



WOLLEGA UNIVERSITY
SCHOOL OF GRADUATE STUDIES
COLLEGE OF NATURAL & COMPUTATIONAL SCIENCES
DEPARTMENT OF CHEMISTRY
M.Sc. THESIS

**INVESTIGATION OF PHYSICOCHEMICAL PROPERTIES,
ANTIOXIDANT AND ANTI-BACTERIAL ACTIVITIES OF APINAE
HONEY AND STINGLESS BEE HONEY PRODUCED IN KONDALA
DISTRICT, OROMIA, ETHIOPIA**

BY: MOAZ ABDULKADIR CHALA

ADVISOR: MELKAMU BIYANA (PhD)

JANUARYR, 2026
NEKEMTE, ETHIOPIA



WALLAGA UNIVERSITY
SCHOOL OF GRADUATE STUDIES
P.O. Box: 395, Nekemte, Ethiopia.

APPROVAL SHEET FOR SUBMITTING THESIS PAPER

Investigation of Physicochemical Properties, Antioxidant and Anti-Bacterial Activities of Apinae Honey and Stingless Bee Honey Produced in Kondala District, Oromia, Ethiopia

Submitted by:

Moaz Abdulkadir Chala

Name of Student

Signature

Date

Approved by:

2 Melkamu Biyana (PhD)

Name of Major Advisor

Signature

Date

3

Name of Co-Advisor

Signature

Date

4

Name of Chairman, DGC

Signature

Date

5

Name of Dean, SGS

Signature

Date

STATEMENT OF THEAUTHOR

I, Moaz Abdulkadir Chala hereby declare that this thesis entitled “ Investigation of Physicochemical Properties, Antioxidant and Anti-Bacterial Activities of Apinae Honey and Stingless Bee Honey Produced in Kondala District, Oromia, Ethiopia" is my original work conducted under the supervision of Melkamu Biyana (PhD). That all references to materials used in it have been properly cited, in other words all scholarly matter that is included in the thesis has been given recognition through citation. This thesis is being submitted to Wollega University in partial fulfillment of the requirements for an MSc in Chemistry. I hereby certify that this thesis has not been submitted to any other university or location for the purpose of conferring a degree or diploma. In accordance with Wollega University Library policies, the thesis was submitted to the university library for use by researchers. As long as the source is properly cited, brief quotations from this thesis are acceptable without specific permission. However, Wollega University may grant requests for permission to use an extended quotation from a reproduction in a portion of this manuscript. In all other cases, the author's permission is required from the School of Graduate Studies.

Name: Moaz Abdulkadir Chala

Signature-

Date_

Department: Chemistry

College: Natural and Computational Sciences

BIOGRAPHICAL SKETCH OF THE AUTHOR

Mr. Moaz Abdulkadir Chala was born on march, 05, 1983, in Likiti kebele, kondala district, West Wollega, Oromia regional state from his father Abdulkadir Chala and mother Kadija Guta He attended primary education at Wanja Likiti primary school and secondary school at Beghi Secondary School (grade 9-10) in 1997-1998 E.C and also joined preparatory school at Kake Preparatory school 2003-2004 E.C. In 2005 E.C he also joined Wallaga University department of chemistry education and received his first degree in educational chemistry in 2007 E.C. Now he is teaching in West Wallaga kondala district kondala Secondary School. He joined Wallaga University in summer program and has been attending his M.Sc. in chemistry.

ACKNOWLEDGEMENTS

First, I would like to thank the Almighty GOD. Secondly, I would like to express my heartfelt gratitude to my advisor Melkamu Biyana (PhD) who gave me a valuable guidance, priceless suggestion, continuous encouragement and professional support from the starting to the end of the whole research work. Also, I would like to acknowledge chemistry department, specially, lab assistants, for their encouragement to do my thesis in whatever direction. I am also deeply thankful to my classmates and friends for their moral support, constructive discussions and encouragement throughout my MSc program. Finally, I would like to extend my deepest gratitude to my family for their unconditional love, patience, financial support and motivation throughout my academic journey.

TABLE OF CONTENTS

CONTENTS	PAGES
APPROVAL SHEET FOR SUBMITTING THESIS PAPER.....	ii
STATEMENT OF THE AUTHOR	iii
BIOGRAPHICAL SKETCH OF THE AUTHOR.....	iv
ACKNOWLEDGEMENTS.....	v
LIST OF TABLES.....	viii
ABBREVIATIONS AND ACRONYMS	xi
<i>Abstract</i>	xii
<i>Keywords:</i>	xii
UNIT ONE.....	1
INTRODUCTION	1
1.1 Back Ground of the Study	1
1.2 Statements of the Problem.....	3
1.3 Objectives of the Study	3
1.3.1 General Objective of the Study.....	3
1.3.2 Specific Objectives of the Study.....	3
1.4 Significance of the Study	4
1.5 Scope and Limitations of the Study	4
UNIT TWO.....	5
LITERATURE REVIEW	5
2.1 Honey Producing Species.....	5
2.2 Global honey production trends	6
2.3 Honey Production of Ethiopia.....	6
2.4 Physical and Chemical Characteristics of Honey	7
2.4.1 Analysis of physicochemical properties of honey	9
2.5 Essential and Non-Essential Metals in Honey	10
2.6 Antioxidant activities of Honey	11
2.7 Antimicrobial Properties of Honey	11
2.7.1 Anti-bacterial-Activities of honey	11
UNIT THREE	14
MATERIALS AND METHODS.....	14
3.1 Description of the Study Area	14
3.2 Chemicals and Apparatus.....	15
3.3 Honey Sampling Technique	15
3.4 Analysis of Physicochemical Properties	16

3.4.1	pH and free acidity.....	16
3.4.2	Determination of the Ash Content	16
3.4.3	Moisture Content	17
3.4.4	Electrical Conductivity	17
3.4.5	Sugar contents.....	18
3.5	Digestion of honey samples	18
3.5.1	Optimization of the working procedure	19
3.5.2	Determination of essential and non-essential metals in honey samples	19
3.6	Investigation of antioxidant activities	20
3.7	Determination of Anti-bacterial Activity of Honey	20
3.7.1	Preparation of Honey Samples for ant- bacterial test	20
3.7.2	Test of Microorganisms used for the Current Study.....	21
3.7.3	Inoculums Preparation	21
3.7.4	Agar Disc Diffusion Assay	21
3.7.5	Minimum Inhibitory Concentration (MIC).....	22
3.7.6	Minimum Bactericidal Concentration (MBC).....	22
3.8	Method validation	22
3.9	Data analysis	23
UNIT FOUR		24
RESULTS AND DISCUSSION		24
4.1	Physicochemical properties of honey samples.....	24
4.1.1	pH and free acidity.....	24
4.1.2	Determination of the ash content	25
4.1.3	Moisture contents of honey.....	25
4.1.4	Electrical Conductivity of honey	26
4.1.5	Sugar content of honey	27
4.2	Optimization of digestion of honey samples.....	28
4.2.1	Method validation through percent recovery study	28
4.2.2	Concentration of essential and nonessential metals.....	29
4.3	Investigation of Antioxidant Activities	30
4.4	Anti-bacterial activity of honey.....	31
UNIT FIVE		34
CONCLUSION AND RECOMANDATION.....		34
5.1	Conclusion.....	34
5.2	Recommendations	35
REFERANCES		36
APPENDIXES		50

LIST OF TABLES

Contents	Pages
Table 1: Physicochemical properties of honey	9
Table 2: Physicochemical properties of honey from Kondala district expressed in (X±SD)...	24
Table 3: Optimization Condition	28
Table 4: The intermediate standard solutions used to prepare working standards and correlation coefficients.....	29
Table 5: Concentrations of Essential and Non-essential Elements in Honey.....	29
Table 6: Antioxidant Activities of honey samples.....	30
Table 7:- Degree of the analysis of the diameter of inhibition zone (DIZ) of Honey and antibiotic used expressed in millimeters	33

LIST OF FIGURE

Contents	Pages
Figure 1. Map of the study area	15
Figure 2. Inhibition zone by disc diffusion	33

LIST OF APPENDEX

Contents	pages
Appendix1: pH, Free Acidity and conductivity of honey of Kondala district.....	50
Appendix 2: Ash content of honey	50
Appendix 3: Moisture Contents.....	51
Appendix 4: Total Sugar contents.....	51
Appendix 5: Optimization of Honey for Metal Analysis.....	51
Appendix 6: Calibration curve for Cadmium concentration in honey vs Absorbance	52
Appendix 7: Calibration curve for Chromium(Cr) concentration in honey vs Absorbance	52
Appendix 8: Calibration curve for Copper(Cu) concentration in honey vs Absorbance.....	52
Appendix 9: Calibration curve for Iron (Fe) concentration in honey vs Absorbance	53
Appendix 10: Calibration curve for Manganese (Mn) concentration in honey vs Absorbance	53
Appendix 11: Calibration curve for Nickel (Ni) concentration in honey vs Absorbance	53
Appendix 12: Calibration curve for Lead (Pb) concentration in honey vs Absorbance	54
Appendix 13: Calibration curve for Zink (Zn) concentration in honey vs Absorbance	54
Appendix 14. Calibration curves for Antioxidant properties of honey	54
Appendix 15: Antibacterial Activities of Honey	55

ABBREVIATIONS AND ACRONYMS

ANOVA	Analysis Of Variance
AR	Analytical Reagents
DIZ	Diameter of Inhibition Zone
DPPH	DiPhenylPicryl Hydrazine
EEC	Eurasian Economic commission
HPLC	High-Performance Liquid Chromatography
LC-MS	Liquid Chromatography Mass Spectroscopy
MBC	Minimal Bactericidal Concentration
MIC	Minimal Inhibitory Concentration
MRSA	<i>Methicillin Resistant Staphylococcus aureus</i>
PCA	Principal Component Analysis
RSA	Radical-Scavenging Activity
UV	Ultra Violet
VRE	<i>Vancomycin Resistant Enterococci</i>

Abstract

Honey is one of the important foods across the world due to its significant roles in maintaining human health. However, its composition and properties differ with plant species on which the bees forage and the climatic conditions of the product areas. This study was aimed to investigate the selected physicochemical, antioxidant properties and antibacterial activities of honey by using standard methods. Three samples of comb honey and one stingless bee honey harvested in Kondala district were studied in this project. Levels of essential and nonessential heavy metals were assessed by optimization of the sample digestion condition and using flame atomic absorption spectrophotometry (FAAS) technique. The antioxidant activity was determined by analyzing the radical-scavenging activity (RSA) using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) while antibacterial activity was evaluated against selected Gram-positive and Gram-negative bacteria using the agar disc diffusion method. The results show the lowest to highest values for pH, free acidity, ash content, moisture content and electrical conductivity 3.30 ± 0.03 to 3.60 ± 0.06 , 37.50 ± 0.50 mEq/kg to 79.50 ± 0.95 mEq/kg, $0.50 \pm 0.02\%$ to $1.00 \pm 0.02\%$, $13.95 \pm 0.05\%$ to $25.00 \pm 0.25\%$ and 0.75 ± 0.03 to 1.50 ± 0.09 , respectively. All honey samples are acidic in nature with no significance difference among the samples while free acidity showed significant difference owing to variations in moisture content of the honey and contamination with microbes or not ripened properly. The results of ash contents and conductivity indicate that there is strong positive correlation between them confirming electrical conductivity is often used as an indirect measure of mineral content. Essential metals such Cu (0.20 ± 0.009 to 1.38 ± 0.009), Fe (0.84 ± 0.049 to 10.3 ± 0.047), Mn (0.19 ± 0.016 to 0.38 ± 0.003) and Zn (0.12 ± 0.003 to 0.23 ± 0.002) as well as none-essential metals like Cd (1.06 ± 0.004) in $\mu\text{g/g}$ were detected but Cr, Ni and Pb were not detected being below the detection limit of the instrument. All honey samples containing significant amount of essential elements making it essential diet for human beings. The antioxidant activities of different honey types show variation, i.e. Damu Honey and Darker honey as in this study (e.g., Likiti honey) tend to have higher antioxidant activity, light honeys (Gararba Honey) shows lower activity, this could be the difference in the nectar type and its storage conditions. The present results revealed that the physicochemical parameters of most samples complied with international honey quality standards with regard to the nutritional value, bioactive potential, and safety of the studied honey samples.

Keywords: - Damu honey, Bee honey, Physicochemical characteristics, Anti-bacterial, Antioxidant activities, heavy metal, essential and non-essential elements

UNIT ONE

INTRODUCTION

1.1 Back Ground of the Study

Honey is a natural sweet substance produced by honeybees from the nectar of plants or from secretions of living plant parts. It is one of the oldest natural foods known to humankind and has long been valued not only as a nutritious sweetener but also for its medicinal and preservative properties. There are two main types of commercially available honey: ‘common’ honey, produced by *Apis mellifera*, and stingless or native bee honey, produced by various stingless bees. Stingless bees, as their name suggests, lack a functional sting and are characterised by their small, compact, dark coloured appearance (Mello dos santos et al., 2024). The composition and quality of honey are strongly influenced by the floral origin, geographical location, bee species, environmental conditions, and post-harvest handling practices. Consequently, its physicochemical characteristics, antioxidant potential, and antimicrobial activities can vary significantly between bee species and regions (Baltrušaitis et al., 2023).

In Ethiopia, honey production plays a vital role in rural livelihoods and traditional medicine. The country is among the leading honey producers in Africa due to its diverse flora and favorable climatic conditions (Bekele et al., 2022). Ethiopian honey is widely used not only as a food source but also as a traditional remedy for wounds, burns, coughs, and gastrointestinal ailments (Daba et al., 2021). In recent years, there has been increasing scientific interest in evaluating the physicochemical and biological properties of honey varieties produced in different Ethiopian regions to verify traditional claims and promote honey as a functional food and natural therapeutic product.

Among the honey-producing bee species, *Apis mellifera* (commonly referred to as *Apis*) and stingless bees (locally known as *Tazma*) are particularly important. Stingless bee honey differs from *Apis mellifera* honey in terms of sensory characteristics, higher moisture content, and enhanced medicinal properties, including antioxidant and antibacterial activities (Tadesse et al., 2020). The physicochemical properties of honey such as moisture content, pH, free acidity, electrical conductivity, and sugar composition are essential indicators of its quality, purity, and freshness (Gebru et al., 2020).

Antioxidant activity, largely associated with phenolic compounds, flavonoids, and enzymes, contributes to honey's ability to neutralize free radicals and protect against oxidative stress (Kek *et al.*, 2014). The antibacterial properties of honey are attributed to its acidity, hydrogen peroxide content, osmotic pressure, and the presence of bioactive phytochemicals, making it a potent natural antimicrobial agent against a wide range of pathogenic bacteria (Nordin *et al.*, 2020). Honey is a very complex natural product that contains sugars, organic acids, amino acids, proteins, minerals, vitamins, lipids, aromatic compounds, flavonoids, pigments, waxes, pollen grains, enzymes and other phytochemicals (Bicudo de Almeida-Muradian *et al.*, 2020).

Medicinal properties of honey have been documented as one of the oldest medical application (Kuropatnicki *et al.*, 2018). Honey has been found to heal surgical wounds, burns, minor cuts, sore throats and laryngitis (Kuropatnicki *et al.*, 2018). It is capable to sterilize the infected wound in human (Taha *et al.*, 2018). This is due to the moisture content, humidity and viscosity in honey that prevent the growth of bacteria on the infected wound (Nezhad-Mokhtari *et al.*, 2021). It is also known to display anti-inflammatory, antioxidant, anti-proliferative and anti-carcinogenic properties (Masad *et al.*, 2021).

The quality of honey being a key factor for both local and international markets is an aspect disregarded by producers and processors in developing countries including Ethiopia (Lewoyehu and Amare, 2019). The composition and quality of honey in general are greatly influenced by geographical and environmental factors and the types of flowers utilized by bees (Lewoyehu and Amare, 2019). To the best of my knowledge, although there are some sorts of reports on the chemical properties of honey produced in the different zones of Oromia Region, there is a limitation of a scientific research on the properties, quality, level of elements, antioxidant, and ant-bacterial activities of honeys produced in the West Wallaga Zone, Kondala district making difficult to judge on the possible reason for the unbalanced preference.

Therefore, this study aims to investigate selected physicochemical properties, level of elements, antioxidant capacity, and antibacterial activities of Apinae and Tazma honeys produced in Kondala District, Oromia, Ethiopia. The findings are expected to provide baseline scientific data on the quality and bioactivity of local honey varieties and contribute to their wider utilization as both food and natural medicine.

1.2 Statements of the Problem

Honey is widely syrupy liquid consumed for its nutritional value and medicinal properties. This importance depends on the chemical composition of the honey and its quality, physical characteristics, antioxidant activities and ant-bacterial activities. The evaluation of the chemical compounds that are decisive for the production of high quality, disease resistant cultivars and its wide spread uses. However, its quality and safety can vary significantly depending on floral source, environmental conditions, and handling practices. Contamination with non-essential or toxic elements poses potential health risks, while variations in antioxidant and antibacterial activities affect its functional value.

Limited comprehensive studies have simultaneously addressed physicochemical quality, elemental composition, and bioactive properties of honey from different regions, creating a gap in knowledge needed for consumer protection, quality control, and market competitiveness. Despite its importance, there is limited scientific information on the physicochemical properties, antioxidant capacity, and antibacterial activity of Apinae and Tazma honeys produced in Kondala District, Oromia, Ethiopia. In particular, comparative data under the same ecological conditions are lacking, making it difficult to validate traditional claims, ensure quality standards, and promote effective utilization of these honeys. Therefore, a systematic investigation of these properties is necessary to generate scientific evidence for quality assessment and value addition of locally produced honey.

1.3 Objectives of the Study

1.3.1 General Objective of the Study

The aim of the current study was to investigate selected physicochemical characteristics, level of heavy metal, antioxidant activities and anti-bacterial activities of honey samples collected from Kondala district, Oromia, Ethiopia.

1.3.2 Specific Objectives of the Study

The specific objectives of the current study were to:

- ✦ Determine selected physicochemical parameters such as pH, free acidity, moisture content, ash contents, electrical conductivity, and sugar content;
- ✦ Determine the levels of selected essential (Fe, Cu, Mn and Zn) and non-essential/toxic (Pb, Cd, Ni, Cr) elements;

- ✦ Determine the antioxidant capacity using the DPPH radical scavenging assay; and
- ✦ Determine antibacterial activity against selected gram-positive (*Staphylococcus aureus* (ATCC-25923)) and gram-negative (*Escherichia coli* (ATCC-25922), *Pseudomonas aeruginosa* (ATCC-27853) Enterobacter Colacae) bacterial strains using the agar disc diffusion method.

1.4 Significance of the Study

This study generally focuses on the physicochemical characteristics, antioxidant activities and anti-bacterial activities of comb honey (*Apis mellifera* honey) and *stingless bee* honey (Damu honey) in Kondala district. The findings will help validate traditional claims, support quality assessment, and promote the effective utilization and value addition of locally produced honey. Additionally, the results may benefit researchers, beekeepers, and policymakers by contributing to honey quality improvement and sustainable apiculture development.

1.5 Scope and Limitations of the Study

This study focused on the investigation of selected physicochemical properties, antioxidant capacity, and antibacterial activities of Apinae (*Apis mellifera*) honey and stingless bee (Tazma) honey produced in Kondala District, Oromia, Ethiopia. Honey samples were collected from the study area and analyzed using standard laboratory methods to compare the quality and bioactive properties of the two honey types under similar environmental conditions.

The study was limited to selected physicochemical parameters and did not include detailed analyses such as vitamin contents, phenolic profiling, or sensory evaluation. In addition, sample collection was restricted to a specific season and geographic area, which may limit the generalization of the findings to other regions or harvesting periods. Resource and time constraints also limited the number of samples and microbial strains tested for antibacterial activity.

UNIT TWO

LITERATURE REVIEW

2.1 Honey Producing Species

Stingless bee (*Meliponini*) and honey bee (*Apini*) are highly eusocial corbiculate bees and common honey producers from the same bee family of Apidae (Agostini *et al.*, 2021). Stingless bees belong to the subfamilies Meliponinae and keeping these bees is known as meliponi culture, while honey bees belonging to the subfamilies Apinae and keeping of these bees is called Apiculture (Hazra *et al.*, 2021). There are approximately 500 species within the stingless bee genus, with the majority of these species being located in Latin America, the mainland of Australia, Africa, and Eastern and Southern Asia. Stingless bees can be classified into 2 genera, namely, the *Melipona* and the *Trigona*. The *Melipona* genus is numerically large, even larger than that of the common honey bee (*Apis mellifera* Linnaeus) (AbdJalil *et al.*, 2017).

Stingless bees also play an important role in the ecology, economy, and culture. They act as the main pollinators for many wild and cultivated tropical plants (Gutiérrez-Arellano and Mulligan, 2020). 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 (Geeraert, 2019) and honey produced is known as pot honey and less yielder than *Apis mellifera* (Gutiérrez-Arellano and Mulligan, 2020).

Based on its entomological derivation, honey may be from the species *Apis mellifera*, which is the most broadly known and commercially accessible around the world, or from meliponines, which are commonly known as stingless bees without sting, or native bees (Temaru *et al.*, 2007). Both the honey types are produced all over the country. Several studies have been conducted to characterize Ethiopian *Apis mellifera* honey depending on its botanical and topographical origins (Belay *et al.*, 2021 and Tesfaye *et al.*, 2016), so that its quality parameters have been standardized both at national and international levels (QSAE, 2005; Alimentarius, 2001). According to Vit, *et al.* (2013) stingless bees construct their nests as spherical pots made of cerumen, that is a mixture of propolis and wax to store their honey

and the honey produced in such a way is called pot honey. The way stingless bee honey ('Damma Daamuu') harvested from feral colonies is absolutely traditional and destructive. The process is not only reducing quality of honey but also endangered the existing local species because of total nest destruction.

2.2 Global honey production trends

Honey is now one of the main ingredients for the thriving natural foods industry. Honey has a variety of flavors and qualities because of which, it is used by a number of end-use industries besides food and beverages, including pharmaceutical, chemicals and personal care among others. Food and beverages has been the dominant end-user of bee products, especially honey, for long and would continue to be so for many more years to come. The industry uses honey as sweetening agent and for flavoring. With adverse effects of sugar sweeteners on health becoming more obvious demand for honey is increasing. Growing liking among consumers for low calorie, healthy and natural sweeteners is anticipated to help demand growth for honey from this end-user industry remain steady.

The quantity of honey produced in Europe, North America and Oceania remained similar to those of 1961 throughout the period of interest. Honey production per capital increased by 42.9% at the global level, from 188 kg per 1000 population in 1961 to 269 kg per 1000 population in 2017 (Phiri, 2022). Furthermore, it was reported that the average the total honey production per year in the world is estimated to be 1.3 million tones (Bogdanov *et al.*, 2008). Of the total production developing countries all to gather produce about 47% of the world total honey production. The major producers are China, Turkey, Argentina, Ukraine, Russian Federation and others (Gebretsadik and Negash, 2016). Between 2001 and 2013, the total cumulative honey produced for some selected countries indicated that, China is the leading honey producing country followed by EU (Nega and Eshete, 2018).

2.3 Honey Production in Ethiopia

Honey, the natural product of honeybee is an excellent energy source because it contains simple sugars that are ready for assimilation immediately on reaching the intestine. Currently, Ethiopia is listed as a third country to export honey to European commission. To export to European Union a number of requirements must be met as the primary and the secondary requirements. The primary requirements listed were: viable offer to the market, listed in the EU inventory of third countries eligible to export honey to EU and clean honey. The

secondary requirements comprised of: Business relation with the buyer, a traceability system for quality control and Hazard analysis and critical control points concept (Sahle *et al.*, 2018).

Honey is harvested two or three times annually at 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 AdeyAbeba in Amharic or dararaa keelloo in Afaan Oromo), *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 (Kumsa *et al.*, 2020). 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 Mono-floral (Literally Damma Tuufoo in Afaan Oromoo) at the studies area.

In addition to this, other honey harvesting season is practiced in February from *Vernonia amaygdalina* L. and *Coffee arabica* L., in the study area. In some places where *Eucalyptus* spp, *Syzygium guinense* L. and *Croton macrostacyus* L. plants are available, there is a possibility of harvesting multi floral honey at the end of May and this might be realized due to the mixture of nectars that is collected from different plants. At the study areas, honey production system is mostly traditional.

2.4 Physical and Chemical Characteristics of Honey

Honey is a natural substance produced by bees and nutritious food of economic importance worldwide. Honey is a natural, sweet, syrupy fluid collected by bees from nectar of flowers. The pleasant aroma and taste of this viscous liquid ranging in color from pale yellow to dark amber varies according to geographical and seasonal conditions. Its use as a sweetener is well known in different parts of the world (Aparna and Rajalkshmi, 1999). It is a sweet viscous liquid that is composed of sugars, amino acids, proline, minerals, aromatic substances, pigment waxes and grains (Almeida-Muradian, *et al.*, 2020) and contains large amount of glucose but low in sucrose (<8%)(Fletcher, *et al.*, 2020).

Honey is easily digestible and a more palatable which supplies substantial energy with 75 to 85% fructose and glucose. The physicochemical composition, flavour and colour of honey vary due to climate, soil, flora, bee species and production methods. The precise composition variation depending on the plant species on which bee forages are the main constituents (Saikia and Sahoo, 2020). Storage conditions may also influence final composition, with the proportion of disaccharides increasing overtime (Kiarie and Mills, 2019). Careless handling

of honey can reduce quality like, high temperature, length of storage and moisture content which lead to fermentation, high levels of Hydroxy-methylfurfural (HMF), loss of enzymatic microbial growth (Mesele, 2021).

Generally speaking, honey contains sugars in high percentages, variable composition of amino acids (*e.g.* aspartic, glutamic, arginine *etc.*), very low proteins, minerals at various levels (*e.g.* Zn, Mg, K *etc.*), highly assimilable vitamins (*e.g.* Vitamin A, Vitamin B1, Vitamin B2, Vitamin B3, Vitamin B5, Vitamin B6, Vitamin B8, Vitamin B9, Vitamin C, Vitamin D and Vitamin K) and enzymes (*e.g.* amylase A and B) (Ball, 2007). Freshly extracted honey is a viscous liquid food, and its viscosity depends on the various honey constituents. Hence, the viscosity is greatly influenced by the composition of honey, mainly its water content (Ajibola, 2015).

In contact with the air, honey absorbs water, a phenomenon known as hygroscopicity. The water content absorbed depends on the relative humidity of the air. The hygroscopicity of honey is conditioned by the large amount of sugar. This process can increase the amount of water in the surface layer of the honey that can affect its quality during storage (fermentation) (Bartulović, 2015). Colour is the physical property perceived most immediately by the consumer. The determination of colour is a useful classification criterion for honeys (Bogdanov *et al.*, 2004). The colour of liquid honey varies from clear and colorless, yellow, amber to dark amber or black. In fact, natural honey becomes dark in colour when heated (Ajibola, 2015).

The electrical conductivity of honey is very small and depends on the mineral, organic acid and protein content of honey. Therefore, electric conductivity is a parameter which is often used in routine honey quality control, and can be considered a valid criterion for determining the botanical origin of a honey sample (Bogdanov *et al.*, 2004). The greater the content of organic and inorganic materials, the greater the value of electrical conductivity is the usual values range from 0.39-0.76 mS/cm. Chestnut honey and honeydew honey have high electrical conductivity, while acacia and meadow honey have lower conductivity (Bartulović, 2015).

Honey has a characteristic micro-flora that can be of primary and secondary origin. It can be said that honey is an environment that allows the growth and reproduction of microorganisms. If the sugar concentration is above 20%, microorganisms do not grow. Under certain conditions, when the water content in honey is above 20%, osmophilic yeasts

can grow and cause fermentation processes. Fermentation happens due to two reasons: hygroscopy and the presence of osmophilic and other types of yeasts that sustain high concentrations of sugar (Bartulović, 2015).

2.4.1 Analysis of 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 (Belay, 2021).

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 (Maione, *et al*, 2019). Properties like pH, free acidity (FA), ash content, EC, HMF, and MC for different types of honey from different countries are shown in table 1.

Table 1: Physicochemical properties of honey

Bee species	Physicochemical parameters						Country	Reference
	pH	FA (meq/kg)	Ash(g /100g)	EC(ms /cm)	HMF (mg/kg)	MC(g/ 100g)		
Melipona (stingless bee)	4.18	5.9	ND	0.66	5.4	27.0	Mexico	Santiesteban-Hernández <i>et al.</i> , 2003
	4.21	11.23	ND	0.238	5.50	13.8	Nigeria	Nweze <i>et al.</i> , 2017
Trigona (stingless)	2.87	36	1.8	3.27	4.3-39.1	25	Ethiopia	Andualem, 2014
	3.75	30.69	ND	0.303	16.58	17.50	Nigeria	Nweze <i>et al.</i> , 2017
Apis mellifera	3.8	6.25	0.2	0.488	ND	18.5	Ethiopia	Andualem, 2014
	3.75	32.43	0.21	0.69	36.35	18.80	Ethiopia	Tesfaye <i>et al.</i> , 2016
	4.24	18.67	ND	0.207	13.75	11.74	Nigeria	Nweze <i>et al.</i> , 2017

Where, 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.

Physicochemical parameters including moisture, reducing sugar, sucrose, water insoluble, ash, free acidity, hydroxyl methyl furfural contents, pH, diastase activity and electrical conductivity (Puścion-Jakubik, *et al*, 2020). The honey samples from Benin showed some parameters such as pH, water content, electrical conductivity, ash content, free acidity, total

sugars, and reducing sugars, respectively, ranging within 3.65–4.09; 12.07–13.16%; 530.25–698.50 $\mu\text{S}/\text{cm}$; 0.42–0.53%; 35.67–40.52 meq/kg; 60–70%; and 58–70% (Azonwade *et al.*, 2018).

In another work, the physico-chemical properties of honey produced from stingless bees (*Meliponula beccarii*) in west Shoa (Wolmera, Jeldu, Toke Kutaye and Chalyia districts) Oromia Region of Ethiopia was reported. The study revealed that the moisture content (MC = 29 ± 1.45), electrical conductivity (EC = 0.22 ± 0.03), pH (3.73 ± 0.13), ash (0.41 ± 1.1), total acidity (TA = 7.3 ± 0.36), hydroxyl methyl furfuraldehyde (HMF = 18 ± 3.45), insoluble materials (IM = 0.70 ± 0.3), and proline (214.5 ± 15) (Gela *et al.*, 2021).

2.5 Essential and Non-Essential Metals in Honey

Humankind uses honey as a popular condiment, natural sweetener and household remedy product (Hak *et al.*, 1998 and Ball & David, W. 2007). The exact composition of honey depends on the floral sources from which the nectar is harvested (White *et al.*, 1962). Trace elements are very important for life but can also potentially have harmful effects. Some metals (Fe, Zn, Cu, and Mn) play important roles in biological systems and are therefore referred to as essential elements, while others are non-essential elements (Pb, Cd, Ni and Cr). The non-essential elements can be toxic even in trace amounts (Teng *et al.*, 1995 and Diyrikli *et al.*, 2006). While, essential metals can also be harmful if taken in the greater amounts than the recommended values (Asraf W. 2005 and Fuh *et al.*, 2003).

Honey possesses a large range of properties and is considered a nutritious food, and therefore it must be free of all toxic elements. The mineral composition of honey differs depending on the plant sources and environments from which it originates. For example, light Blossom honeys have a lower content of trace elements than darker honeys such as honey dew, chestnut and heather (Demalsy *et al.*, 1989, Miret *et al.*, 2005 and Sevimli *et al.*, 1992). Analyses of trace elements in honey samples provide a more accurate indication for the presence of contaminants in environments. In Turkey, 21 honey samples have been collected and analyzed for Ni, Mn, Fe, Cu, Cd and Mg using Atomic Absorption Spectroscopy (AAS) Erbilir, and Erdogrul, (2005). Trace element analyses are extremely important in regards to the quality control of food and diet.

2.6 Antioxidant activities of Honey

Honey is a natural dietary antioxidant whose components are responsible for the redox properties are likely to be flavonoids, phenolic acids, enzymes, vitamins and minerals such as copper and iron (Chua *et al.*, 2013). Polyphenols are currently of particular interest to medical and food nutrition research, mainly because of their functional properties. Besides being radical scavenger, polyphenol could be an effective immune modulator and hormone action inhibitor (Havsteen, 2002). Polyphenols are believed to be potent scavengers of peroxy radicals, mainly because of the presence of high mobility of hydrogens in their molecular structures (Al-Mamary *et al.*, 2002). Of the polyphenols, phenolic acids are likely to be the major group in honey. The antioxidant properties of different honey samples were determined using total phenolic content, 1,1-diphenyl-2-picrylhydrazyl (DPPH), iron reduction power and β -carotene linoleic acid emulsion methods (Gül and Pehlivan, 2018).

2.7 Antimicrobial Properties of Honey

Several *in vitro* and *in vivo* studies and researches have been conducted on the properties of honey: antibacterial, antifungal, antiviral and antiprotozoal (Guttentag *et al.*, 2021). The antimicrobial potential of honey varies considerably; it mostly depends on the botanical origin of the flowers, geographic source, season, harvest, storage conditions (Anthimidou and Mossialos, 2013), age and the health of bee colonies (Mathialagan *et al.*, 2018). Honey composition and physicochemical properties differ according to the floral source used by the bees (Feknous *et al.*, 2021).

A number of studies have found a relationship between the floral origin of some honeys and their microbial activities (Haderbache *et al.*, 2020) because according to Molan (1992) the botanical origin plays a key role in the various activities of honey. The antibacterial activity might differ within the same floral source. This relates as per Basualdo *et al.* (2007) to soil composition, climate, honey processing and propolis concentration. Honeys of various geographical and floral origins can have different antibacterial properties, according to their chemical composition (Irish *et al.*, 2011; Moussa *et al.*, 2012).

2.7.1 Anti-bacterial-Activities of honey

The anti-bacterial activity of honey was first recognized in 1892, by Van Ketel (Albaridi, 2019). Two millennia before bacteria were identified as the cause of disease, physicians at

that time were aware that certain types of honey are the best therapy for particular ailment and infections (Elijah *et al.*, 2015). Honey was used to treat infected wounds as long ago as 2000 years before bacteria were discovered to be the cause of infection (Mirzaei *et al.*, 2018). When applied topically to wounds, osmosis would be expected to draw water from the wound into the honey helping to dry the infected tissue and reduce bacteria growth (Weissenstein *et al.*, 2018). Laboratory studies and clinical trials have shown that honey is an effective broad-spectrum antimicrobial agent. Honey has been reported to have inhibitory effect on several bacteria including aerobes and anaerobes, Gram-positive and Gram-negative and is effective against *methicillin resistant Staphylococcus aureus* (MRSA), β -hemolytic *Streptococci* and *vancomycin-resistant enterococci* (VRE) (Wasihun and Kasa, 2016).

Pure honey has been shown to be bactericidal to many pathogenic microorganisms including *Salmonella spp*, *Shigella spp* and other entero pathogenic like *Escherichia coli*, *Vibrocholerae* and other gram-negative and gram-positive organisms (Balogun and Tunde, 2020). The ability of honey to kill microorganisms has been attributed to its high osmotic effect, high acidic nature, hydrogen peroxide concentration and its chemical nature, which include its content of tetracycline derivatives, peroxides, amylose, fatty acids, phenols, ascorbic acid, terpenes, benzyl alcohol and benzoic acid (Elijah *et al.*, 2015). Most types of honey generate hydrogen peroxide when diluted because of the activation of the enzyme glucose oxidase, which oxidize glucose to gluconic acid and hydrogen peroxide (Albaridi, 2019).

Hydrogen peroxide is the major contributor to the antimicrobial activity of honey, and the different concentrations of this compound in different honeys result in their varying antimicrobial effects (Fernandes *et al.*, 2021). It has further been reported that physical property along with geographical distribution and floral sources may play an important role in the antimicrobial activity of honey (Villacrés-Granda *et al.*, 2021). The antibacterial activity screening and phytochemical analysis of essential plants has been of great interest in the discovery of drugs effective in the treatment of several diseases (Fentahun *et al.*, 2017).

In general, Previous studies taken place on Physicochemical Properties have demonstrated that moisture content below 20% is essential to prevent fermentation, while pH values typically range from 3.2 to 4.5 (Finola *et al.*, 2007). Electrical conductivity is an indicator of botanical origin, with higher values often linked to honeydew honeys (Persano Oddo & Piro, 2004). Honey is a source of essential minerals such as potassium, sodium, calcium, and

magnesium, which are derived from nectar and soil composition (Conti, 2000). However, contamination by non-essential metals like lead and cadmium may occur through environmental pollution or poor handling practices, necessitating regular monitoring (Silva *et al.*, 2009). The antioxidant potential of honey varies with floral origin, correlating strongly with total phenolic content (Al-Mamary *et al.*, 2002). DPPH radical scavenging assays have shown that darker honeys often exhibit higher antioxidant activity (Gheldof & Engeseth, 2002).

Honey's antibacterial efficacy has been documented against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and other pathogens (Molan, 1992; Irish *et al.*, 2011). Its action is attributed to synergistic effects of hydrogen peroxide, osmotic pressure, low pH, and plant-derived phytochemicals. Given these attributes, comprehensive evaluation of honey's physicochemical properties, essential and non-essential element content, antioxidant capacity, and antibacterial activity is important for assessing its nutritional value, safety, and therapeutic potential. The present study aims to investigate these parameters in honey samples from different floral and geographical origins, contributing to the understanding of their quality and bioactive properties.

UNIT THREE

MATERIALS AND METHODS

3.1 Description of the Study Area

The sample were collected in Kondala district West Wollega Zone of Western Oromia located at 633 km South West of Addis Ababa at 09° 23' 11" N Latitude and 34° 37' 15" E Longitude. Kondala district is part of the West Wollega zone which was established in December 2005 after being separated from the Begi district. It is bounded by Benishangul-Gumuz region and Mana-sibu in the north, Kellem Wollega zone in the south and south east, Begi in the west and Babo Gambel in north east. Geba dafino is the administrative center. The study district is naturally gifted with suitable climate and natural resources which provide favorable environment for beekeeping.

The area is covered with virgin forests like ‘Gaararbaa’, ‘Yabaloo Dhaatii’, ‘Caakkaa Liqixii,’ 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 between April and October. The study area is also characterized by two dry seasons or dearth periods from beekeeping point of view. The main drought period starts from the middle of February to end of March where flowering plants scarce and dry and the second starts droughts period from July to August due to long rainy, which is not conducive for honeybees to forage.

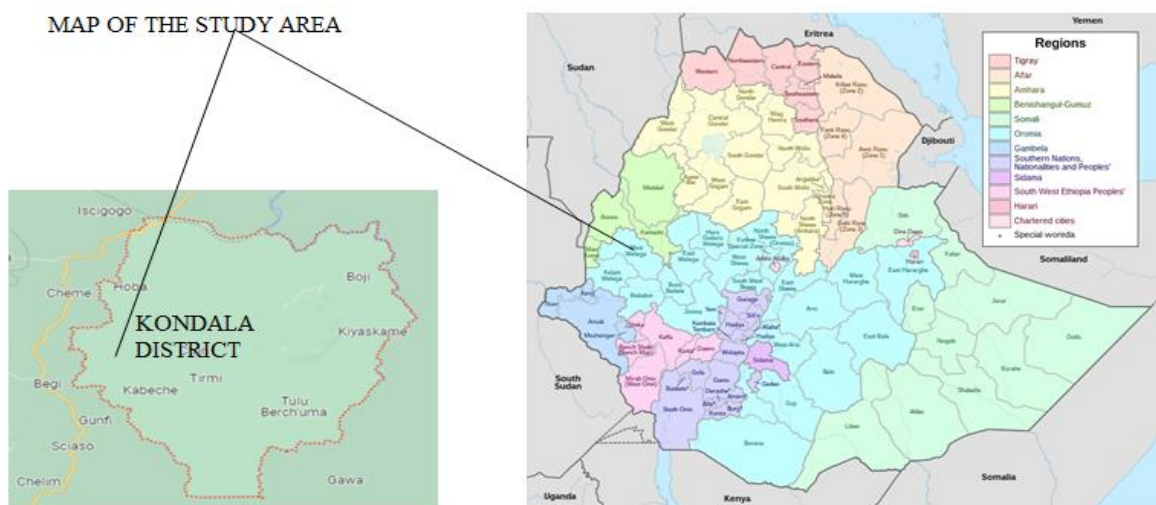


Figure 1. Map of the study area

3.2 Chemicals and Apparatus

All the chemicals and reagents used for this study were analytical grade. Chemicals that were used for the study include; Sodium Hydroxide Nitric Acid(69%) (Loba Chemia), Hydrogen peroxide(30%) (Addis Ababa), Olive oil, Manganese chloride, Zinc sulphate, Cupric Chloride, Nickel Chloride, Cadmium nitrate, lead nitrate, Iron(II) sulphate all were from Lobe Chemia, PVT. LTD, Chromic sulphate from Nice Chemicals Pvt. Ltd, distilled water, Bi-sulphate, Barium Chloride, Sulphuric Acid, HydroChloric Acid, HPLC-grade of methanol(MeOH), saline solution, chloramphenicol, Cipro, Muller Hinton powder, Nutrient agar, Mannitol salt agar, Glucose agar, peptone source of nitrogen, Sodium Chloride, bile salt, Sodium Carbonate, Lactose, peptic digest of animal tissue, and tap water.

The apparatus and instruments used for this study were, Air tight glass jar, Sensitive balance, Refrigerator, Glass centrifuging tube, Micro spatula, Electric muffle furnace, desiccators, conductivity meter, Thermostat controlling water, measured beaker, Micro pipette, UV spectrophotometer, pH meter, digestive furnace and digestive tube were used to digest honey samples, A 50 mL volumetric flask, Dropper and Micropipette was used for measuring volumes of standard solution, Plastic funnel and Filter paper and Flame atomic absorption spectroscopy FAAS (Savant AA 8350, Australia) were used for this study.

3.3 Honey Sampling Technique

Following the honey flow season (February – April, 2025), the samples of Apinae honeys were collected carefully from producers without contamination in sterile screwed-cup container and were kept in a cool and dry place in the laboratory before processing. The representative samples were collected in accordance by identifying the area where produce more honey from the district. Fresh honey samples were collected from samples plots to make representative sample using purposive sampling approach. Totally, three samples for each site around 1kg were collected and mixed thoroughly to get representative sample from the districts honey producers area of selected site and kept in a clean container.

Stingless bee honey was also taken as a representative from three area of the district honey producers point of view. To collect Tazma honey sample (stingless bee honey), the feral stingless bee colony nests were first located by the help of experienced local traditional hunters. Then, the underground nests were then carefully excavated without damaging the nest chambers containing both honey and pollen stores. Directly from the honey chamber,

honey samples were harvested from seal honey pots using disposable syringes (10 mL) and pooling in to air tighten glass jars for sampling area. Accordingly, about 1Kg of each honey representative samples pooling in to 2 groups were collected and stored in refrigerator (4 °C) until laboratory analysis.

3.4 Analysis of Physicochemical Properties

Chemical compositions (pH, free acidity, ash content, sugar contents) and physical compositions including Moisture content and electrical conductivity of the bees honey samples were determined according to the standards protocols for each respective parameter. Each physicochemical property was analyzed with three replications under each respective procedure (Ismail, *et al*, 2021).

3.4.1 pH and free acidity

The honey samples pH and free acidity were considered according to the procedure of Bogdanov (2009). For the measure of pH value, a solution of 10 g honey was dissolved in 75 mL of distilled water, then homogenized and subjected to reading in a pH meter (3100 Janeway, England) that was calibrate at pH 4.0 and 7.0. Furthermore, the honey solution was titrated with 0.1 M sodium hydroxide (NaOH) to the value of pH 8.30 at a steady reading after 2 min of the starting of titration. The readings were then recorded to the closest 0.2 mL employing a 10 mL burette to obtained precision value.

The free acidity of the honey was expressed in mill equivalents of acid per kg of honey sample, which is equivalent to ml of 0.1M NaOH x 10 (Eq. 1). Then, the value was expressed to the nearest one decimal point according to the standard procedure the Harmonized Methods of International Honey Commission (Bicudo de Almeida-Muradian *et al*, 2020).

$$Acidity = 10V \text{ --- (1)}$$

Where, V = the volume of 0.1M of NaOH in a 10g of honey sample.

3.4.2 Determination of the Ash Content

The contents of ashes in the selected honey samples were determined based on the standard procedure of (Gela, *et al*, 2021) with minor modification. First, the selected crucibles were heated in a furnace for about 25 min at a temperature of 300 °C. Then they were transferred to the desiccators for 20 min to cool down and weighed separately to 0.001g (M1). Then, 15 g of honey sample was accurately measured (M) and put into an ignited and pre-weighed

crucible. In each sample, two droplets of olive oil was added to prevent frothing, and then the honey samples were incinerated in an electric muffle furnace (Electric muffle furnace- Nabertherm Model No B-150, German) (550°C) for about 4 hours until complete incineration (white to light gray color) to reach the constant weight. The crucibles were then taken out and cooled in the desiccators and weighted for constant weight (M2). Finally, the ash content were calculated in percent in g/100g mass using the formula (Eq. 2), (Gela *et al*, 2021).

$$Ash\%_{by\ mass} = \frac{M2 - M1}{M} \times 100 \text{ ----- (2)}$$

Where, M1 = Weight of empty crucible, M2 = Weight of the ash and crucible, M = Mass of the sample taken for the test.

3.4.3 Moisture Content

Moisture content was determined using a standard method described by AOAC (2000). About 2 ml of honey samples in triplicates were put in pre- weighed dried crucibles, kept overnight in an oven at 110 °C and weighed. The loss in weight was taken as a measure of moisture content (Shahnawaz *et al.*, 2013) and calculated by the following formula (Eq. 3)

$$Moisture(\%) = \left[\frac{\text{weight of fresh sample} - \text{weight of dry sample}}{\text{weight of fresh sample}} \right] * 100 \text{ ----- (5)}$$

3.4.4 Electrical Conductivity

The electrical conductivity of honey samples were measured using a conductivity meter. 20 g of an anhydrous honey sample was diluted in 100 mL distilled water and from the dissolved solution; 40 mL was poured into a measured beaker and placed in a thermostat controlling water bath at 20°C. Then, the measurement of electrical conductivity was acquired by conductivity meter with 1 cell constant. After the conductivity cell was immersed in to the honey solution, the conductance read was recorded in mS at an equilibrium temperature. Finally, the value of electrical conductivity was calculated by the following equation Eq. (4) as described in International Honey Commission (Gela *et al*, 2021).

$$SH = K \times G \text{ ----- (6)}$$

Where: SH =electrical conductivity in mScm⁻¹, K = cell constant in cm⁻¹, G = conductance in mS

3.4.5 Sugar contents

Determination of sugar contents (total sugar, reducing sugar and non-reducing sugar) was carried out using the Layne- Eynon method as described in AOAC (2005). About 5 g of honey sample was taken into a beaker and 100 ml of warm water was added to it. The solution was stirred until all the soluble matters were dissolved and filtered through Whatman filter paper into a 250 volumetric flask. 100 ml of the solution was pipetted and prepared into a conical flask, after which 10 mL of diluted hydrogen chloride (HCl) was added and boiled for 5 min. On cooling, the solution was neutralized to phenolphthalein with 10% NaOH and kept in a 250 volumetric flask. This solution was used for titration against Fehling's solution and the reading was calculated as follows:

$$\text{Total sugar} = \frac{\text{dilution}(250) \times 2.5 \times S}{\text{Titre} \times \text{weight of sample} \times 10} \text{ --- (3)}$$

Apparent reducing sugars and apparent sucrose content of honey are most commonly determined for honey quality control purposes. The Lane and Eynon method (Codex Alimentarius Standard, 1969) is used for the determination of sugars. Apparent reducing sugars are defined as those sugars that reduce a Fehling's reagent under the conditions specified (Bogdanov et al., 1997; Bogdanov & Martin, 2002; Bogdanov, 2009; Granato & Nunes, 2016). The reducing sugar was calculated using the calculation given next.

$$\text{Reducing sugar} = \frac{250 \times 100 \times S}{H \times M} \text{ --- (4)}$$

where, S = Fehling's factor (as obtained from standardization procedure of CuSO₄ undertaken in step 1 of the procedure),

Strength of CuSO₄ solution /Fehling factor (S) = Titre value of standard invert sugar solution × 0.001 , H = Volume of honey solution required (burette reading), M = Mass of honey

Non-reducing sugar was estimated as the difference between the total sugar content and reducing sugar content on subtraction (Total sugar-Reducing sugar). The test was done in triplicate. The concentration of total sugars was deduced from the standard curve with sucrose as the reference sugar.

3.5 Digestion of honey samples

The essential and non-essential metals contents of honey samples collected from Kondala district were determined by Flame Atomic Absorption Spectrophotometer. The samples were

digested to obtain the essential and non-essential metals by optimum digestion condition. The digestion method for honey analysis was found to be efficient for all in the analyzed metals.

In this study, wet (acid) digestion method was used (Pisani *et al*, 2008) with some modifications. First, to minimize contamination glass wares were washed with 10% HNO₃ and then all materials were rinsed with distilled water and dried in an oven before use. One gram of representative of each honey sample was accurately weighed and has been taken in to beakers. Then 8 mL grade 69% HNO₃ and 4 mL 30% H₂O₂ is added to each sample. The solution is then stirred carefully and put in digestive furnace for heating by adjusting the temperature at 90°C for 60 minutes.

Organic parts of the sample are evaporated in the form of red gases. During this time the color of the gas gradually changed from red to yellow and then to white. After the color of the samples has changed near to dry and white, samples are then cooled to room temperature. The solution form of the samples was filtered with filter paper and transferred to a volumetric flask and diluted to 50 mL with distilled water. The digestion gave a clear colorless solution. In addition to sample digestion, digestion of blank was also prepared for correcting the effect of blank, keeping all digestion parameter the same.

3.5.1 Optimization of the working procedure

To prepare clear and colorless sample solution that is suitable for the analysis, different working procedures for the digestion of honey samples were assessed using mixture of HNO₃ and H₂O₂ by varying parameters such as volume of reagents, digestion time and digestion temperature. By examining the nature of the final digested honey samples, the optimized procedure was selected based on clearness of the digested sample, less digestion time, minimum temperature applied, less reagent consumption and simplicity. A series of procedures involving some changes in reagent volume, digestion time and temperature were tested. Total of nine trials were tested for digestion of the honey samples.

3.5.2 Determination of essential and non-essential metals in honey samples

Standard solutions which used for calibration were prepared from an intermediate standard solution 100ppm which was prepared from the standard stock solutions that contained 1000ppm for each of the metals. Secondary standards for instrument calibration were prepared by diluting with deionized water to obtain eight working standards for each metal

included in this work. Ni, Cu, Cr, Mn, Fe, Zn, Cd and Pb were analyzed using FAAS (Savant AA 8350) equipped by diluting with deuterium air background corrector and standard air-acetylene flame system using external calibration. The acetylene and air flow rates was managed to ensure suitable flame conditions. Five replicate determinations were carried out on each sample.

The concentration of all eight metals was determined by FAAS and the instrument readout were recorded for each solution manually. The same analytical procedure was employed for the determination of elements in digested blank solutions. The operating conditions FAAS was used for metal analysis in honey samples. The parameters of operating conditions are depends on metals, concentrations, lamp current, wave length slight width, slit height and flame type.

3.6 Investigation of antioxidant activities

The free radical scavenging activity of honey samples was determined by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay as described by Isla *et al.* (2011), with minor modification. The DPPH $\cdot$ solution (20 mg/L) was prepared by dissolving 2 mg of DPPH $\cdot$ in methanol (100 mL). A 0.75 mL of methanolic honey solution at different concentrations, ranging from 20 to 40mg/mL, was added to 1.5 mL of DPPH $\cdot$ solution. The natural antioxidant, ascorbic acid was used as a positive control (Ferreira *et al.*, 2009). The absorbance was measured at 517 nm after 15 min of incubation at 25 °C. The ability to scavenge the DPPH $\cdot$ was calculated using equation (5), The concentration of honey sample required to scavenge 50% of DPPH $\cdot$ (EC₅₀) was determined based on the ascorbic acid calibration curve (0–10mg/L) (Barbosa-Pereira *et al.*, 2013). The experiment was performed in triplicate;

$$\text{DPPH Scavenging actiVity(\%)} = (A_{\text{control}} - A_{\text{sample}}) * \frac{100}{A_{\text{control}}} \dots \dots \dots (7)$$

where A_{control} and A_{sample} are the absorbance's of control and sample, respectively.

3.7 Determination of Anti-bacterial Activity of Honey

Honey has many antimicrobial activities through our life. In this study its antibacterial activities was analyzed by Agar disc diffusion assay.

3.7.1 Preparation of Honey Samples for ant- bacterial test

The honey samples were 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 milliliter of pure honey was transferred into 1 mL of sterile distilled water during the preparation of honey samples for antibacterial test.

3.7.2 Test of Microorganisms used for the Current Study

For this work, the standard bacterial strains gram positive (*Staphylococcus aureus*(ATCC-25923)), gram negative(*Escherichia coli*(ATCC-25922), *Pseudomonas aeruginosa*(ATCC-27853) and Enterobacter Colacae) were obtained from Wollega University, Biology department.

3.7.3 Inoculums Preparation

Standard 0.5 McFarland will be prepared by mixing 0.5 mL of 0.048M BaCl₂ (1.175%W/V BaCl₂.2H₂O) with 99.5 mL of 0.18 M H₂SO₄ (1% V/V) and thoroughly mix to ensure that it is evenly suspended (Howe and Andrews, 2012). Then, the standard will be distributed in to a capped test tube of the same size and volume as those using to prepare the test inoculums. Inoculums will be prepared by picking 2-3 colonies from 24-hour old culture that grown on their selective media (*Staphylococcus aureus* on mannitol salt agar, *Pseudomonas aeruginosa* on *pseudomonas* agar and *Escherichia coli* on MacConkey agar) and suspended in 5 mL saline solution (0.85% saline). The suspend inoculums was vortexes for 15 seconds and their turbidity was adjusted by adding colony or saline solution into microbial stock solution and compared visually with standardizing 0.5 McFarland (turbidity adjust spectrophotometrically to 0.08-0.12 at OD₆₂₅ nm) against a white paper background and contrasting black lines.

3.7.4 Agar Disc Diffusion Assay

Agar disc diffusion test was carried out according to (Moussa, *et al*, 2012). The solidified Muller Hinton agar plate for bacteria was 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 prepared from what man no. 1 and sterilized to be placed on the seeded plates. A 30 µL of the prepared honey solution was pipetted on the surface of the disc. Ciprofloxacin (5 µg) (25µg were dissolved in 25 µL sterile distilled water for a single disc) was used as a positive control for bacterial strains, while 30 µL sterile distilled water was used as a negative control. All the assays were replicated three times.

3.7.5 Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) was determined by broth dilution method (Owuama, 2017). To determine the lowest concentration of anti-bacterial 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 bacterial was used. A two-fold serial dilution was prepared from the stock solution. A 18-24 h bacterial cultures was used and standardized by comparing its turbidity with McFarland 0.5 standard.

Three sterile capped test tubes were prepared and labeled for each sample. Then 3 mL (75%), 2 mL (50%), 1 mL (25%), was pipetted from stock honey solution (75%) with in a series of tubes. Then to each test tube, 1 mL of the standardized inoculums suspensions was pipetted while negative control tubes contained only broth and the positive control tubes contained one milliliter tested bacteria plus broth for comparison. The tube was vortexed and incubated for 24 h at 37°C. Finally, by comparing with control tube which showed clear tubes were used as the least honey concentration that inhibits bacterial growth.

3.7.6 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 will be recorded as MBC (Mama *et al*, 2019).

3.8 Method validation

The Method validation was performed according to the European Commission Decision 2002/657/EC concerning the performance of methods in the official control of residues in products of animal origin including honey. Parameters such as specificity, accuracy, precision and limits of detection/quantification were used in this process.

3.9 Data analysis

One-way ANOVA was employed to compute the variations between the means of each variable in every parameter of the analyzed honey samples. For all the computations, SPSS version-21 statistical software was employed and tests were made at 5% level of significance. Inhibition zone, antioxidant activity, level of essential and non-essential metals and physicochemical parameters of the tested honey samples were used for mean separation. Complete randomized experimental design was used. All the tests were performed in triplicates and standard deviation was calculated.

UNIT FOUR

RESULTS AND DISCUSSION

4.1 Physicochemical properties of honey samples

Selected physicochemical properties of honey samples were investigated and given in table 2. These include pH, free acidity, conductivity, moisture content and ash content.

Table 2: Physicochemical properties of honey from Kondala district expressed in ($\bar{X} \pm SD$)

Parameters	Types and locations of honey			
	Gararba honey	Yabalo honey	Likiti honey	Damu(Stingless bee honey)
pH	3.42 $\pm$ 0.03	3.53 $\pm$ 0.05	3.60 $\pm$ 0.06	3.30 $\pm$ 0.03
Free Acidity	39 $\pm$ 1.20	37.50 $\pm$ 0.50	40 $\pm$ 0.50	79.50 $\pm$ 0.95
Ash content (%)	0.50 $\pm$ 0.02	0.55 $\pm$ 0.03	0.71 $\pm$ 0.02	1.00 $\pm$ 0.02
Moisture content (%)	16.86 $\pm$ 0.12	13.95 $\pm$ 0.05	17.31 $\pm$ 0.12	25.00 $\pm$ 0.25
Conductivity	0.75 $\pm$ 0.03	0.80 $\pm$ 0.03	0.90 $\pm$ 0.04	1.50 $\pm$ 0.09

4.1.1 pH and free acidity

Honey is naturally acidic irrespective of its geographic origin, which may be due to the presence of organic acids that contribute its flavor and its stability against microbial spoilage (Khahl, 2012). The pH and free acidity of the honey samples were studied as shown in table 2. In this work, pH values of honey samples ranged from pH 3.30 $\pm$ 0.03 to pH 3.60 $\pm$ 0.06. Honey sample from stingless bees was found to be more acidic (pH 3.30 $\pm$ 0.03) than honeybees from three honey samples (pH 3.42 $\pm$ 0.03 to 3.60 $\pm$ 0.06). pH of honey samples in the current study ranged from 3.42 $\pm$ 0.03 to 3.60 $\pm$ 0.06 (Table 2). The pH values obtained are typical of honey samples reported in literature (Kayacier and Karaman 2008; Kinoo *et al.*, 2012; WONGSA *et al.*, 2023; Khahl *et al.*, 2012).

Codex Alimentarius commission (2001), sets a maximum free acidity of 50 meq/kg for most honeys (except baker's honey). Higher acidity is common in stingless bee honey, which may naturally exceed 50 meq/kg due to the fermentation and organic acids. The results obtained in the present study suggest that the free acidity of all honey is in average and the Likiti honey is higher from *Apis mellifera* honey. But higher in stingless bee honey than all *Apis mellifera* honey studied in the study area. This may be the cause of stingless bee honey contains higher moisture content(table 2) encourages yeast growth and producing acetic and lactic acids

which increase free acidity and *Apis mellifera* honey is drier and less prone to fermentation. For the case of Likiti honey it may be contaminated with microbes or not ripened properly.

4.1.2 Determination of the ash content

It was known that the ash content of honey depends on the material contained in the pollen collected by the bees during foraging on the flora, geographical area, botanical origin of the honey and soil type where the nectar-producing plants are located (Kumar *et al.*, 2018). The minimum, maximum and mean ash contents of the honey samples analyzed in the present study was in average with the maximum limits (0.6%) set for ash content of the honey by EU, CA and QSAE (Codex Alimentarius commission, 2001) and the average was also greater than the national and international limits for ash content of honey.

Typically, the ash content of honey ranges from 0.02% to 1.0%, with blossom honeys showing lower values compared to honeydew honeys, which tend to be richer in minerals (White, 1978). A higher ash content often correlates with darker color and stronger flavor characteristics (Lachman, Koliňová, Miholová, Kořata, Titěra, & Kult, 2007). For example, forest and buckwheat honeys usually contain higher ash values than acacia or citrus honeys.

It is possible to generalize that honey of the study area is in the average with national and international standards in its ash content. Deviations from this threshold may suggest the addition of mineral-rich substances or exposure to external contaminants during processing or storage. Based on this information, the difference in ash content might be due to the difference in flora type, geographical area, and soil type and physiology of each plant. Hence, the results of present study suggest that honey produced from native stingless bees honey and *Apis mellifera* honey in study area are richer in mineral contents, since the ash contents depends largely on the mineral content of the nectar source and surrounding soil.

4.1.3 Moisture contents of honey

The moisture content of honey is one of the criteria that determine its shelf life and ability to resist spoilage by microbial fermentation. Thus, the higher the moisture, the higher the probability that honey will ferment upon storage. The moisture content reported in this study for honeybees honey ranged between 13.95 ± 0.05 up to $17.31 \pm 0.12\%$ and $25.00 \pm 0.25\%$ for stingless bees honey (Table 2).

The recommended limits by international and national honey quality regulations are that moisture content should not exceed 20% (Codex 1982) or 21% (EU Council 2001 and Tanzanian Honey Quality Guidelines 2007). Of the 3 honeybees honey samples studied, 3 of them had moisture content levels below 20%. These results indicate that the study sites honey is thicker, more stable and standard for commercial honey.

On the other hand, stingless bees' honey sample reported in this study had in middle set moisture content limits. Its high moisture content than honey bees honey indicates stingless bee honey is more acidic and more conductive. This honey is thinner and contains tangier taste. The reason why the studied area stingless honey contains more moisture could be; the combination of open storage in permeable pots, contamination during the collection, humidity of the environment and nectar composition of the honey.

4.1.4 Electrical Conductivity of honey

Electrical conductivity (EC) measures a honey sample's ability to conduct an electric current, which reflects the concentration of ionizable minerals, organic acids and proteins present in it. It is expressed in millisiemens per centimeter (mS/cm) (Vit *et al.*, 2013). Several studies have demonstrated strong positive correlations between EC and ash content in honey ($r > 0.90$), confirming that both parameters can effectively distinguish between different honey types (Terrab *et al.*, 2002; Conti *et al.*, 2007). Electrical conductivity also serves as an indicator of adulteration, since diluted or sugar-syrup-adulterated honeys tend to exhibit significantly reduced conductivity values compared to genuine samples (Anklam, 1998).

In other word, according to Nordin *et al.* (2018), who conducted a comprehensive review of the physicochemical properties of stingless bee honey globally, the electrical conductivity of such honey varies widely, ranging from 0.102 mS/cm to 8.77 mS/cm, with an average of 1.16 ± 0.16 mS/cm. This broad range indicates substantial variability among stingless bee honey samples. The findings of the study highlight that the International Codex Alimentarius Commission guidelines for honey, which require an electrical conductivity of less than 0.8 mS/cm for most kinds of honey, except for those produced from honeydew, chestnut flowers, and mixtures of these might not be suitable for all stingless bee honeys.

In this work, the EC values of honey samples ranged from 0.75 ± 0.03 to 0.9 ± 0.04 for *Apis mellifera* honey (table 2). Honey sample from stingless bees was found to be containing more conductivity (1.5 ± 0.09 mS/cm) than honeybees from three honey samples. These values

obtained are in average with standard requirement included in codex Alimentarius and EU Directive 2001/110/EC. However, the conductivity of Likiti honey (0.90 ± 0.04 mS/cm) is above the average. According to (Escuredo, Dobre, Fernández-González, & Seijo, 2014), the higher EC of honeydew honey reflects its greater mineral and ash content, particularly of potassium, calcium, and magnesium ions. As such, EC is often preferred over direct ash measurement because it is rapid, non-destructive and highly correlated with mineral content (Pohl, 2009). The Values exceeding the limit may suggest adulteration, such as the addition of mineral-rich syrups, or contamination during processing or storage (Anklam, 1998) and these reason may be why the results of EC of Likiti honey is higher.

4.1.5 Sugar content of honey

The total reducing sugar in the honey samples varied for *Apis Mellifera* honey and Stingless bee honey from $76.1 \pm 0.03\%$ to $81 \pm 0.04\%$ and from $66.2 \pm 0.02\%$, respectively. The total reducing sugar of the honey samples were above 65%, the minimum limit set by Eurasian Economic commission (EEC) regulations for reducing sugars. The mean percentages of sucrose of the studied honey samples were all below 5% which the maximum limit proposed by FAO /WHO standards of honey. The findings of this study are further validated by Codex Alimentarius, given the fact that the range of non-reducing sugar in honey is 1.15 to 12%. Besides, these results are also in agreement with those of Kamal *et al.* (2002) who have reported a range of 1.115 to 12.135% in honey varieties.

The likiti honey sample recorded the highest value of 81g/100g followed by Yabalo honey (77.50 g/100g) and Gararba Honey (76.1 g/100g) which had significantly higher ($p < 0.05$) total reducing sugar content than other honey samples. The current result is above the value (Awraaris, *et al.* 2014) reported previously on the total reducing sugar value ranging from 64.78 to 69.27% in Ethiopian honey analyzed from Southwestern Ethiopia. In addition, similar findings were reported by (Tewodros, *et al.* 2013) for honey samples collected from Sekota district. Similarly, (Tewodros, *et al.* 2013) reported the sucrose level of honey ranged between 1.0 to 5.2% and (Awraaris, *et al.* 2014) that ranged between 1.68 to 6.37% in previous studies of Ethiopian honey.

According to the findings of (Bogdanov, *et al.* 2004) sucrose content in the honey sample is a significant criterion to determine the honey purity. This is because the sucrose present in natural or pure honey is little because of the activity of invertase enzyme present in honey.

These enzymes are responsible for the breakdown of sucrose. Thus, the content of sucrose in pure honey is low. In general, since Pure, good-quality honey typically shows $\geq 65\%$ sugars, all the honeys of the study area are preferable.

4.2 Optimization of digestion of honey samples

To optimize the digestion of honey samples nine different trials were tested. In this study the optimal digestion procedure chosen was the trial that required 1 hour for complete digestion of 1g of honey sample, temperature of 90°C and with 8mL 69% HNO_3 : 4mL of 30% H_2O_2 reagent ration (Table 3) was accepted.

Table 3 Optimization conditions for Sample digestion

Parameters	Trial	Reagents used	Volume ratio (mL) Total volume =12 mL	Temperature (°C)	Digestion Time (Hour)	Color
Temperature	1.	$\text{HNO}_3:\text{H}_2\text{O}_2$	1:1	50	0:30	Yellow
	2.	$\text{HNO}_3:\text{H}_2\text{O}_2$	1:1	70	0:30	Clear yellow
	3.	$\text{HNO}_3:\text{H}_2\text{O}_2$	1:1	90	0:30	Light yellow
Digestion time	4.	$\text{HNO}_3:\text{H}_2\text{O}_2$	1:1	90	0:30	Light yellow
	5.	$\text{HNO}_3:\text{H}_2\text{O}_2$	1:1	90	1:00	Colorless
	6.	$\text{HNO}_3:\text{H}_2\text{O}_2$	1:1	90	1:30	Clear
Reagent volume	7.	$\text{HNO}_3:\text{H}_2\text{O}_2$	1:1	90	1:00	Clear
	8.	$\text{HNO}_3:\text{H}_2\text{O}_2$	2:1	90	1:00	Clear colorless
	9.	$\text{HNO}_3:\text{H}_2\text{O}_2$	1:2	90	1:00	Yellow

Because of the drawback; like the higher chemical composition, longer duration of complete digestion and observation of less clear colors in some of the tested trials. The trial number 8 is but preferred for this experiment.

4.2.1 Method validation through percent recovery study

The instrument was calibrated using five series of working standards. The working standard solutions of each metal were prepared by diluting the intermediate standard solution. The concentrations of the un-spiked samples, spiked samples, the recovery percent, the amount of samples added and the value of the correlation coefficient of the Calibration for each metals was shown in Table 4.

Table 4: The intermediate standard solutions used to prepare working standards and correlation coefficients ($\bar{X} \pm SD$, for n = 4).

Metal	Concentration un-spiked samples ($\mu\text{g/mL}$)	The amount added ($\mu\text{g/mL}$)	Concentration in spiked samples ($\mu\text{g/mL}$)	Recovery (%)	R ²
Cd	0.941	1.000	1.798	85.70	0.992
Cr	ND	1.000	ND	-	0.993
Cu	0.63 $\pm$ 0.050	1.000	1.63 $\pm$ 0.050	102.00	0.997
Fe	3.26 $\pm$ 0.049	1.000	4.4 $\pm$ 0.050	114.00	0.999
Mn	0.52 $\pm$ 0.041	1.000	1.61 $\pm$ 0.049	109.00	0.997
Ni	ND	1.000	0.2 $\pm$ 0.048	-	0.995
Pb	ND	1.000	6.4 $\pm$ 0.045	-	0.994
Zn	-0.15 $\pm$ 0.022	1.000	0.63 $\pm$ 0.031	78.00	0.991

The result of method validation expressed in table 4 indicated that recovery and calibration of the instruments are acceptable. The result of precision can be expressed by standard deviation value, it was reliable and below $p \leq 0.05$, percentage of recovery becomes on the range (78-114) and correlation coefficient also above 0.99. The results of analysis of heavy metals are greater than limit of detection for essential elements and below the detection limit for non-essential elements. Therefore, the obtained results of heavy metals in honey by this method are accepted and reasonable.

4.2.2 Concentration of essential and nonessential metals

The results of this experiment showed that the concentration of the essential metals (Fe, Zn, Cu, and Mn) and non-essential metals (Ni, Pb, Cd, and Cr) in the digested honey samples were determined. The results indicate that, the *Apis mellifera* honey or commonly called comb honey and stingless bee honey (Damu) samples collected from Kondala districts had different concentration of metals. The average concentration of the analyzed heavy and trace metals in all honey samples with their corresponding standard deviation is summarized in Table 5.

Table 5: Concentrations of essential and non-essential elements in honey ($\bar{X} \pm SD$, for n = 5).

Metal type	Element	Damu Honey	Gararba Honey	Yabalo Honey	Likiti Honey
Essential	Cu	0.21 $\pm$ 0.004	0.20 $\pm$ 0.009	1.38 $\pm$ 0.009	0.17 $\pm$ 0.007
	Fe	10.3 $\pm$ 0.047	1.12 $\pm$ 0.044	1.22 $\pm$ 0.050	0.84 $\pm$ 0.049
	Mn	0.19 $\pm$ 0.016	0.25 $\pm$ 0.004	0.34 $\pm$ 0.007	0.38 $\pm$ 0.003
	Zn	0.15 $\pm$ 0.003	0.14 $\pm$ 0.008	0.23 $\pm$ 0.002	0.12 $\pm$ 0.003
Non-essential	Cd	1.06 $\pm$ 0.004	---	---	---
	Cr	----	---	---	---
	Ni	---	---	---	---
	Pb	---	---	---	---

Among the metals studied in this work, Iron has higher concentration. On the other hand, Cr, Pb and Ni were not detected which might be their existence below the instrumental detection limit. Heavy metals usually present in nature are not dangerous, because they are present in very small amounts. However if their levels are higher, then they can show harmful effects. The main sources of heavy metal ions are directly foods, water, and indirect industrial product. Elements like Copper, Iron, Zink, and Manganese are essential metals play an important role in biological process. But when their intakes exceed the recommended quantities they can have side effects.

In other case Pb, Cd, Ni and Cr are non-essential elements. Soil and water conditions, type of plants bee can forage, the use of fertilizers, all affects the level of trace metal components of honey. Take of food is an obvious means of supply to metals. Not only, are many metals natural components of foodstuffs, but also because of environmental contamination and contamination during processing (Zahrah, 2014). It is possible that the contaminations occur in minerals and elements of heavy metals by bad storage of honey or using vessels inappropriate. Lead and cadmium are known as toxic elements. Both metals cause adverse health effects in humans, and their widespread presence in the human environment comes from anthropogenic activities (Niloofer Khoshbakht Fahim, 2013).

However, the concentration of Cd was 1.06 ± 0.004 ($\mu\text{g/mL}$) in 'Damu' honey samples. The main source of this element can be anthropogenic like the combustion of some coals and oils, sludge-based fertilizers and phosphate fertilizers are important source of cadmium in agriculture and the other reason why Damu honey contains this element could be contamination during the harvesting of the honey. The present investigation of heavy metals in honey samples of Kondala district revealed that the area is less contaminated with heavy metals. The district has considerable area of natural forests and plantation crops. Hence, the honey samples collected in the selected areas has less heavy metal but is at the acceptable levels for human consumption.

4.3 Investigation of Antioxidant Activities

In the present study, the antioxidant activity of four samples of honey were evaluated using the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay. The radical scavenging activity (%RSA) increased with concentration, indicating moderate antioxidant potential. The antioxidant activity increases with honey concentration, reaching 26.34% RSA for comb

Honey (Likiti Honey) and 33.4% for Stingless bee Honey (Damu honey) at 40 mg/mL (Table 6).

Table 6 Antioxidant Activities of honey samples ($\bar{X} \pm SD$, n = 4).

Concentrations (mg/mL)	Absorbance (517nm)					Radical Scavenging activity %			
	Damu	Gararba	Yabalo	Likiti	Ascorbic Acid	Damu	Gararba	Yabalo	Likiti
25	0.90±0.002	0.92±0.010	0.91±0.012	0.88±0.010	0.95±0.001	5.400	3.360	4.412	7.560
30	0.85±0.002	0.90±0.005	0.88±0.020	0.85±0.015	0.97±0.003	12.50	7.31	9.17	12.46
35	0.80±0.010	0.87±0.005	0.84±0.004	0.74±0.010	0.98±0.002	17.94	10.77	14.36	24.10
40	0.66±0.010	0.84±0.004	0.82±0.015	0.73±0.015	0.99±0.002	33.40	15.14	17.86	26.34

These results suggest that the tested honey contains significant bioactive compounds like phenolics and flavonoids (Brand-Williams *et al.*, 1995). As literature shows, darker honeys (e.g., manuka, buckwheat) typically exhibit stronger antioxidant capacity than lighter ones like acacia. The tested honey is consistent with moderate–high activity likely due to phenolics/flavonoids. High %RSA indicates strong antioxidant activity. Different honey types shows variable antioxidant capacity, Damu Honey and Darker honey as in this study (e.g., Likiti honey) tend to have higher antioxidant activity, Light honeys (Gararba Honey) shows lower activity. In general, this result indicate that the Likiti honey shows strong bioactive properties than the others samples. This indicates the presence of phenolic/flavonoids compounds in Likiti honey more than the others.

4.4 Anti-bacterial activity of honey

Antibacterial activity correlates with total phenolic content and flavonoids levels, especially for Gram-negative strains. Gram-positive bacteria (e.g., *S. aureus*) are usually more sensitive to honey than Gram-negative bacteria (e.g., *E. coli*, *P. aeruginosa* and *Enterobacter Cloacae*). Floral source matters: darker honeys (honeydew, buckwheat, Gelam) show larger inhibition zones than lighter honeys (acacia, citrus). Manuka and other polyphenol-rich honeys produce zones comparable to or exceeding 20 mm at moderate concentrations (Kwakman & Zaat, 2012; Almasaudi *et al.*, 2017).

Honey inhibits bacterial growth through multiple factors:

- ✓ Osmotic effect: High sugar content (~80%) draws water from bacterial cells, dehydrating and killing them. Low pH: Typical pH (3.2–4.5) inhibits many pathogens.
- ✓ Hydrogen peroxide: Produced enzymatically when honey is diluted; acts as an antimicrobial.
- ✓ Non-peroxide factors: Includes methylglyoxal (notably in manuka honey), bee defensin-1, phenolic compounds, and flavonoids.
- ✓ Synergistic action: Combination of factors enhances overall efficacy. (Kwakman & Zaat, 2012 and Almasaudi *et al.* 2017).

Assay of honey against *S. aureus* *E. coli* Crude, unprocessed and undiluted honey samples from Honey bee honey three samples and one from stingless bee honey were taken for the study. Ciprofloxacin showed a zone of inhibition of 26 mm, 20mm, 18mm, and 16mm against *Staphylococcus aureus*, *E. Coli*, *P. aeruginosa* and *E. Cloaces* respectively. These result indicating moderate susceptibility against the three later mentioned. Overall, the results indicate that the same antibiotics have varying degrees of efficacy against *Staphylococcus aureus*, *E.Coli*, *P. aeruginosa* and *E. Cloaceas*.

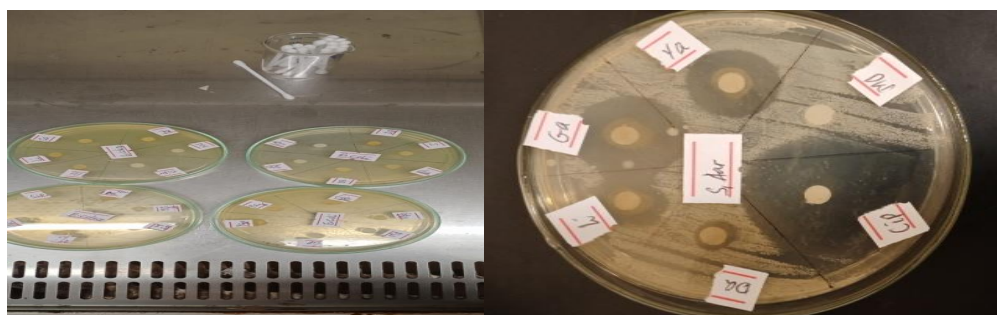


Figure 2: Inhibition zone by disc diffusion

Numerous studies carried out have shown that honey possess antibacterial against several bacterial pathogens, such as: *E. coli*, *S. Typhi*, *P. aeruginosa*, *K. pneumoniae*, *B. cereus*, and *S. Epidermidis* (Mandal & Mandal 2011; Feás *et al.*, 2013; Combarros-Fuertes *et al.*, 2019).

The present research work investigated the antibacterial activity of honey on gram-positive bacteria (*Staphylococcus aureus*) and the three mentioned above. The results obtained from our study demonstrate that honey exhibits a significant antibacterial effect against

Staphylococcus aureus bacteria, but on the other three types of bacteria no significant effect. The bacteria isolates were susceptible to organic honey at 100% concentration.

Table 7: Degree of the analysis of the diameter of inhibition zone (DIZ) of Honey and antibiotic used expressed in millimeters

No	Honey samples and analysed antibiotic	DIZ (mm) Against S.Aureus	DIZ (mm) Against E.coli	DIZ (mm) Against P. aeruginosa	DIZ (mm) Against E.colaces
1	DAMU	10	NSE	NSE	NSE
2	GARARBA	20	NSE	NSE	NSE
3	YABALO	18	NSE	NSE	NSE
4	LIKITI	15	NSE	NSE	NSE
5	Ciprofloxacin	26	20	18	16

NSE- No Significant Effect, **DIZ** diameter of inhibition zone

A recent study by Kim *et al.* (2021) investigated the antibacterial activity of Korean honey on *Enterococcus faecalis* reported that honey exhibited a significant inhibitory effect against *Enterococcus faecalis*. The researchers suggested that the antibacterial activity of honey may be attributed to the presence of various bioactive compounds, including polyphenols and flavonoids, which could have possess antibacterial properties.

The analysis of the diameter of inhibition zone (DIZ) values (Table 7) showed that the tested honey sample had potent antibacterial activity against *Staphylococcus aureus*, the diameters of inhibition for each of the isolate varied between 10 and 20 mm (mean inhibition value) (see also Appendix 6) this results are in agreement with the study of Bouacha *et al* (2020), which reported high antibacterial activity concentration of ranging from 25% - 5% of Honey against *Enterococcus faecalis* isolated from the urine of pregnant women (GSC Biological and Pharmaceutical Sciences, 2024). The results of our study clearly demonstrate the significant antibacterial activity of honey against the tested bacterial strains. This finding supports the potential use of honey as an alternative antibacterial for bacterial infections. However, further investigations are needed to deeper into the mechanisms of action and optimize the use of honey. Ultimately, the findings of this study provide valuable insights into the potential application of honey in the field of biochemistry research and offer promising avenues for future studies and clinical applications.

UNIT FIVE

CONCLUSION AND RECOMANDATION

5.1 Conclusion

This study investigated selected physicochemical properties, antioxidant capacity, and antibacterial activities of Apinae (*Apis mellifera*) honey and stingless bee (*Tazma*) honey produced in Kondala District, Oromia, Ethiopia. The results demonstrated that both honey types possess acceptable physicochemical quality parameters and exhibit notable antioxidant and antibacterial activities, although variations were observed between Apinae and Tazma honeys.

The observed differences in physicochemical characteristics and biological activities can be attributed to differences in bee species and floral sources within the same ecological setting. Overall, the findings provide scientific evidence supporting the nutritional and medicinal potential of both Apinae and stingless bee honeys. The study contributes valuable baseline data for future research, quality control, and value addition of locally produced honey, and supports the promotion of these honeys for food and therapeutic applications.

5.2 Recommendations

The following recommendations are considered based on the results obtained from this study:-

- There is a need for further studies to be done on various methods and systems to be used to reduce elemental contents like Cd found by this study in Damu honey (stingless bee honey).
- The current traditional way of honey harvesting from feral colonies is destructive for Stingless bee honey and needs to provide better method of harvesting.
- Further research is needed to determine the botanical origin, pollen analysis, phenol, flavonoids and vitamin contents for Kondala district's honeys from different seasons and floral sources.
- Relevant stakeholders, including agricultural offices, and research institutions, should support quality control and value addition of locally produced Apinae and stingless honeys to enhance their market potential.

REFERENCES

- AbdJalil, M. A., Kasmuri, A. R., Hadi, H. (2017). Stingless bee honey, the natural wound healer: A review. *Skin Pharmacology and Physiology*, 30(2), 66-75.
- Adane A. (2015). Studies on the levels of essential and non-essential metals in the raw and processed food (kolo and bread) of maize/corn (*zea mays* L.) cultivated in selected areas of Ethiopia degree of Master of Science in chemistry Addis Ababa University. 1-64
- Addis G. and Malede B. (2014). Chemical analysis of honey production challenges in and around Gonder, Ethiopia *Academic Journal of Nutrition*. 6–14
- Agostini, J. C., Costa, C. P., Ferreira, J. T., Dias, A. C., Guerra, N. P., Simões, Z. L. P., Lourenço, A. P. (2021). Vitellogenin of the solitary bees *Centristarsata* and *Centrisanalis* (Hymenoptera: Apidae): DNA structural analysis and gene expression. *Apidologie*, 52(1), 292-307.
- Ajibola, A. (2015). Physico-Chemical and Physiological Values of Honey and Its Importance as a Functional Food. *International Journal of Food Nutrition Science*, 2(2), 180-188.
- Albaridi, N. A. (2019). Antibacterial potency of honey. *International journal of microbiology*.
- Al-Mamary, M., Al-Meeri, A., Al-Habori, M. (2002). Antioxidant activities and total phenolics of different types of honey. *Nutrition Research*. 22(9), 1041–1047.
- Almasaudi *et al.* (2017). Antimicrobial potential of honey. *Saudi Journal of Biological Science*.
- Anklam, E. (1998). A review of the analytical methods to determine the geographical and botanical origin of honey. *Food Chemistry*, 63(4), 549–562. [https://doi.org/10.1016/S0308-8146\(98\)00057-0](https://doi.org/10.1016/S0308-8146(98)00057-0)
- Anthimidou E., Mossialos D. (2013). Antibacterial activity of Greek and Cypriot honeys against *Staphylococcus aureus* and *Pseudomonas aeruginosa* comparison to Manuka honey. *Journal of Medicinal Food*, 16: 42–47

- AOAC (2000). Sugars and sugar products. In official methods of analysis. Horwitz, W. (ed). *Association of official analytical chemist international*, 2 No.44. 16th Edition. Washington, DC 22-33
- AOAC (2005). Official Methods of Analysis of the Association of Analytical Chemists International, 18th edition, Gathersburg, *MD U.S.A Official methods*. 2200.
- AOAC Official Method (2005) 930.37 – Reducing and Total Sugars in Honey (Lane & Eynon method). *AOAC International*.
- AOAC Official Methods (for antioxidant assays in honey).
- Aparna, A.R., Rajalkshmi, D. (2009). Honey and its characteristics, sensory aspects, and applications. *Food Reviews International*. 15(4), 455-471.
- Ashenafi, M. (1994). The in vitro antibacterial activity of "tazma mar" honey produced by the stingless bee (*Apis mellipodae*). *The Ethiopian Journal of Health Development*, 8(2).
- Asraf, W. (2005). "Accumulation of heavy metals in kidney and heart tissues of *Epinephelus Microdon* fish from the Arabian Gulf" *Environmental Monitoring Assessment* 101, 311-316.
- Awraris Getachew Shenkutie, Hailemariam Gizaw, Dejen Aseffa. (2014). Physico-chemical Properties of Honey Produced in Masha, Gesha, and Sheko Districts in South-western Ethiopia. *Current Research in Agricultural Sciences* 1, 110 – 116.
- Azonwade, E.E., Paraiso, A., Dossa, C.P.A., Dougnon, V.T., N'tcha, C., Mousse, W., Baba-Moussa, L. (2018). Physicochemical Characteristics and Microbiological Quality of Honey Produced in Benin. *Journal of Food Quality*. 1896057.
- Ball, David, W. (2007). "The chemical composition of honey" *Chemical education* 84, 1643-1646.
- Balogun, O. D., Tunde, G. S. (2020). Antimicrobial activities of pure honey against some bacteria in wound infection. *International Journal of Innovation Research Advertising Studies (IJIRAS)*, 7, 146-149.

- Baltrušaitis, J., Kumari, A., & Singh, P. (2023). Composition, functional properties and safety of honey: A review. *Food Science and Technology*, 43(2), 453–468.
<https://doi.org/10.1590/fst.47122>
- Baltrušaitytė, V., Venskutonis, P. R., & Čeksterytė, V. (2007). Radical scavenging activity of different floral origin honey and beebread phenolic extracts. *Food Chemistry*, 101, 502–514.
- Barbosa-Pereira, L., Angulo, I., Paseiro-Losada, P. and Cruz, J. M. (2013). “Phenolic profile and antioxidant properties of a crude extract obtained from a brewery waste stream,” *Food Research International*, 51, 663–669.
- Bartulović, M. (2015). Proizvodnja plasma meda OPG-a Antunović .Diplomskirad, Poljoprivrednifakultet – *Osijek, Sveučilište JosipaJurja Strossmayera*.
- Basualdo. C., Sgroy, V.N., Finola, M.S., Marioli, J.M. (2007). Comparison of the antibacterial activity of honey from different provenance against bacteria usually isolated from skin wounds. *Veterinary Microbiology*, 124: 375–81.
- Bekele, T., Lemma, D., & Tufa, A. (2022). Contribution of apiculture to livelihoods in Ethiopia: A review. *Agricultural Reviews*, 43(4), 437–445. <https://doi.org/10.18805/ag.RF-340>
- Belay, A. (2021). Sheka forest biosphere reserve beekeeping practices and characteristics of *Schefflera abyssinica* honey, Ethiopia. *Environment, Development and Sustainability*, 1-19.
- Bicudo de Almeida-Muradian, L., Monika Barth, O., Dietemann, V., Eyer, M., Freitas, A. D. S. D., Martel, A. C., Gasparotto Sattler, J. A. (2020). Standard methods for *Apis mellifera* honey research. *Journal of Apicultural Research*, 59(3), 1-62.
- Bogdanov, S. (2003). Honey composition and properties. *Bee Product Science*. Retrieved from <http://www.bee-hexagon.net>
- Bogdanov, S. 2009. Physical properties of honey. In: Book of Honey, Chapter 4. *Bee Product Science*. www.beehexagon.net.
- Bogdanov, S., Jurendic, T., Sieber, R., Gallmann, P. (2008). Honey for Nutrition and Health: a review. *American Journal of the College of Nutrition*. 27, 677-689.

- Bogdanov, S., Ruoff, K. & Oddo, L. (2004). Physico-chemical methods for the characterization of unifl oral honeys: a review. *Apidologie*, 35, S4–S17.
- Bogdanov, S., Ruoff, K., & Oddo, L. P. (2004). Physico-chemical methods for the characterisation of unifloral honeys: A review. *Apidologie*, 35(Suppl. 1), S4–S17. <https://doi.org/10.1051/apido:2004047>
- Bouacha, M., Ayed, H., & Grara, N. (2020). Antibacterial properties of honey from different Algerian regions against staphylococcus aureus straina from wounds. *Microbiology Research Journal International*, 30(6), 14-23.
- Brand-Williams, W., Cuvelier, M. E., & Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity. *LWT - Food Science and Technology*, 28(1), 25–30.
- Chua, L.S., Rahaman, N.L.A., Adnan, N.A., Tan, T.T.E. (2013). Antioxidant Activity of Three Honey Samples in relation with Their Biochemical Components. *Journal of Analytical Methods in Chemistry*: 313798.
- Codex Alimentarius Commission. (2001). Revised Codex Standard for Honey (Codex Stan 12-1981, Rev.2-2001). Rome: FAO/WHO.
- Codex Alimentarius Commission. (2022). Food and Agriculture Organization.
- Commission Decision 2002/657/EC of (12 August 2002). Off. J. Eur. Commun. L221, 8.
- Conti, M. E. (2000). Lazio region (central Italy) honeys: A survey of mineral content and typical quality parameters. *Food Control*, 11(6), 459–463.
- Conti, M. E., Stripeikis, J., Campanella, L., Cucina, D., Tudino, M. B., & D’Arco, G. (2007). Characterization of Italian honeys (Marche Region) on the basis of their mineral content and some typical quality parameters. *Chemosphere*, 69(4), 528–534. <https://doi.org/10.1016/j.chemosphere.2007.05.078>
- Daba, A., Fekadu, E., & Gemed, B. (2021). Indigenous knowledge and practices of apitherapy in Oromia Region, Ethiopia. *Open Journal of Biological Sciences*, 7(3), 129–136. <https://www.biolscigroup.us/articles/OJBS-7-129.php>
- Demalsy, F., Vincet, B., Beaulieu, F. (1989) “Mineral content and geographical origin of Canadian honey” *Apidologie* 20, 77-91.

- Diyrikli, U., Mendil, D., Tuzen, M., Soylak, M, Elci, L. (2006). “Trace metal pollution from traffic in Denizli-Turkey during dry season” *Biomedical Environmental Sciences* 19, 254-261.
- EEC (1974). Council directive of 22 July 1974 on the harmonization of the laws of the member states relating to honey. Official Journal of European Communities, L221: 1014.
- Elijah, M. I., Imohiosen, O., Lamidi, B. T., Umar, M. S. (2015). Biochemical screening of pure honey and its antibacterial activity on some bacterial isolates compared with a common antibiotic. *International Journal of Pharmaceutical Science Innovation*, 4, 15-20.
- Erbilir, F., Erdogrul, O. (2005). “Determination of heavy metals in honey in Kahramanmaras city, Turkey” *Environmental Monitoring and Assessment* 109, 1-3.
- Escuredo, O., Dobre, I., Fernández-González, M., & Seijo, M. C. (2014). Contribution of electrical conductivity and pollen analysis to the differentiation of chestnut and eucalyptus honeys. *Food Chemistry*, 142, 243–249. <https://doi.org/10.1016/j.foodchem.2013.07.063>
- Feknous, N., Ouchene, L.L., Boumendjel, M., Mekhancha, D.E., Boudida, Y., Chettoum, A., Boumendjel, A., Messarah, M. (2021). Local honey goat milk yoghurt production. Process and quality control. *Food Science and Technology*, 42: 1–10.
- Fentahun, M., Ayele, Y., Amsalu, N., Alemayehu, A., Amsalu, G. (2017). Antibacterial evaluation and phytochemical analysis of selected medicinal plants against some pathogenic enteric bacteria in Gozamin District, Ethiopia. *Journal of Pharmacovigilance*, 5(5).
- Fernandes, L., Ribeiro, H., Oliveira, A., Silva, A. S., Freitas, A., Henriques, M., Rodrigues, M. E. (2021). Portuguese honeys as antimicrobial agents against *Candida* species. *Journal of traditional and complementary medicine*, 11(2), 130-136.
- Ferreira, I. C., Aires, E., Barreira, J. C. M., Estevinho, L. M. (2009). “Antioxidant activity of Portuguese honey samples: different contributions of the entire honey and phenolic extract,” *Food Chemistry*, 114, (4), 1438–1443.

- Finola, M. S., Lasagno, M. C., & Marioli, J. M. (2007). Microbiological and chemical characterization of honeys from central Argentina. *Food Chemistry*, 100(4), 1649–1653.
- Fletcher, M. T., Hungerford, N. L., Webber, D., de Jesus, M. C., Zhang, J., Stone, I. S., Zawawi, N. (2020). Stingless bee honey, a novel source of trehalulose: a biologically active disaccharide with health benefits. *Scientific reports*, 10(1), 1-8.
- Fuh, C.B., Lin, H.I, Tsai, H.Y. (2003). “Determination of lead, cadmium, chromium and arsenic in 13 herbs of tocolysis formulation using atomic absorption spectrometry” *Food eheheh and Drug Analysis* 11, 39-45.
- Gebretsadik, T., Negash, D. (2016). Honeybee Production System, Challenges And Opportunities In Selected Districts Of Gedeo Zone, Southern Nation, Nationalities And Peoples Regional State, Ethiopia. *International Journal of Research-Granthaalayah*. 4(4), 49-63.
- Gebru, E., Asres, D., & Tola, Y. (2020). Physicochemical characteristics of honey obtained from traditional and modern hive production systems in Tigray Region, Northern Ethiopia. *Momona Ethiopian Journal of Science*, 12(1), 1–12.
<https://journal.mu.edu.et/index.php/mejs/article/view/134>
- Geeraert, L. (2019). Intensification of Arabica coffee agro forestry systems in southwestern Ethiopia: Effects on coffee pollinator communities and coffee quality.
- Gela, A., Hora, Z. A., Kebebe, D., Gebresilassie, A. (2021). Physico-chemical characteristics of honey produced by stingless bees (*Meliponula beccarii*) from West Showa zone of Oromia Region, Ethiopia. *Heliyon*, 7(1), 05875.
- Gheldof, N., & Engeseth, N. J. (2002). Antioxidant capacity of honeys from various floral sources. *Journal of Agricultural and Food Chemistry*, 50(11), 3050–3055.
- GSC Biological and Pharmaceutical Sciences, (2024), 26(03), 113–122, 119
- Gül, A., Pehlivan, T. (2018). Antioxidant activities of some mono floral honey types produced across Turkey. *Saudi Journal of Biological Sciences*. 25(6), 1056-1065.

- Gutiérrez-Arellano, C., Mulligan, M. (2020). Small-sized protected areas contribute more per unit area to tropical crop pollination than large protected areas. *Ecosystem Services*, 44, 101137.
- Guttentag, A., Krishnakumar, K., Cokcetin, N., Hainsworth, S., Harry, E., Carter, D. (2021). Inhibition of dermatophyte *fungi* by Australian jarrah honey. *Pathogens*, 10: 194.
- Haderbache, L., Annou, S., Mohammedi, A. (2020). Antimicrobial potential of Ziziphus and Euphorbia honeys harvested in semi-arid region of Algeria and their possible use in soft medicine. *Journal of Microbiology, Biotechnology and Food Sciences*, 9: 1114–1118.
- Hak, C.G., Kyoo, H.M., Gil, K.J. (1998). “The chemical composition of Korean honey” *Korean Food Science and Technology* 20, 631-636.
- Havsteen, B.H. (2002). The biochemistry and medical significance of the flavonoids. *Pharmacology and Therapeutics*. 96(2-3), 67–202.
- Hazra, T., More, S., Murmu, M., Mahato, S., Ghorai, N., Bera, S., Khan, M. A. (2021). Palynological Characterization of Stingless Bee *Tetragona gonulairidipennis* Smith (Hymenoptera: Apidae: Meliponini) Honey from Chotanagpur Plateau, Eastern India. *Bee World*, 1-5.
- Howe, R. A., Andrews, J. M. (2012). BSAC standardized disc susceptibility testing method (version 11). *Journal of antimicrobial chemotherapy*, 67(12), 2783-2784.
- Ibrahim M., Vildana A., Stela J., Emdzad G ., Dilajla J., and Damirseulja M. (2011). Determination of Pesticides, Heavy Metals, Radioactive Substances, and Antibiotic in Honey. *Polish Journal of Environmental Study*, 719–72
- Irish, J., Blair, S., Carter, D.A. (2011). The antibacterial activity of honey derived from Australian flora. *PLoS One*, 6: 18229.
- Isla, M. I., Craig, A., Ordoñez *et al.* (2011). “Physico chemical and bioactive properties of honeys from Northwestern Argentina,” *Lebensmittel-Wissenschaft & Technologie*, 44(9), 1922–1930.

- Ismail, N. I., Kadir, M. R. A., Zulkifli, R. M., Mohamed, M. (2021). Comparison of physicochemical, total protein and antioxidant profiles between Malaysian Apis and Trigona honeys. *Malaysian Journal of Analytical Sciences*, 25(2), 243-256.
- Kamal, A., Raza, S., Rashid, N., Hameed, T., Gilani, M., Qureshi, M. A. and Nasim, K. (2002). Comparative study of honey collected from different flora of Pakistan. *Journal of Biological Sciences*, 2(9): 626–627.
- Kayacier, A and Karaman S. (2008). Rheological and some physicochemical characteristics of selected Turkish honeys. *J. Texture Stu.*39: 17-29. 21.
- Kek, S. P., Chin, N. L., & Yusof, Y. A. (2014). Antioxidant capacity and total phenolic content of honey from various floral sources in Malaysia. *Food Chemistry*, 152, 557–563. <https://doi.org/10.1016/j.foodchem.2013.11.031>
- Khahl I, Moniruzzaman, M., Boukraa L., Benhanifia, M. Islam A, Islam N, Sulaiman S.A, and Gan S.H. (2012). Physiochemico and antioxidant properties of Algerian Honey. *Molecules*, 17:11199-11215.
- Khalil, M.I., Moniruzzaman, M., Boukraa, L. (2012). “Physicochemical and Antioxidant Properties of Different Floral Honeys.” *Food and Chemical Toxicology*, 50(10): 3224-3233.
- Kiarie, E. G., Mills, A. (2019). Role of feed processing on gut health and function in pigs and poultry: conundrum of optimal particle size and hydrothermal regimens. *Frontiers in Veterinary Science*, 6, 19.
- Kinoo, M.S, Mahomoodally, M.F and Puchooa, D. (2012). Anti-Microbial and physicochemical properties of processed and raw honeys of Mauritius. *Advances in infectious Diseases*,2: 25-36
- Kumsa, T., Bareke, T., Addi, A. (2020). Migratory Beekeeping as Strategy to Harvest Multiseason Honey in Ethiopia. *Bee World*, 97(4), 105-108.
- Kuropatnicki, A. K., Kłósek, M., Kucharzewski, M. (2018). Honey as medicine: historical perspectives. *Journal of Apicultural Research*, 57(1), 113-118.
- Kwakman & Zaat (2012). Antibacterial components of honey. *FEMS Microbiology Rev.*

- Lachman, J., Kolihová, D., Miholová, D., Košata, J., Titěra, D., & Kult, K. (2007). Analysis of minority honey components: Possible use for the evaluation of honey quality. *Food Chemistry*, 101(3), 973–979. <https://doi.org/10.1016/j.foodchem.2006.02.049>
- Lane, J.H., & Eynon, L. (1923). Determination of reducing sugars by titration with Fehling's solution using methylene blue indicator.
- Legesse, T., Wubie, A., & Birhanu, M. (2021). Physicochemical properties and antioxidant activity of stingless bee honey from selected districts of Ethiopia. *Ethiopian Journal of Applied Science and Technology*, 12(2), 23–35.
- Lewoyehu, M., Amare, M. (2019). Comparative assessment on selected physicochemical parameters and antioxidant and antimicrobial activities of honey samples from selected districts of the Amhara and Tigray regions, Ethiopia. *International journal of food science*.
- Maione, C., Barbosa Jr, F., Barbosa, R. M. (2019). Predicting the botanical and geographical origin of honey with multivariate data analysis and machine learning techniques: a review. *Computers and Electronics in Agriculture*, 157, 436-446.
- Maiyo W., Kituyi J . L ., Mitei Y., and Kagwanja S. (2014) . Heavy Metal Contamination in Raw Honey, Soil and Flower Samples. *International Journal of Emerging Science and Engineering*.6-9.
- Mama, M., Teshome, T., Detamo, J. (2019). Antibacterial activity of honey against methicillin-resistant staphylococcus aureus: a laboratory-based experimental study. *International journal of microbiology*.
- Mandal, M.D., & Mandal, S. (2011). Honey: Its medicinal property and anti bacterial activity. *Asian pacific Journal of Tropical biomedicine*, 1(2), 154-160.
- Marcovecchio JE, Botte SE, Freije RH. (2007). Heavy metals, major metals, trace elements. *In: Nollet LML, editor. Handbook of water analysis. 2nd ed. Boca Raton: CRC Press.* 275–311
- Martinello, M., Mutinelli, F. (2021). Antioxidant activity in bee products: A review. *Antioxidants*, 10(1), 71.

- Masad, R. J., Haneefa, S. M., Mohamed, Y. A., Al-Sbiei, A., Bashir, G., Fernandez-Cabezudo, M. J., Al-Ramadi, B. K. (2021). The Immunomodulatory Effects of Honey and Associated Flavonoids in Cancer. *Nutrients*, 13(4), 1269.
- Mathialagan M., Johnson Thangaraj Edward Y.S., David P.M.M., Senthilkumar M., Srinivasan M.R., Mohankumar S. (2018). Isolation, characterization and identification of probiotic lactic acid bacteria (LAB) from honey bees. *International Journal of Current Microbiology and Applied Sciences*, 7: 894–906.
- Mello dos Santos, M., Khan, N., Lim, L.Y., Locher, C. (2024). Antioxidant Activity, Physicochemical and Sensory Properties of Stingless Bee Honey from Australia. *Foods*, 13, 1657
- Mesele, T. L. (2021). Review on physico-chemical properties of honey in Eastern Africa. *Journal of Apicultural Research*, 60(1), 33-45.
- Miret, G., Terrab, M.L., Hernanz, A., Recamales, D.F., Heredia, M.A. (2005). “Multivariate correlation between color and mineral composition of honeys and by their botanical origin” *Agriculture and Food Chemistry* 53, 2574-2580.
- Mirzaei, B., Etemadian, S., Goli, H. R., Bahonar, S., Gholami, S. A., Karami, P., Tavakoli, R. (2018). Construction and analysis of alginate-based honey hydrogel as an ointment to heal of rat burn wound related infections. *International journal of burns and trauma*, 8(4), 88.
- Mohammed, S. O. N. (2018). In-Vitro Antibacterial Activity of crude Garlic (*Allium Sativum* Extract Against Clinical Isolate of Methicillin Resistant *Staphylococcus aureus* from Elribat university hospital, Sudan (*Doctoral dissertation, Nadir Musa Khalil Abuzeid*).
- Molan P.C. (1992), The antibacterial activity of honey: 1. The nature of the antibacterial activity. *Bee World*, 73: 5–28.
- Moussa, A., Noureddine, D., Saad, A., Abdelmelek, M., Abdelkader, B. (2012). Antifungal activity of four honeys of different types from Algeria against pathogenic yeast: *Candida albicans* and *Rhodotorula*. *Special Asian Pacific journal of tropical biomedicine*, 2(7), 554-557.

- Nega, T., Eshete, Y. (2018). Review of Ethiopia's Global Position in Honey and Other Bee Products Production and Marketing: Analysis of Sectoral Opportunities and Limitations. *Biomedicine Journal of Science & Technology Research*. 10(3), 7879-7883.
- Nezhad-Mokhtari, P., Javanbakht, S., Asadi, N., Ghorbani, M., Milani, M., Hanifehpour, Y., Akbarzadeh, A. (2021). Recent advances in honey-based hydrogels for wound healing applications: Towards natural therapeutics. *Journal of Drug Delivery Science and Technology*, 102789.
- Nordin, A., Sainik, N. Q. A. V., Chowdhury, S. R., & Saim, A. B. (2020). Stingless bee honey: Evaluating its antibacterial activity and bacterial diversity. *Insects*, 11(8), 500. <https://doi.org/10.3390/insects11080500>
- Nordin, A., Sainik, N.Q.A.V., Chowdhury, S.R., Saim, A.B., Idrus, R.B.H. (2018). Physicochemical properties of stingless bee honey from around the globe: A comprehensive review. *J. Food Compos. Anal.* 73, 91–102.
- Official methods of analysis of the Association of *Official Analytical Chemists 13th Ed.*, (1980): 289, 508. See 18.025, 31.012.
- Owuama, C. I. (2017). Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) using a novel dilution tube method. *African journal of microbiology research*, 11(23), 977-980.
- Persano Oddo, L., & Piro, R. (2004). Main European unifloral honeys: Descriptive sheets. *Apidologie*, 35, S38–S81.
- Phiri, B.J. (2022). Upward global trends in managed honey bee colonies and production: a six-decade viewpoint, 1961-2017. Research Square.
- Pisani, A., Protano, G., & Riccobono, F. (2008). Minor and trace elements in different honey types produced in siena Country (Italy). *Food Chemistry*, 107(4), 1553-1560.
- Pohl, P. (2009). Determination of metal content in honey by atomic absorption and emission spectrometries. *TrAC Trends in Analytical Chemistry*, 28(1), 117–128. <https://doi.org/10.1016/j.trac.2008.09.015>
- Puścion-Jakubik, A., Borawska, M. H., Socha, K. (2020). Modern Methods for Assessing the Quality of Bee Honey and Botanical Origin Identification. *Foods*, 9(8), 1028.

- Sahle, H., Enbiyale, G., Negash, A., Neges, T. (2018). Assessment of honey production system, constraints and opportunities in Ethiopia. *Pharmaceutical Pharmacology International Journal*, 6(1), 42-47.
- Saikia, D., Das, R., Sahoo, S. (2020). An Overview of the Digital India Programme with Special Reference to Agriculture. *Agriculture & Food E-news letter*.
- Sevlimli, H., Bayulgen, H., Varinioglu, N. (1992). "Determination of trace elements in honey by INAA in Turkey" *Radio analytical Nuclear Chemistry Letter* 165, 319-325.
- Shahnawaz, M., Sheikh, S.A, Hussain, M., Razaq, A, and Khan, S.S (2013). A study of the determination of the physiochemical properties of honey from different valleys of Gilgit – Baltistan. *International Journal of Agriculture Science Research*, 2(2): 049-05-3.
- Sharma, O. P., Bhat, T.K. (2009). "DPPH antioxidant assay revisited," *Food Chemistry*, 113(4), 1202–1205.
- Silva, L. R., et al. (2009). Honey from northeast Portugal: Physicochemical characteristics and mineral contents. *Microchemical Journal*, 93(1), 73–77.
- Tadesse, M., Hussen, A., & Negash, D. (2020). Physicochemical characteristics of honey produced by stingless bees (*Meliponula beccarii*) from West Shoa Zone, Oromia Region, Ethiopia. *BMC Chemistry*, 14(8), 1–9. <https://doi.org/10.1186/s13065-020-00688-z>
- Taha, M., Abdelbary, H., Ross, F. P., Carli, A. V. (2018). New innovations in the treatment of PJI and biofilms-clinical and preclinical topics. *Current reviews in musculoskeletal medicine*, 11(3), 380-388.
- Temaru, E., Shimura, S., Amano, K., Karasawa, T. (2007). Antibacterial activity of honey from stingless honeybees (Hymenoptera; Apidae; Meliponinae). *Polymer Journal of Microbiology*. 56(4), 281.
- Teng, J.H., Wang, T.L., Lin, W.C., Chin, M.T., Chen, Z.S. (1995). "Investigation on four heavy metal constituents of commercial restorative Chinese medicines" *Food and Drug Analysis* 3, 193-202.

- Terrab, A., Díez, M. J., & Heredia, F. J. (2002). Characterisation of Moroccan unifloral honeys by their physicochemical characteristics. *Food Chemistry*, 79(3), 373–379. [https://doi.org/10.1016/S0308-8146\(02\)00189-9](https://doi.org/10.1016/S0308-8146(02)00189-9)
- Terrab, A., *et al.* (2003). Mineral content and electrical conductivity of the honeys produced in northwest Morocco and their contribution to the characterization of unifloral honeys. *Journal of the Science of Food and Agriculture*, 83(7), 637–643.
- Terrab, A., Recamales, Á. F., Hernanz, D., & Heredia, F. J. (2004). Contribution to the study of avocado honeys by their mineral contents using inductively coupled plasma optical emission spectrometry. *Food Chemistry*, 92(2), 305–309. <https://doi.org/10.1016/j.foodchem.2004.07.029>
- Tesfaye, B., Begna, D., Eshetu, M. (2016). Evaluation of physico-chemical properties of honey produced in Bale natural forest, Southeastern Ethiopia. *International Journal of Agricultural Science Food Technology*. 2 (1), 21–27.
- Tewodros Alemu, Eyassu, S., Amsalu Bezabih, (2013). Physicochemical Properties of Honey Produced in Sekota District, Northern Ethiopia. *International Food Research Journal* 20, 3061–3067.
- Villacrés-Granda, I., Coello, D., Proaño, A., Ballesteros, I., Roubik, D. W., Jijón, G., Alvarez-Suarez, J. M. (2021). Honey quality parameters, chemical composition and antimicrobial activity in twelve Ecuadorian stingless bees (Apidae: Apinae: Meliponini) tested against multi resistant human pathogens. *LWT*, 140, 110737.
- Vit, P., Pedro, S.R., Roubik, D.(2013). Pot-honey: a Legacy of Stingless Bees. *Springer Science & Business Media*.
- Wasihun, A. G., Kasa, B. G. (2016). Evaluation of antibacterial activity of honey against multidrug resistant bacteria in Ayder Referral and Teaching Hospital, Northern Ethiopia. *Springer Plus*, 5(1), 1-8.
- Weissenstein, A., Villalon, G., Luchter, E., Bittmann, S. (2018). Medical honey and the role in pediatric emergency wound management. *Journal of Pedestrian Diseases*, 2, 1-7.

- Weldegebriel Y. (2009). Determination of Selected Trace Metals and Physicochemical Parameters Commercially available Honey in Ethiopia. *Addis Ababa University. Msc. thesis.* 1-53
- White, J. W. (1978). Honey. *Advances in Food Research*, 24, 287–374.
[https://doi.org/10.1016/S0065-2628\(08\)60189-3](https://doi.org/10.1016/S0065-2628(08)60189-3)
- White, J.W., Reithof, M.L., Subers, M.H., Kushnir, I. (1962). “Composition of American honey” *US Department of agriculture technical bulletin* 1261, 11-24.
- WONGSA *et al.*, (2023). *Curr. Res. Nutritional Food Science Journal*. 11(1) 246-257
- Zahrah, (2014). *Al- Engineering and Technology*, 3(2), 8977-8989,

APPENDIXES

Appendix1: pH, Free Acidity and conductivity of honey of Kondala district



Appendix 2: Ash content of honey



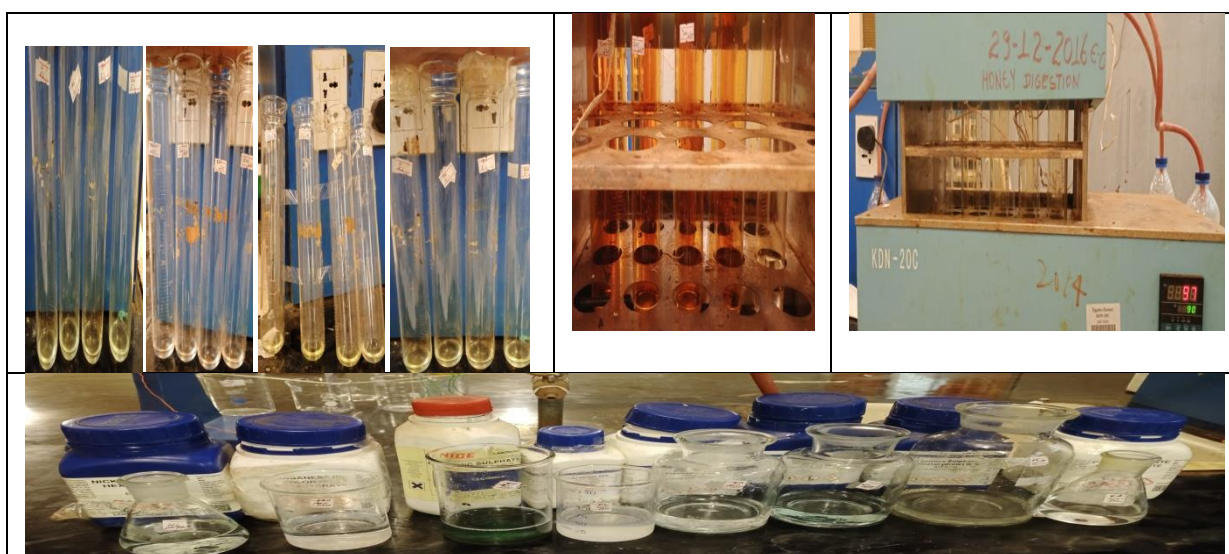
Appendix 3: Moisture Contents



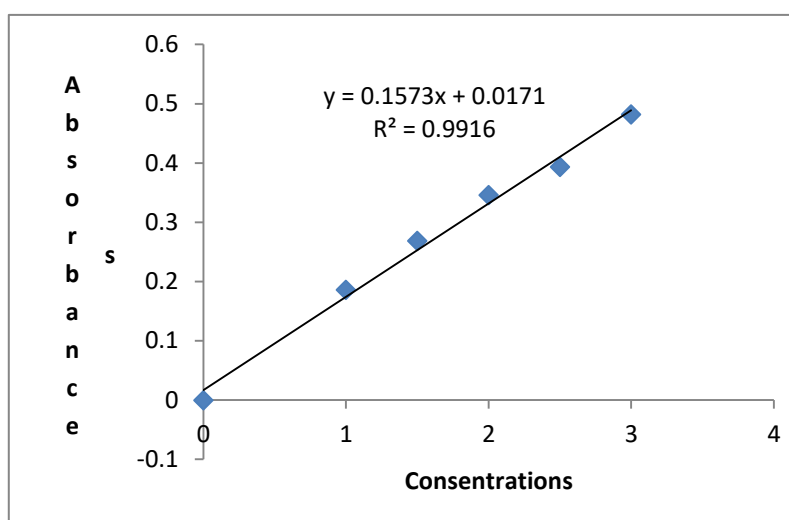
Appendix 4: Total Sugar contents



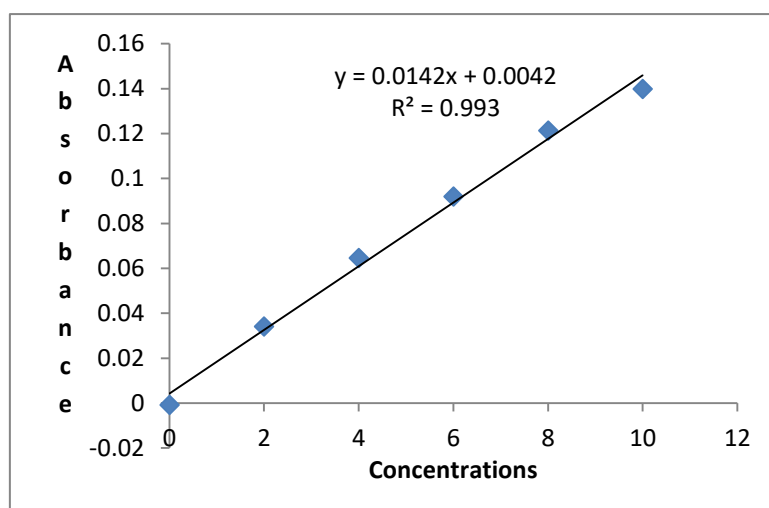
Appendix 5: Optimization of Honey for Metal Analysis



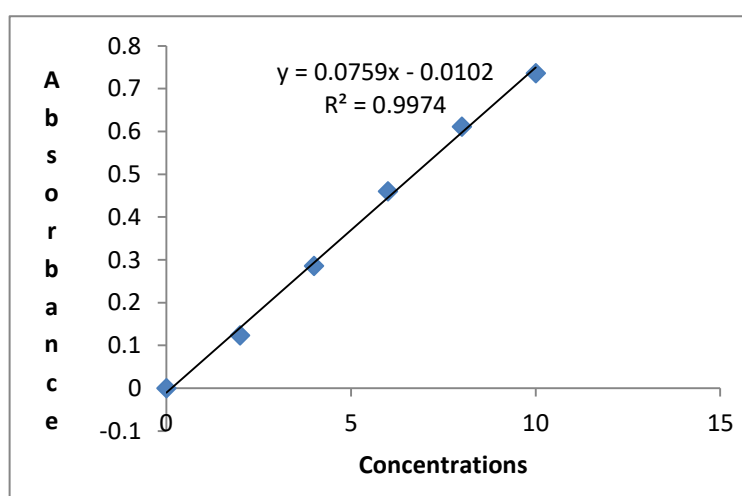
Appendix 6: Calibration curve for Cadmium concentration in honey vs Absorbance



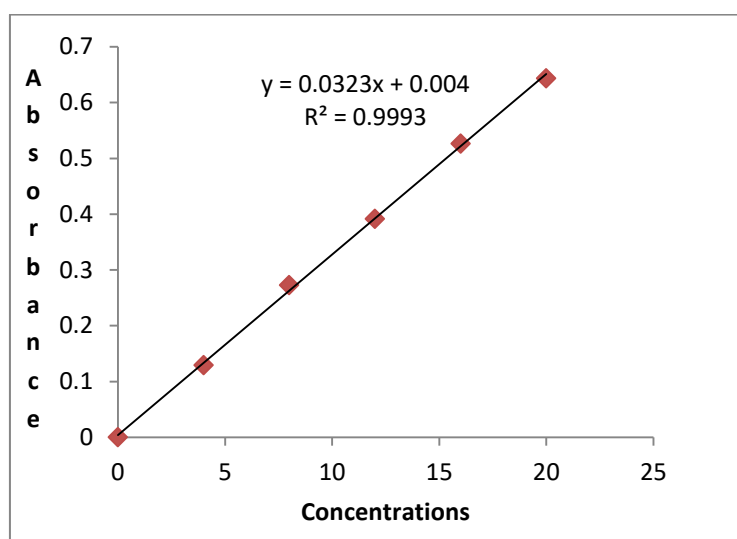
Appendix 7: Calibration curve for Chromium(Cr) concentration in honey vs Absorbance



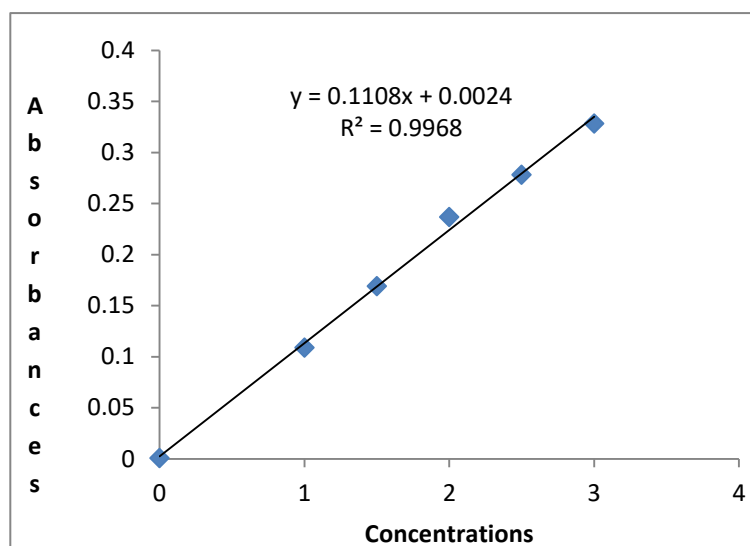
Appendix 8: Calibration curve for Copper(Cu) concentration in honey vs Absorbance



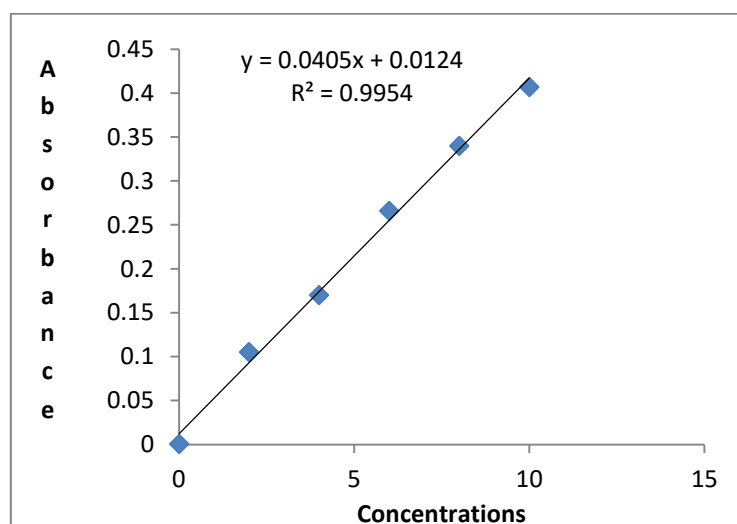
Appendix 9: Calibration curve for Iron (Fe) concentration in honey vs Absorbance



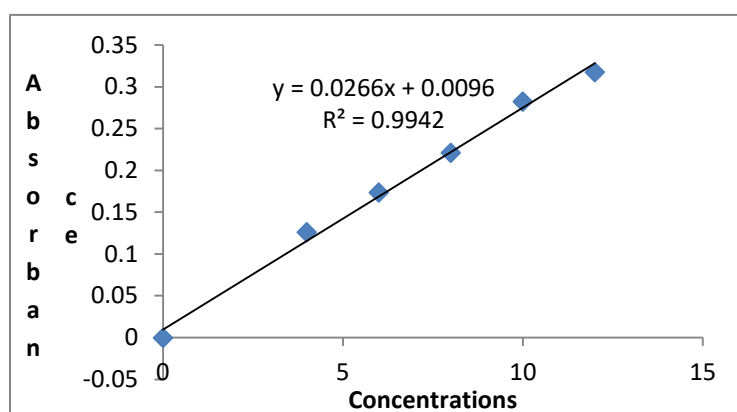
Appendix 10: Calibration curve for Manganese (Mn) concentration in honey vs Absorbance



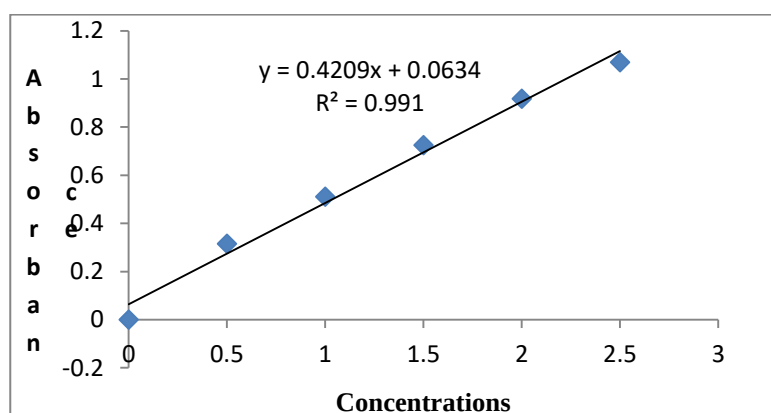
Appendix 11: Calibration curve for Nickel (Ni) concentration in honey vs Absorbance



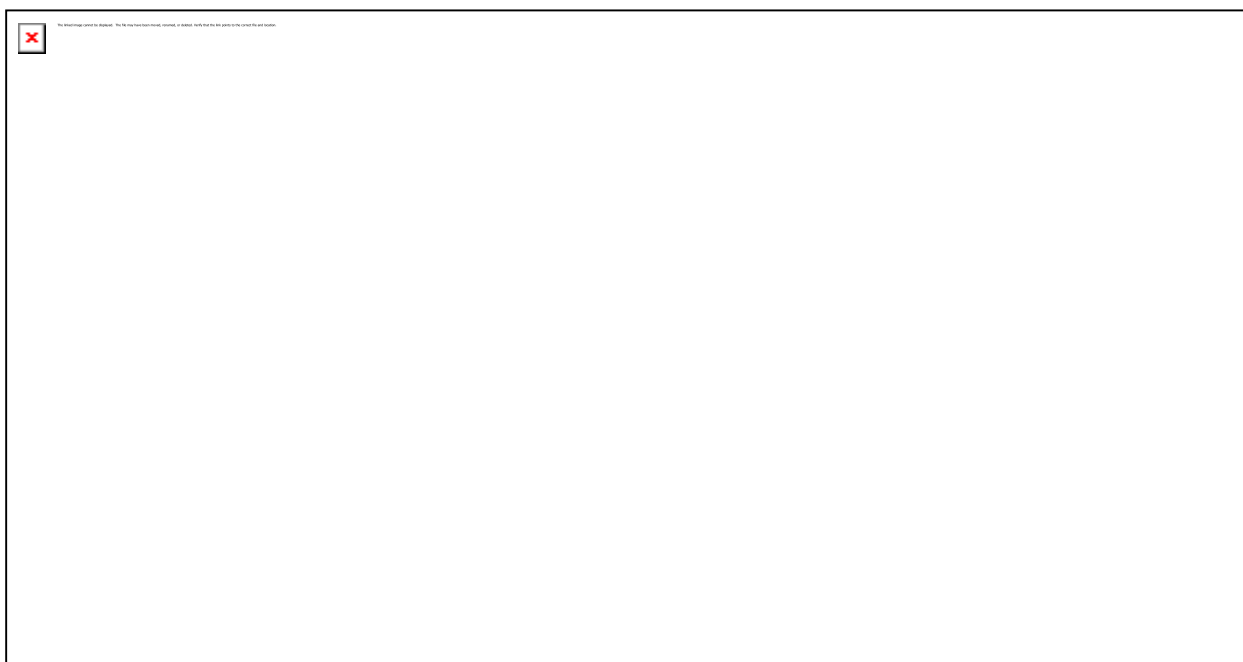
Appendix 12: Calibration curve for Lead (Pb) concentration in honey vs Absorbance



Appendix 13: Calibration curve for Zink (Zn) concentration in honey vs Absorbance



Appendix 14. Calibration curves for Antioxidant properties of honey



Appendix 15: Antibacterial Activities of Honey

