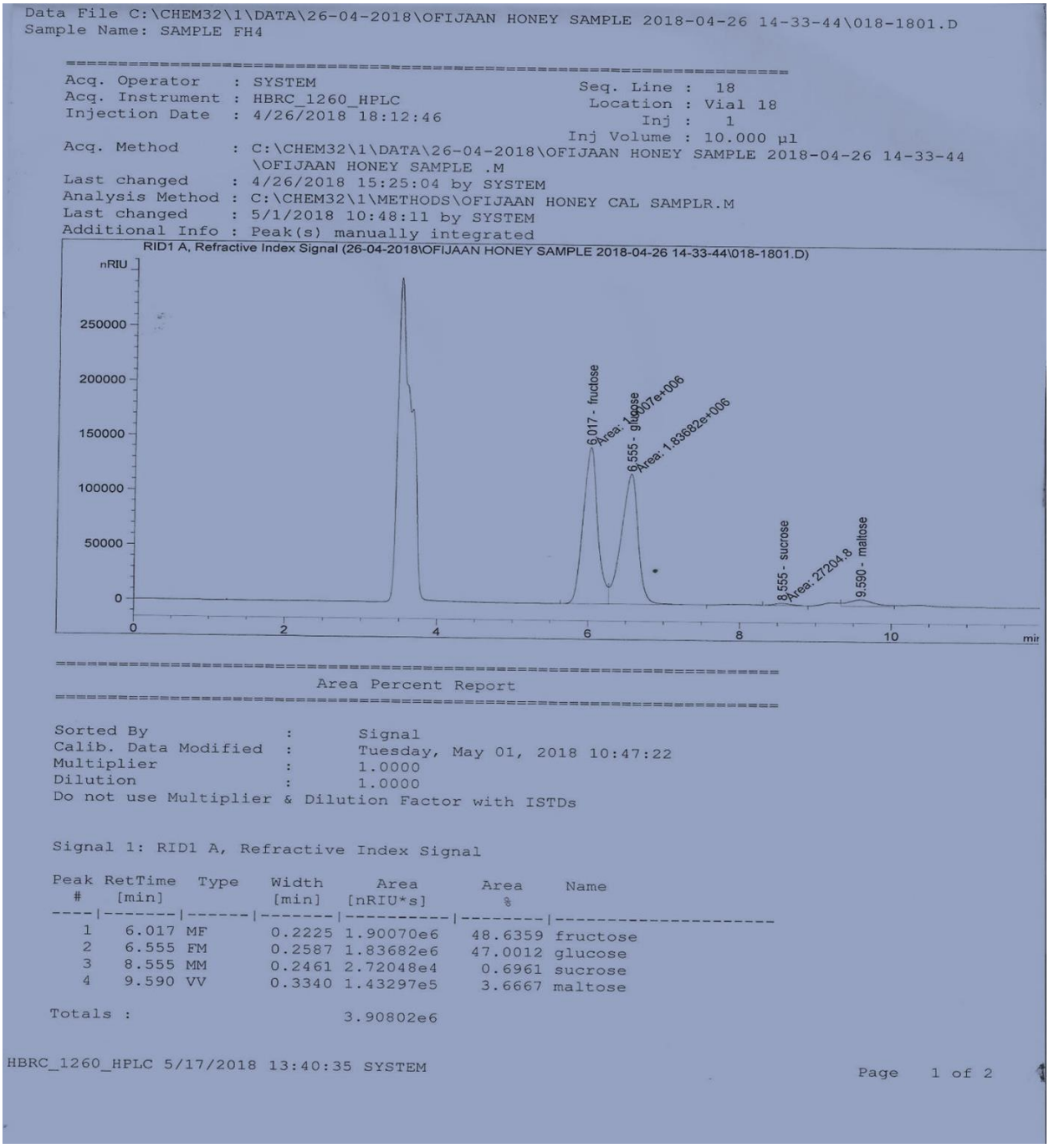
Sorted By : Signal
Calib. Data Modified : Tuesday, May 01, 2018 10:47:22
Multiplier : 1.0000
Dilution : 1.0000
Do not use Multiplier & Dilution Factor with ISTDs

Signal 1: RID1 A, Refractive Index Signal

Peak #	RetTime [min]	Type	Width [min]	Area [nRIU*s]	Area %	Name
1	6.012	MF	0.2215	1.95603e6	52.5047	fructose
2	6.543	FM	0.2560	1.72272e6	46.2421	glucose
3	8.572	BB	0.1643	4942.97803	0.1327	sucrose
4	9.595	MM	0.2730	4.17444e4	1.1205	maltose
Totals :				3.72544e6		

HBRC_1260_HPLC 5/17/2018 13:39:57 SYSTEM Page 1 of 2

Annex 3: Chromatogram by HPLC, of fructose, glucose, sucrose and maltose by *Apis mellifera* market honey sample number five.



Data File C:\CHEM32\1\DATA\26-04-2018\OFIJAAN HONEY SAMPLE 2018-04-26 14-33-44\018-1801.D
Sample Name: SAMPLE FH4

Acq. Operator : SYSTEM
Acq. Instrument : HBRC_1260_HPLC
Injection Date : 4/26/2018 18:12:46
Seq. Line : 18
Location : Vial 18
Inj : 1
Inj Volume : 10.000 µl
Acq. Method : C:\CHEM32\1\DATA\26-04-2018\OFIJAAN HONEY SAMPLE 2018-04-26 14-33-44\OFIJAAN HONEY SAMPLE .M
Last changed : 4/26/2018 15:25:04 by SYSTEM
Analysis Method : C:\CHEM32\1\METHODS\OFIJAAN HONEY CAL SAMPLR.M
Last changed : 5/1/2018 10:48:11 by SYSTEM
Additional Info : Peak(s) manually integrated

RID1 A, Refractive Index Signal (26-04-2018\OFIJAAN HONEY SAMPLE 2018-04-26 14-33-44\018-1801.D)

Area Percent Report

Sorted By : Signal
Calib. Data Modified : Tuesday, May 01, 2018 10:47:22
Multiplier : 1.0000
Dilution : 1.0000
Do not use Multiplier & Dilution Factor with ISTDs

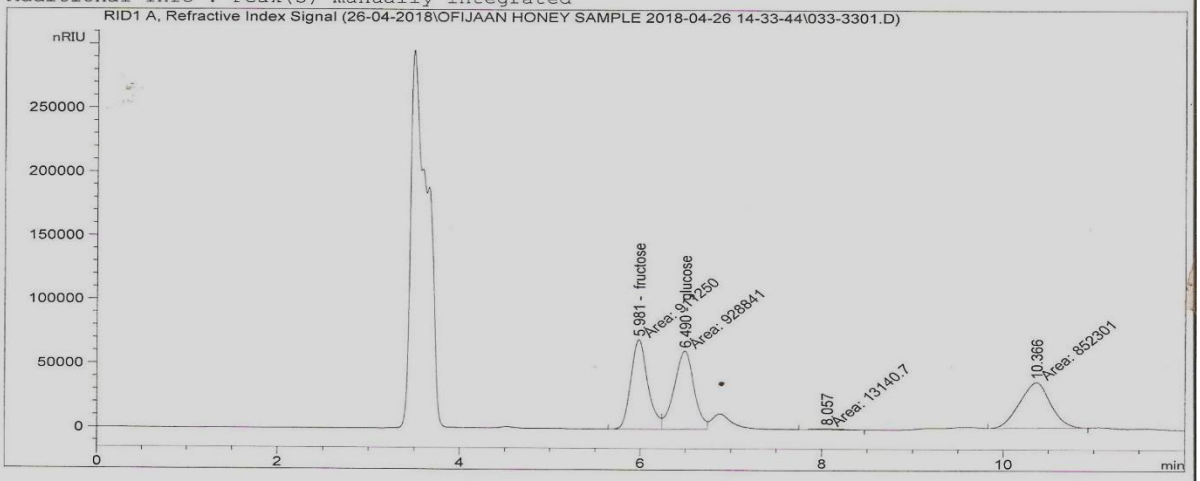
Signal 1: RID1 A, Refractive Index Signal

Peak #	RetTime [min]	Type	Width [min]	Area [nRIU*s]	Area %	Name
1	6.017	MF	0.2225	1.90070e6	48.6359	fructose
2	6.555	FM	0.2587	1.83682e6	47.0012	glucose
3	8.555	MM	0.2461	2.72048e4	0.6961	sucrose
4	9.590	VV	0.3340	1.43297e5	3.6667	maltose
Totals :				3.90802e6		

Annex 4: Chromatogram by HPLC, of fructose, glucose, sucrose and maltose by *Apis mellifera* Fresh honey sample number four.

Data File C:\CHEM32\1\DATA\26-04-2018\OFIJAAN HONEY SAMPLE 2018-04-26 14-33-44\033-3301.D
Sample Name: SAMPLE TH10

Acq. Operator	: SYSTEM	Seq. Line	: 33
Acq. Instrument	: HBRC_1260_HPLC	Location	: Vial 33
Injection Date	: 4/26/2018 21:25:27	Inj	: 1
		Inj Volume	: 10.000 µl
Acq. Method	: C:\CHEM32\1\DATA\26-04-2018\OFIJAAN HONEY SAMPLE 2018-04-26 14-33-44 \OFIJAAN HONEY SAMPLE .M		
Last changed	: 4/26/2018 15:25:04 by SYSTEM		
Analysis Method	: C:\CHEM32\1\METHODS\OFIJAAN HONEY CAL SAMPLR.M		
Last changed	: 5/1/2018 10:48:11 by SYSTEM		
Additional Info	: Peak(s) manually integrated		



Area Percent Report

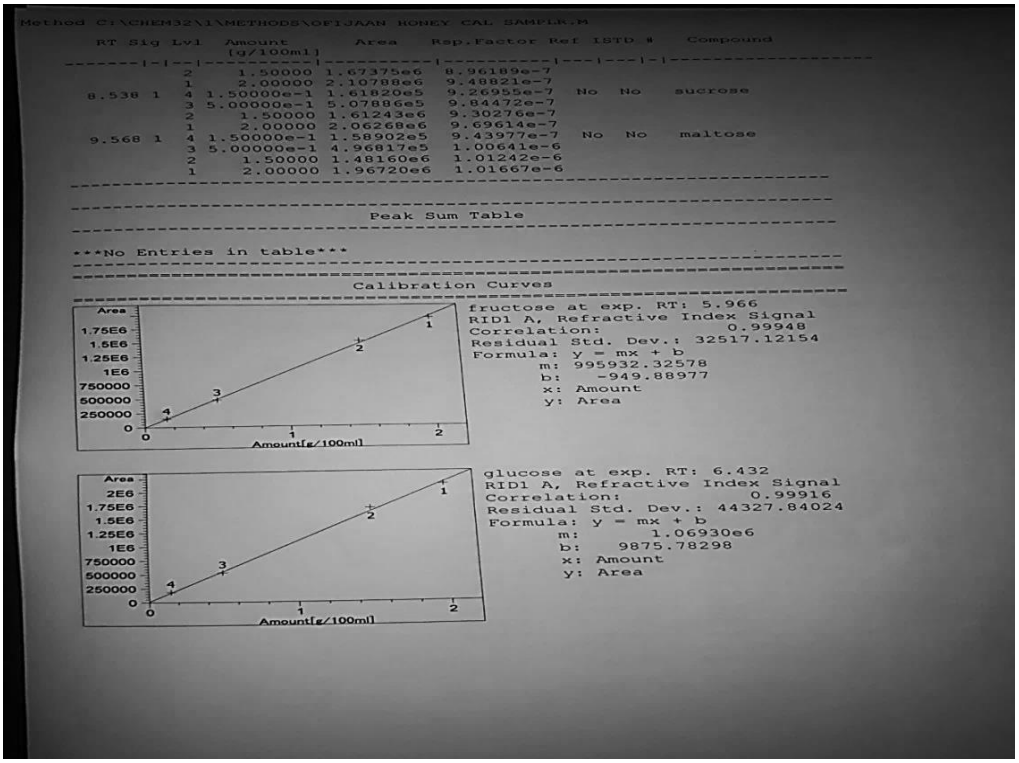
Sorted By	:	Signal
Calib. Data Modified	:	Tuesday, May 01, 2018 10:47:22
Multiplier	:	1.0000
Dilution	:	1.0000

Do not use Multiplier & Dilution Factor with ISTDs

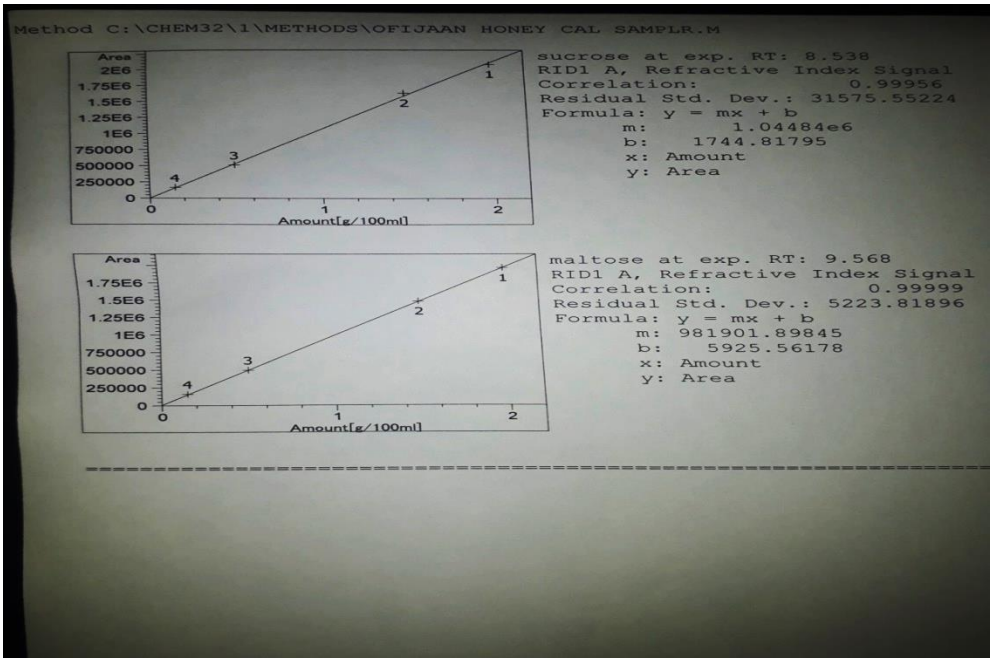
Signal 1: RID1 A, Refractive Index Signal

Peak #	RetTime [min]	Type	Width [min]	Area [nRIU*s]	Area %	Name
1	5.981	MF	0.2176	9.11250e5	33.6810	fructose
2	6.490	FM	0.2536	9.28841e5	34.3312	glucose
3	8.057	MM	0.3426	1.31407e4	0.4857	?
4	8.538		0.0000	0.00000	0.0000	sucrose
5	9.568		0.0000	0.00000	0.0000	maltose
6	10.366	MM	0.3969	8.52301e5	31.5021	?

Annex 5: Chromatogram by HPLC, of fructose, glucose, sucrose and maltose by *Meliponula beccarii* market honey sample number ten.



Annex 6: Calibration curve of fructose and glucose



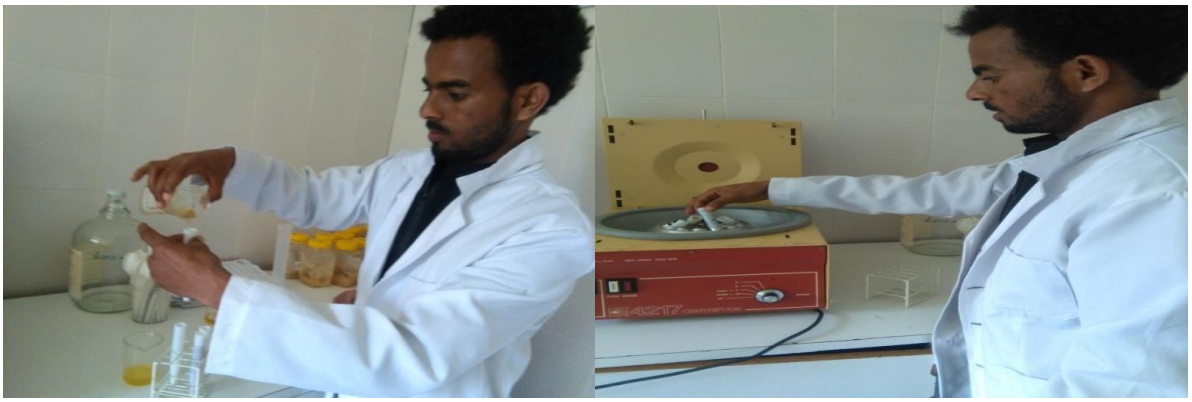
Annex 7: Calibration curve of sucrose and maltose

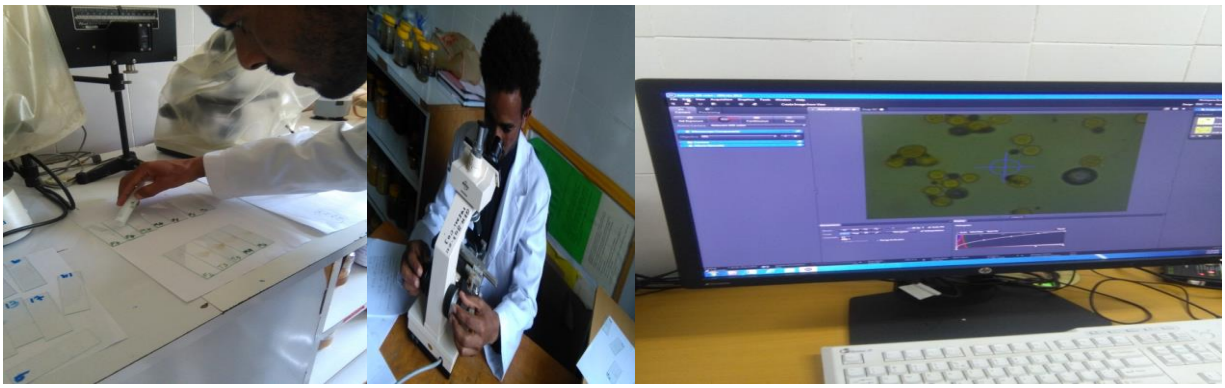
Annex 8: HMF content determination using UV- Spectrophotometer

Annex 9: ASH Analysis using muffle furnace.

Carbonation of honey samples using not plate Ash of honey samples

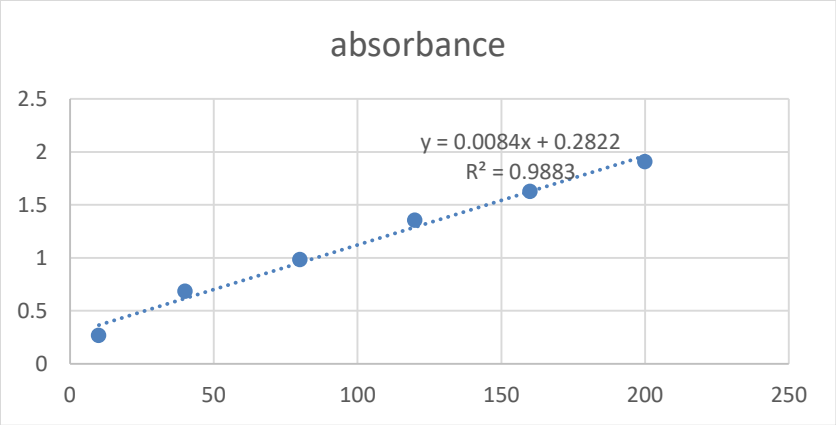
Annex 10: Analyzing free acidity and pH content of the honey samples by using pH meter



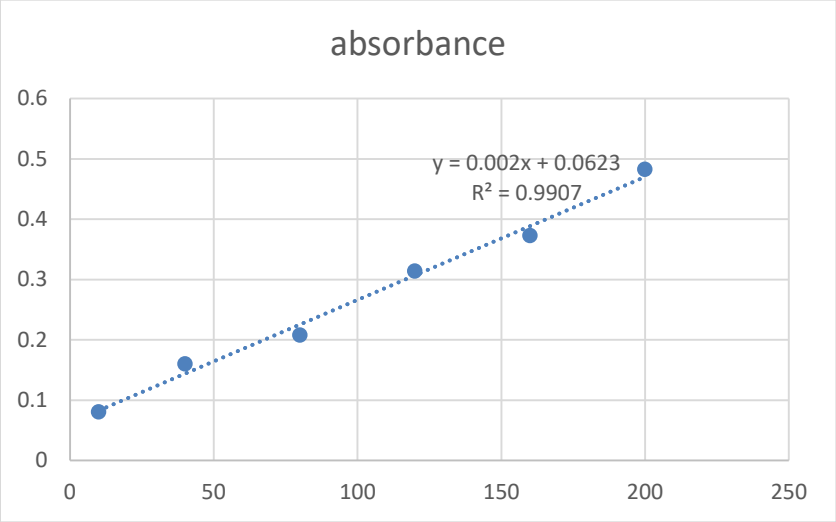


Annex 11: Pollen grain analysis of honey sample.

Sample preparation, centrifuging, fixing on the slide, counting on microscope connected with computer.

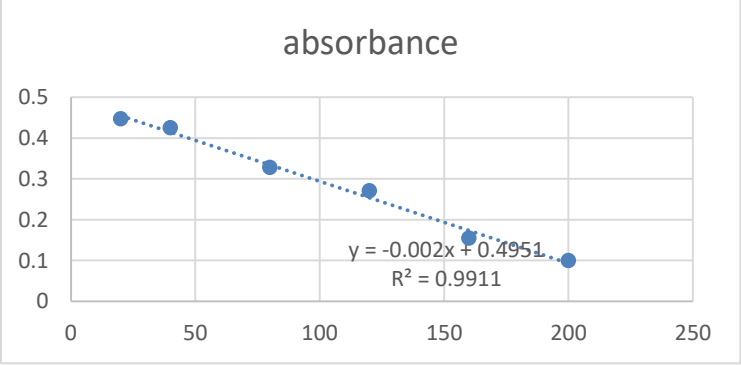


Annex 12: Calibration curve of phenol



Concentration (mg/l)

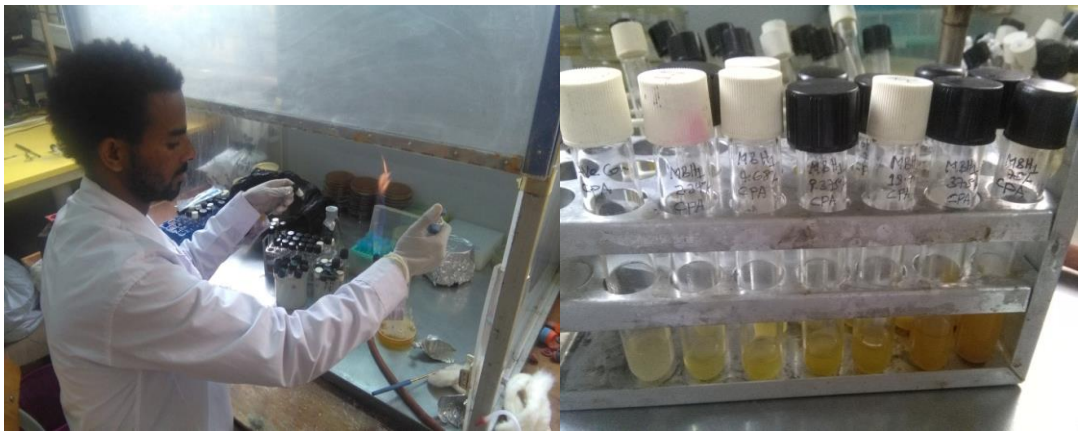
Annex 13: Calibration curve of flavonoid



Annex 14: Calibration curve of Ascorbic acid

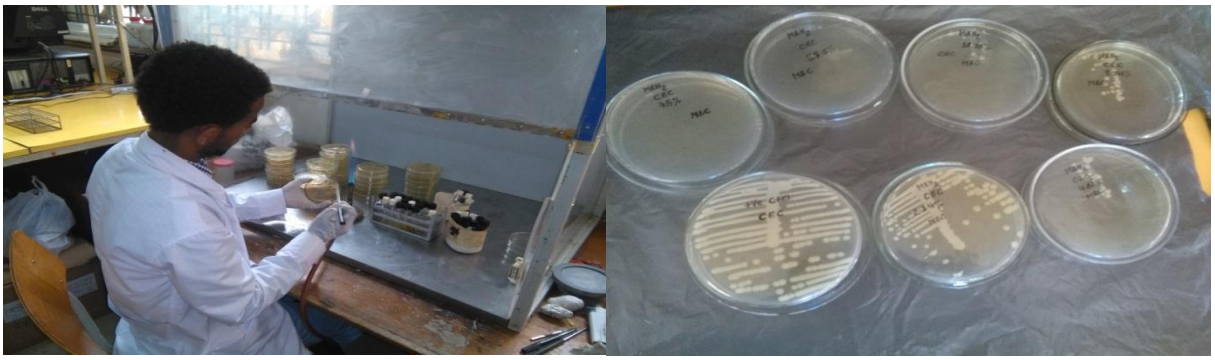
Annex 15: **Susceptibility testing by well diffusion method**. Spreading the inoculum on the solidified plate, dropping the antimicrobial agent in to the well and measuring the inhibition zone of CSA (Clinical *Staphlococcus aureus)* by Caliper. AMMH *(Apis mellifera L.* honey), AMFH (*Apis mellifera L.* fresh honey*)* and MBH *(Meliponula beccarii L.*honey). Chl (Chloramphenicol which is positive control) and H_2O (water which is as negative control).

Annex 16: **Inhibition zone diameter (mm) by Agar Disc diffusion**. Inhibition zone of CSA (Clinical *Staphlococcus aureus*), CPA (Clinical *Pseudomonas aeruginosa*), CEC (*Clinical Esherichia coli*) and CST (Clinical *Salmonella typhi*). AMMH (*Apis mellifera* L. honey), AMFH (*Apis mellifera* L. fresh honey) and MBH (*Meliponula beccarii* L. honey), Chl (Chloramphenicol which is positive control) and H_2O (water which is as negative control).

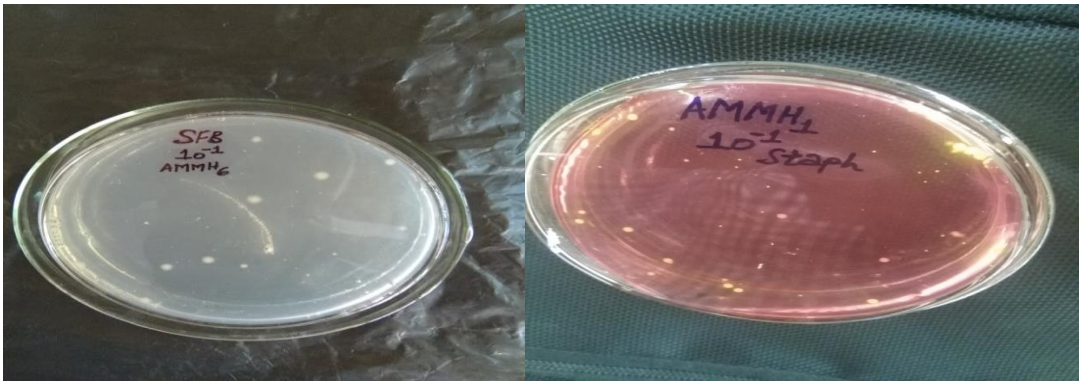


Annex 17: Determination of minimum inhibitory concentration

Making serial dilution of honey sample for minimum inhibitory concentration



Annex 18: **Determination of minimum bactericidal concentration**. Streaking from MIC to get MBC on to the plate



Annex 19: **Microbial growth on plate**. $AMMH_{1\ or\ 6}$: *Apis mellifera* market honey sample 1st and 6th, SFB: spore forming bacteria, staph: staphylococcus, 10^{-1}: first serial dilution.

Annex 20: Antibiogram data of commonly used commercial drugs against pathogens obtained from Ethiopian Public Health Institute (EPHI), Bacteriology laboratory.

Organisms	Antimicrobial agent	µg/disc	Zone of diameter in mm	Category
Staphylococcus aureus isolated from pus	Tetracycline	30	24	Susceptible
	Clarithromycin	15	27	Susceptible
	Clindamycin	2	21	Susceptible
	Oxacillin	30	23	Susceptible
	Cotrimoxazole		22	Susceptible
	Cefoxitin	30	23	Susceptible
Pseudomonas aeruginosa isolated from fluid	Amikacin	30	17	Susceptible
	Ceftazidime	30	6	Resistant
	Ciprofloxacin	5	7	Resistant
	Doripenem	10	6	Resistant
	Gentamicin	10	12	Resistant
	Piperacillin-tazopactam	100	6	Resistant
	Tobramycin	10	7	Resistant
Escherichia coli from urine	Gentamicin	10	6	Resistant
	Tetracycline	30	6	Resistant
	Cefazolin	30	6	Resistant
	Doripenem	10	26	Resistant
	Tobramycin	10	7	Resistant
	Piperacillin-tazopactam	100	24	Resistant
	Ciprofloxacin	5	6	Resistant
	Ceftriaxone	30	8	Resistant
Salmonella typhi from blood	Ciprofloxacin	5	6	Resistant
	Amikacin	30	17	Susceptible
	Gentamicin	10	6	Resistant
	Tetracycline	30	6	Resistant
	Ceftazidime	30	6	Resistant
	Chloramphenicol	20	23	Susceptible