JIMMA UNIVERSITY

SCHOOL OF GRADUATE STUDIES

COLLEGE OF NATURAL SCIENCE

DEPARTMENT OF BIOLOGY (APPLIED MICROBIOLOGY)

Physio-Chemical properties, Microbial safety and Anti microbial Activities of Honey Collected from Kuyu Woreda , North Shewa, Ethiopia.

By: Teshome Debelie

A Thesis Submitted To the Department of Biology, College Of Natural Science, Jimma University, in partial Fulfillment of the Requirement for the Degree of Master of Science in Biology (Applied Microbiology)

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Jimma, Ethiopia

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Advisor: Ketema Bacha (PhD)

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LIST OF ABBREVIATION/ACRONYMS

AMB	Aerobic mesophilic bacteria
AOAS	Association of Official Analytical Chemists
APHA	America Public Health Association
BAM	Bacteriological Analytical Manual
CFU	Colony forming unit
EAR	Estimated Average Requirement
MIC	Minimum inhibitory concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
PCA	Plate Count Agar
PDA	Potato Dextrose Agar
PRI	Population Reference Intake
RDA	Recommended Dietary Allowances
RNI	Reference Nutrient Intake
TAPC	Total aerobic plate count
USDA	United State Department of Agriculture
VRBA	Violet red bile agar
XLD	Xylose Lysine Deoxycholate Agar

ABSTRACT

Honey is a natural sweetener with various beneficial properties including anti-oxidant, nutritional, anti-microbial and industrial application. Among the traditional application of honey is its common role in wound healing as it has antibacterial activity. However, there are no data on the microbial quality and the significance of honey in traditional healing of infectious diseases in the study area. To this effect, this study was designed to document the physico-chemical properties, microbial quality and antimicrobial activities of honey samples collected from North Shewa, Kuyu woreda, Garba Guracha Town. Standard microbiological methods were followed for microbiological analysis. The disc diffusion assay method was implemented for antimicrobial activity tests, while the heavy metal content analysis was carried out using Flame Atomic Absorption Spectrophotometer. Other physicochemical parameters including pH and moisture content were also determined. Socio-demographic data related to the traditional use, storage, harvest, and others practices were gathered using structured questionnaire. Result of the current study indicated that the honey samples had mean pH, % ash and moisture contents of 4.1±0.13, 0.84%± 021%, and 16.78%±1.37%, respectively. Furthermore, mean zinc and lead contents of the honey samples were 0.45 ±0.06 mg/l and 0.08± 0.04 mg/l, respectively. Microbiologically, Salmonella sp, Shigella spp, Lactic acid bacteria, and Enterobacteriaceae were not detected in all studied honey samples. Only aerobic mesophilic bacteria, yeasts and molds were encountered in the samples with variable counts among the groups. The number of Aerobic mesophilic bacteria (AMB) varied between 3.94 Log cfu/ml -5.95 log CFU/ml with mean count of 5.00 ± 0.55 log cfu/ml. The number of yeasts and moulds were usually below detectable level with mean counts of 1.87± 0.32log cfu/ml and 1.16± 0.21Log cfu/ml, respectively. Anti-microbial activity of honey revealed some degree of activity only to gram negative bacteria (Escherichia coli and S. typhimurium) but no effect on Staphylococcus aureus using honey concentration up to 50%. The inhibition zone observed for S. typhimurium using honey concentration of 50% and 10% were 16.11±0.70mm and 9.03± 0.94mm, respectively. Likewise, the Inhibition zone diameters recorded for Escherichia coli using concentration of 50% and 10% were 13.60±2.1 and 8.08±0.64, respectively. These findings provide supportive evidence of the application of honey for the treatment of infectious diseases besides indication of the low level of contamination of honey at the study area with respect to Salmonella and Shigella. This does not, however ensure guaranty for microbial safety of honey since aerobic mesophilic bacteria, yeasts and molds were detected at lower level. Thus, it calls for better storage, preservation, and use of honey for nutritional and medicinal applications.

***Key words**: Antimicrobial activity, Garba Guracha, Honey,Kuyu woreda, N/Shewa*

1. Introduction

Antimicrobial agents are essentially important in reducing the global burden of infectious diseases. However, as resistant pathogens develop and spread, the effectiveness of the antibiotics was diminished. This type of bacterial resistance to the antimicrobial agents poses a very serious threat to public health and all kinds of antibiotics, including the major last-resort drugs, as the frequencies of resistance are increased worldwide (Levy and Marshall, 2004;Mandal *et al.,* 2009).

The use of honey as a traditional remedy for microbial infections dates back to ancient times. The ability of honey to kill microorganisms has been attributed to its high osmotic effect, high acidic nature, hydrogen peroxide concentration and its phytochemical nature (Molan, 1992). Honey has previously been shown to have wound-healing and antimicrobial properties, but this is dependent on the type of honey, geographical location and flower from which the final product is derived (Cooper and Molan, 2000).

Honey is the sugary substance produced from the nectar of flowers by the worker bees. As defined by the Codex Alimentarius Commission (Codex, 2001) honey is the natural sweet substance produced by honeybees from the nectar of blossoms or from the secretion of living parts of plants or excretions of plant-sucking insects living on parts of plants, which honeybees collect, transform and combine with specific substances of their own, store and leave in the honey comb to ripen and mature.

Physico-chemical properties of honey, such a low water activity and high sugar concentration prevent the growth or even survival of different types of bacteria. However, honey is not sterile. Primary sources of microorganisms in honey may be pollen, the digestive tract of honey bees, soil, water, air, and nectar. These natural sources are very difficult to control. Secondary sources are closely connected with hygiene of processing, handling, and storage of honey. Different microorganisms were isolated from honey. Anaerobes are reported as the predominant microflora, including *Clostridium botulinum*, which is responsible for causes of infant botulism.

Several species of *Bacillus* sp. are also detected in honey (Bogdanov, 2006). Other significant groups of microbes are yeasts and moulds, responsible for fermentation and spoilage of hone (Tanzi, 2002).

Micro-organisms in honey may influence the stability of the products and its hygienic quality (Snowdon and Cliver, 1996). Due to the natural properties of honey and control measures in the honey industry, honey is a product with minimal types and levels of microbes. The use of honey as a traditional remedy for microbial infections dates back to ancient times (Molan, 1992).

Research has been conducted on manuka honey which has been demonstrated to be effective against several human pathogens, including *Escherichia coli, Enterobacter aerogenes, Salmonella typhimurium, S. aureus* (Lusby *et al.,* 2000). As a natural product, honey is famous for its richness in nutrients and being a valuable remedy as it was used to treat diseases (e.g. for gastrointestinal disorders or wound healing) in Egypt and Greece (Inoue *et al*.,2005).

The use of traditional medicine to treat infection has been practiced since the origin of man kind (Sofowora, 1987), and past it was the only method available. Currently, due to absence of sufficient modern health care system, particularly in rural areas, people prefer to visit traditional healer and herbal medicines (Abebe, 1996). The integration of traditional and modern medicines was gaining increased recognition globally.

Food – borne illnesses are global problem in developing countries. Food spoilage or deterioration is predominantly caused by the growth of microorganism including *Escherichia coli (E. coli), Enterobacter aerogenes, Salmonella typhimurium, S. aureus.* In Ethiopia health and health related indicators of the Ministry of Health published in 2004 shows that among the ten leading causes of outbreak visiting health institutions are all forms of diarrhea which are directly or indirectly related to food (Mulata, 2011). The use of honey as a traditional remedy for gastrointestinal disorders, wound healing, diarrhea and allergic throughout Ethiopia. Furthermore, the chemical profile of honey, hence its nutritional and antimicrobial activities,

could vary considerably, depending upon the floral origin of the nectar and even on the time of year in which the honey is collected (Mato *et al.,* 2003). However, scientific information on the microbial safety and antimicrobial activities of honey from the study site is scanty. The current study was designed to address the gap

2. Objectives

2.1. General Objective

The general objective of this work was:

- To determine the physico-chemical properties, microbial safety and anti microbial activities of honey.

2.2. Specific Objective

The specific objectives of the current study were:

- To determine physico-chemical properties of honey samples collected from Garba Guracha town
- To evaluate the microbial load and prevalence of pathogens in different honey samples
- To isolate and characterize dominant microorganism from honey sample
- To assess the antimicrobial activities of honey.

3. Literature Review

3.1. Honey

Honey is the sugary substance produced from the nectar of flowers by the worker bees. As defined by the Codex Alimentarius Commission (Codex, 2001) honey is the natural sweet substance produced by honeybees from the nectar of blossoms or from the secretion of living parts of plants or excretions of plant-sucking insects living on parts of plants, which honeybees collect, transform and combine with specific substances of their own, store and leave in the honey comb to ripen and mature. There is a growing consumption of honey because of its high values in maintaining good health and in treatment of various diseases. With many therapeutic claims about honey, it implies that honey could play a very effective role in health care delivery in Nigeria (Mato *et al.,* 2003). It therefore becomes necessary to monitor the hygienic quality of honey being sold to consumers in our local markets.

Honey has been suggested as the quality assessment and botanical origin indicator (Gupta *et al*., 1992). It has also been reported that some aroma compounds can be correlated with the geographical origin of honey. Indeed, 1-penten-3-ol, detected in English honeys, has been considered as a specific compound for this region (Ceyhan and Ugur, 2001). According to Mato *et al* (2003), certain osmophilic yeasts, when present in honey, multiply as moisture increases, facilitating the fermentation process. These microorganisms are present in the body of bees, in nectar, in the soil, and in honey extraction and storage Honey stored at adequate moisture content is unquestionably a guaranteed and extremely durable product, which can be exposed on store shelves without problem until consumption (Moraes *et al.,* 1989).

Honey is increasingly being used in the management of infected wounds where conventional pharmaceutical products are failing, especially now that CE-marked sterile honey and honey-impregnated dressings are available (Molan and Betts, 2004). Honey was used in the medicine of many ancient communities (Molan, 2006), including the ancient Egyptians. The ancient Chinese

and Sumerians provided the first written prescriptions relating to the medical use of honey, found as clay tablets, dating back to 2000 B.C.

3.2. Physico-chemical property of Honey

Many authors have reported studies on honeys, some based on physico-chemical and sensorial analysis (Gupta *et al*., 1992; Singh and Kuar, 1997) .Honey is a nectar and sweet deposit from plants which is gathered, modified and stored in the honey comb by honey bees (Snowdon and Cliver, 1996).Liquid honey does not spoil because of its high sugar concentration and low moisture content, which kills most bacteria by plasmolysis and impedes the development of airborne yeasts. The moisture content of natural raw honey varies from 14% to 18%. As long as the moisture content remains below 18%, virtually no microorganisms can successfully multiply in honey.

3.2.1. Chemical Characteristics of Honey

Honey is a complex substance, made up of at least 181 different substances known at present (Jones, 2001). 107 researchers believing that the number is actually closer to 600 different substances (Bogdanov *et al.,* 2004). Honey is a saturated or supersaturated sugar solution, meaning that it possesses a high concentration of sugar (with approximately 17% water on average). The main sugars present in honey are fructose (an average of 38%), glucose (31%) and disaccharides like maltose (7.3%) and sucrose (1.3%), and higher sugars (1.5%) (White, 1994).The presence of fructose and glucose in honey is due to the action of the bee enzyme invertase on the sucrose molecules contained in nectar, producing a ratio between glucose and fructose of 2: 1 (Anklam, 1998).

Starch has also been recovered from honey, and this is solely a product of the processing of the nectar, as it is not present in the raw nectar. Honey is also rich in organic acids, with at least 30 different organic acids being recovered from this product, among the most common are gluconic

acid, acetic acid, citric acid, lactic acid, succinic acid and formic acid (Mato *et al.,* 2003). These acids are the results of the action of enzymes like glucose oxidase on the sugars present in honey and makeup an average of 0.50% of the honey by weight (Mato *et al.,* 2003). The organic acids present in honey are believed to contribute to the organoleptic properties such as flavor and color as well contributing to physical and chemical characteristics such as pH, acidity and electrical conductivity (Mato *et al.,* 2003). In the 1930s citric and malic acid were thought to be the predominant acids in honey, but in 1960 gluconic acid was shown to be the predominant form. This organic acid is derived from two sources, the action of the enzyme glucose oxidase and the metabolic activity of certain *Glucobacter* spp bacteria (present in the bee's gut). The concentration of this organic acid depends on the time needed for the manufacture of the honey, strength of the bee colony and the quality of the nectar to be transformed. Other organic acids are either intermediates of the Krebs cycle or products of enzymatic pathways, and as such can differ significantly from honey to honey. Hence, variations in the organic acids present in honey can be used as indicators of deterioration, authenticity and purity (Mato *et al.,* 2003).

Another important characteristic of honey is its low pH, the normal pH ranges between 3-6. This acidity is thought to be caused by the presence of the different organic acids in the honey, and is one of the factors limiting the growth of microorganisms (Ceyhan and Ugur, 2001). Honey possesses a low amount of nitrogen (0.041% w/v) that is part of proteins, enzymes and free amino acids. The amount of nitrogenous compounds present in honey may affect its characteristics; the high protein concentration (2% w/v) in heather honey, for example is responsible for its viscous characteristics. Estimation of protein in honey by the volume of precipitate with tannin has been used in the past to distinguish between honey and artificial blends (Mato *et al.,* 2003). The presence of enzymes in honey is important, as these aid the transformation of the nectar into honey, some of the most common enzymes recovered in honey are invertase, catalase, phosphatase glucose Oxidase and diastase (Crane , 2001).

The mineral component of honey is referred to as the ash portion and makes up about 0.1% of all the components in honey. Ash is more abundant in darker honeys and mono floral honeys tend to have lower ash content (Crane, 2001). Potassium makes up around half of the total ash content in honey, and other minerals found are calcium, copper, sodium, magnesium, manganese and chlorine salts. Another 30 different mineral complexes may be used to determine floral origin of the honeys as they are characteristic for each plant (Anklam, 1998). Honey contains trace amount of vitamins, flavonoids, antioxidant components and unidentified plant derived elements phyto chemical components (Sato and Miyata, 2000). It also possessestr ace amounts of other beehive products like propolis, royal jelly and wax of which the first two are recognized as antimicrobial agents as well (Anklam, 1998).

The actual chemical profile of honey varies considerably from honey to honey, depending upon the floral origin of the nectar and even on the year and time of year in which the honey is collected (Mato *et al.,* 2003). The type of bee producing the honey also changes its chemical characteristics (Demera and Angert, 2004), and there are certain chemical markers that are used to determine if the honey is authentic (that is if it has been altered in any way, like the addition of extra sugar or water), like protein, moisture, sugar, and hydroxyl rnethyl furfural (HMF) content (Mateo and Bosch, 1998). HMF is a product of sugar breakdown in honey as a result of heating or storage, so this substance is useful to determine if the honey was heat processed or aged. Usually honeys used for medical purposes, since the first recordings, tended to be local honeys that were produced mainly from the nectar collected from one predominant flower source. Analysis of the pollen content of honey is mainly used to determine the predominant floral species that the bee has foraged, however as the composition of honeys is further characterized chemically, other markers, like specific proteins or organic acids are being used for honey classification (Mato *et al.,* 2003). Unifloral honeys are largely derived from a single floral source and useful because their characteristics can be better defined than those of honeys that come from more than one floral source (multifloral do not demonstrate a predominant floral source when the pollen is analyzed).

3.2.2. Degree of Variance Observed in Honey

In almost all studies in which more than one type of honey has been used, differences in the antibacterial activity of the honeys have been observed. The degree of difference observed has in some cases been very large, and in many others where it has been smaller this possibly is the result of a more limited range of testing rather than of less variance in the activity of the honeys. In many studies the antibacterial activity of different honeys has been compared by way of the inhibited number determined by the method devised by such comparisons (Duo et al., 1997). Coined the term 'inhibition number' in to describe the degree of dilution to which a honey will retain its antibacterial activity. This is a term that has been widely used since as a measure of the antibacterial activity of honey. The inhibition number involves a scale of 1 to 5 representing sequential dilutions of honey in 5% steps, from 25% to 5%. There have since been various minor modifications to this method so that the actual concentration corresponding to the inhibition number reported may vary. One modification has been to estimate fractional inhibition numbers by visual assessment of partial inhibition on the agar plate with the concentration of honey that just allows growth. The effect of differences between methods on the comparability of the inhibited numbers from different studies has been discussed to range over the five-fold difference in concentration in the dilution series (Sato and Miyata, 2000).

In three other studies activity was found to range over a four-fold difference in concentration in the dilution series. With some honeys not active at the highest concentration tested in some of the studies, and others still active at the greatest dilutions, it is possible that if greater and lesser degrees of dilution had been included in the testing then a wider range of activities would have been detected. One study using a wider range of dilutions (honey from 50-0.25%) found the minimum inhibitory concentrations of the honeys tested to range from 25% to 0.25%3. Another, testing from 50% to 0.4% found the minimum inhibitory concentrations to range from greater than 50% (i.e. not active at 50%) to 1.5% (Sato and Miyata, 2000)

Other studies with wide ranges tested also found some honeys without activity at the highest concentration tested, and other honey with activity at the lowest concentration tested: the ranges were from 20-0.6% and 50-1.5%. When the data are examined, activities are seen to be fairly well spread over these ranges. Plotted the distribution of the activity of 131 samples of honey tested, and found that it deviated from a normal Gaussian distribution because of the large number of samples with low activity. (In 7% of the samples the activity was below the level of detection.) They attributed this to destruction of activity by exposure to heat and light, and estimated that 50% of the samples had lost more than half of their original activity, and 22% had lost more than three-quarters. Another study of 345 samples of honeys4 also found a large number with low activity (36% of the samples had activity near or below the level of detection), the rest having almost a Gaussian distribution over a twenty-fold range of activity.

3.2.3. Heavy Metals

Considered as an analytical sample, honey is one of the most complex mixtures of carbohydrates produced in nature. In common honey, mono- and disaccharides constitute 80– 85 % (w/w), water is around 15–20% (w/w) and other organic compounds and inorganic ionsbeing present to a minor extent (Sanna *et al.,* 2000).

The heavy metals cadmium, lead and mercury are common air pollutants, being emitted mainly as a result of various industrial activities. Although the atmospheric levels are low, they contribute to the deposition and build-up in soils. Heavy metals are persistent in the environment and are subject of bioaccumulation in food-chains. Emissions of heavy metals (HM) in Slovak republic from industry have exerted declining trend since 1990. Declining trend of emissions of most heavy metals affected the shut-down of some productions, large reconstruction of separating devices and change of used raw materials. However, in 2006 in comparison to previous years, an increase of emissions of Pb, Hg, Cr, As, Ni, Cu and Zn in combustion processes in industry as well as an increase of emissions of Pb, Cd, As, Ni, Cu and Zn in

industrial technologies were recorded. Motivations for controlling of HM concentrations are diverse. Some of these concentrations are dangerous to health or to the environment (e.g. mercury, cadmium, lead, chromium), some may cause corrosion (e.g. zinc, lead), some are harmful in other ways (e.g. arsenic may pollute catalysts).

Within the European community the eleven elements of highest concern are arsenic, cadmium, cobalt, chromium, copper, mercury, manganese, nickel, lead, tin, and thallium, the emissions of which are regulated in waste incinerators (Hogan, 2010). Some of these elements are actually necessary for humans in minute amounts (cobalt, copper, chromium, manganese, nickel) while others are carcinogenic or toxic, affecting, among others, the central nervous system (manganese, mercury, lead, arsenic), the kidneys or liver (mercury, lead, cadmium, copper) or skin, bones, or teeth (nickel, cadmium, copper, chromium).

The heavy metals Pb, Cd and Hg are not part of the family of trace elements which in small quantities are essential for human health e.g. copper or zinc. The examination of humanotoxic effects in the context of the critical loads concept focuses in respect of each particular heavy metal (Pb, Cd, Hg) on its transfer via soil and/or water into human nutrition or drinking water systems. Other sources of pollution with consequences for human health such as house dust or paints are not taken into consideration. Honey is an important food for the human nutrition. Honey possesses valuable nourishing, healing and prophylactic properties which result from its chemical composition (Tuzen and Duran, 2002).

Honey bees are good biological indicators because they indicate the chemical impairment of the environment they live in through two signals: the high mortality and the residues present in their bodies or in beehive products (in the elements and other contaminants like heavy metals and radionuclides) that may be detected by means of suitable laboratory analyses (Celli, 1994).

However, the minor components are often of great importance from many points of view. The evaluation of heavy metals content in honey has a twofold significance: the former one lies in the toxicity of theses metals, with the consequent necessity to develop adequate analytical procedures for their monitoring; the latter one is suggested by the possibility of using bees and their products as bio-indicator of possible environmental pollution, taking advantage of both the large covering area where they live and of the concentration effect of the possibly present environmental pollutants into the "products" of bees (Rowarth, 1990).

3.3. Microbiology of Honey

Micro-organisms in honey may influence the stability of the products and its hygienic quality (Snowdon and Cliver, 1996). Due to the natural properties of honey and control measures in the honey industry, honey is a product with minimal types and levels of microbes. Microbes of concern in post-harvest handling are those that are commonly found in honey (i.e., yeasts and spore-forming bacteria), those that indicate the sanitary or commercial quality of honey (i.e., coliforms and yeasts), and those that are under certain conditions could cause human illness. Primary sources of microbial contamination are likely to include pollen, the digestive tracts of honey bees, dust, air, earth and nectar sources which are very difficult to control (Popa, *et al*.,2009). The same secondary (after-harvest) sources that influence any food product are also sources of contamination for honey. These include air, food handlers, cross-contamination, equipment and buildings. Secondary sources of contamination are controlled by hygienic processing practice (Snowdon and Cliver, 1996). The filamentous fungi, being more spread in nature and having thermal resistant spores, with a great capacity of surviving, can be introduced in honey even by man, through dust, through the water installations or containers or even by the bees through pollen (Marghitas, 2008). Most microbes found in honey are not dangerous for the consumer's health. However, normal honey must lack pathogenic micro-organisms that produce enteric illnesses (Tchoumboue *et al*.,2007). It has been discovered that microbial contamination of honey do occur mostly in honey marketed in local markets (Popa *et al.*,2009). For instance in Cameroon (Tchoumboue *et al*.,2007) isolated bacteria and fungi from honey samples collected

from local markets but isolated none from honey samples collected from the Bee research farm of University of Dschang. In honey samples collected from local markets in Romania (Popa *et al.,*2009) reported the presence of *Bacillus* sp. and eight types of fungi. While microbiological screening is going on in different parts of the world, there is dearth of information on the microbiological status of honey samples collected from local markets in Nigeria. This explains the rationale behind the present study.

3.4. Antimicrobial Characteristics of Honey

Antimicrobial agents are essentially important in reducing the global burden of infectious diseases. However, as resistant pathogens develop and spread, the effectiveness of the antibiotics is diminished. This type of bacterial resistance to the antimicrobial agents poses a very serious threat to public health and all kinds of antibiotics, including the major last-resort drugs, as the frequencies of resistance are increased worldwide (Levy and Marshall, 2004; Mandal *et al.,* 2009). The use of honey as a traditional remedy for microbial infections dates back to ancient times. The ability of honey to kill microorganisms has been attributed to its high osmotic effect, high acidic nature, hydrogen peroxide concentration and its phytochemical nature (Molan, 1992). Honey has previously been shown to have wound healing and antimicrobial properties, but this is dependent on the type of honey, geographical location and flower from which the final product is derived (Molan and Cooper, 2000). It is well established that honey inhibits a broad spectrum of bacterial species. More recently, honey has been reported to have an inhibitory effect to around 60 species of bacteria including aerobes and anaerobes, Gram positives, and Gram negatives (Hannan *et al.*, 2004). There are many reports of bactericidal as well as bacteriostatic activity of honey and the antibacterial properties of honey may be particularly useful against bacteria, which have developed resistance to many antibiotics (Alandejani *et al.,* 2009). Sidr honey is made from bees who feed only on the nectar of the Sidr tree, which is native to the South Saudi Arabia and Yemen regions. The Sidr tree is considered sacred and has been used as a Natural medicine for centuries. Sidr honey is a "monofloral honey", a type of honey which has a high value in the

market place because it has a distinctive flavor or other attribute due to its being predominantly from the nectar of one plant species.

Sidr honey has wide medicinal applications and uses which include: liver diseases treatment, stomach ulcers, respiratory infections, diseases resulting from malnutrition, digestive problems, constipation, eye diseases, infected wounds and burns, surgical wounds (caesarian section), speedy recovery after childbirth, general health and vitality. Sidr honey has strong antioxidant and antibacterial properties (Alandejani *et al.,* 2009) .Mountain honey has high antibacterial activity against gram positive and gram negative bacteria (Mekawey, 2010). A large number of honeys are available in the Saudi market and are either locally produced or imported from different countries. Some of them are traditionally used as remedy for several ailments. The antibacterial efficiency of local Saudi honeys has not been thoroughly evaluated (Eman and Mohamed, 2011).Therefore, the purpose of the present study was to evaluate and compare the *in vitro* inhibitory effect of Sidr and mountain Saudi honeys against the growth of four different gram negative bacteria.

Many authors have reported studies on honeys, some based on physico-chemical and sensorial analysis (Gupta *et al*., 1992; Esti *et al.,* 1997), the chemical identification of certain compounds in honeys from different sources and regions (Aray *et al*., 1997; Martos *et al*., 2000) and, in recent years, studies on the antimicrobial properties of honeys (Allen *et al.,* 1991; Taormina *et al.,* 2001). However, except for *Clostridium botulinum* limited quantitative data of bacteria in honeys have been reported in scientific literature (Tysset and Rousseau, 1981; Root, 1983; Nakano *et al.,* 1990; Piana *et al.,* 1991). According to White *et al.* (1963) honey is a mixture of fructose (average 38.4%), glucose (average 30.3%), sucrose (average 1.3%) and other carbohydrates (about 12%), minerals (average 0.169%) and proteins (169 mg/100g), with a water content of about 17.2%.

The pH of honey ranges from 3.4 to 6.1 with an average of 3.9, while the water activity varies between 0.5 and 0.6. Osmolarity, pH and hydrogen peroxide are the major factors in honey that may be considered for its antimicrobial activity. The principal factor responsible for this activity is hydrogen peroxide produced by the oxidation of glucose by the enzyme glucose-oxidase, which is activated by successive dilutions of honey. In addition, a residual nonperoxide antibacterial activity attributed to phenolic components (phenolic derivatives of benzoic acid, cinnamic acid and flavonoids) was detected in several honeys (Molan and Russell, 1988; Weston *et al*., 1999). These intrinsic properties of honey affect the growth and survival of microorganisms by bacteriostatic or bactericidal action and, in particular, the low pH and high sugar content ofundiluted honeys prevent the growth of many species of microorganisms. In consequence, honey can be expected to contain a small number and a limited variety of microorganisms. Vegetative forms of human disease-causing bacteria have not been found in honey and, as bacteria do not replicate in honey, a high count of vegetative bacteria is indicative of a recent contamination from a secondary source. Thus, the microorganisms of interest are those that with stand the concentrated sugar, acidity and antimicrobial character of honey. These microorganisms include certain yeasts and spore-forming bacteria; coliforms or yeasts indicative of sanitary or commercial quality, and microorganisms such as Bacillus cereus, Clostridium perfringes or C. botulinum, which under certain conditions (e.g. germination and growth in a non-heated-treated product) could cause illnesses in humans (Snowdon and Cliver, 1996). Moreover, microorganisms that cause diseases in honey bees are also of interest. Paenibacillus larvae, a spore-forming bacterium, is one of the major pathogens of Apis mellifera responsible for an infectious disease known as American foulbrood (Alippi *et al*., 2002; Lauro *et al*., 2003). However, P. larvae have never been associated with illness in humans. The aim of this work was to determine and characterize microbial populations in Argentinean honeys obtained from commercial sources, apiaries and bulk containers.

3.4.1. Potential Antimicrobial Agents

The use of honey as a traditional remedy for microbial infections dates back to ancient times (Molan, 1992). Research has been conducted on manuka (L. scoparium) honey (Visavadia *et al.,*2006), which has been demonstrated to be effective against several human pathogens, including *Escherichia coli (E. coli), Enterobacter aerogenes, Salmonella typhimurium, S. aureus* (Lusby *et al*.,2005).Laboratory studies have revealed that the honey is effective against methicillin-resistant S. aureus (MRSA), haemolytic streptococci and vancomycin resistant Enterococci (VRE)(Allene and Molan,2000). However, the newly identified honeys may have advantages over or similarities with manuka honey due to enhanced antimicrobial activity, local production (thus availability), and greater selectivity against medically important organisms (Lusby *et al.,*2005). The coagulasenegative staphylococci are very similar to *S. aureus* (Cooder *et al*.,2002;Abhisk *et al.*,2010) in their susceptibility to honey of similar antibacterial potency and more susceptible than *Pseudomonas aeruginosa (P. aeruginosa) and Enterococcus spec*ies (Cooder *et al.*,2002).

3.4.2. Honeys in Wound Healing

Honey used in wound dressing either produces radicals (H_20_2) at low concentrations, to fight invading pathogens, or due the presence of the enzyme catalase converts excess H_20_2 to water and oxygen, preventing the formation of free radicals. Secondly, the availability of polyphenolic antioxidants in honey detoxity lipid peroxides and interfere with the Fenton reaction. Furthermore, the acidic pH of honey serves an antiseptic function. All these benefits of honey make it ideal for wound repair and therefore ideal for treatment of burns and specifically wound dressings. Honey has been described to have antibacterial effects and this also promotes wound healing. Molan (1999), reported on infected wounds treated with honey and observed complete inhibition of a collection of strains of Methicillin-resistant *Staphylococcus aureus* (MRSA) and sterile wounds within 3 - 6 days when I - 4% *(v/v)* honey was used. When a 2 - *4% (vlv)* honey

was used, there was complete inhibition of 58 strains of coagulase positive *staphylococcus aureus* and wounds were sterile within 7 days. When a 5.5 - 8.7% v/v honey used, there was complete inhibition of 20 strains of pseudomonas and wounds were sterile within 7-10 days.

The high osmolarity of honey leads to inhibition of microbial growth through sugar molecules 'tying up' water molecules so that bacteria have insufficient water to support their growth. Even when honey is diluted down to an extent that osmolanty is reduced to a level that ceases to inhibit bacterial growth, honeys additional antibacterial components still ensure sterility. This is due to the presence of H_20_2 as well as the presence of polyphenolic compounds. Honey contains both catalase (EC 1.11.1.6), which is a peroxidase that converts H_20_2 to H_20 and 0_2 and glucose oxidase (GOD, -D- glucose:oxygen-1- oxidoreductase EC 1.13.4) which is an oxido-reductase that catalyses the oxidation of glucose to H_20_2 and D-glucono-ô-lactone. The rate at which catalase, calatyses the conversion of H_20_2 produced by glucose oxidase to H_20 and 0_2 will be a function of factors such as substrate concentration, density due to high sugar content and pH. As H_2O_2 accumulates due to wound derived inflammation the forward reaction catalysed by catalase with the production of H_20 and 0_2 is favoured without subsequent tissue damage that can occur at high H202 concentrations which includes lipid peroxidation, DNA and protein modification that can lead to cellular dysfunction or death (Molan, 1999).

Other effects of honey are its deodorizing effect (Molan, 1999) whereby malodours that are caused by the bacterial metabolism of amino acids into ammonia, amines, and sulphur compounds. Honey has a high glucose content which is used by infecting bacteria in preference to amino acids resulting in the formation of lactic acids as opposed to the malodorous products. Honey also has a degrading action which removes debris from wounds. This occurs via an osmotic action which causes an outflow of lymph and wound fluid which causes the debris from the wound bed to lift. A further benefit is that the wound remains moist which prevents the tearing away of newly formed tissue when the dressing is removed (Molan, 2001). Furthermore, honey has been found to stimulate the growth of new tissue growth and this includes the formation of new healthy granulation tissue and epithelium. Low concentrations of H_20_2

stimulate angiogenesis and the growth of fibroblasts which results in increased collagen production. Angiogenesis then provides more oxygen and nutrients to the wound site and tissue regeneration can occur.

3.5. Food Borne Pathogenic Microorganisms

3.5.1. *Escherichia Coli*

The family of *enterobacteriaceae* .Gram negative, non spore formingbacteria. *Escherichia coli (E. coli)* is the only member of the total coliform group of bacteria that is found only in the intestines of mammals, including humans. The presence of *E. coli* in water indicates recent fecal contamination and may indicate the possible presence of disease-causing pathogens, such as bacteria, viruses, and parasites. Although most strains of *E. coli* are harmless, certain strains, such as *E. coli O157:H7*, may cause illness (Wilshaw *et al*., 2000).

3.5.2. *Salmonella typhimurium*

Salmonella typhimurium is a rod shaped gram-negative ,non-spore bacillus,motile. *Salmonella* species are the causative agents of typhoid fever and diarrheal diseases in humans, responsible for an estimated 16 million cases of systemic typhoid fever worldwide each year (Pang *et al.,* 1995). Orally ingested bacteria penetrate the intestinal mucosa andmigrate via the lymph nodes to the spleen and liver to cause systemicdisease (Finlay and Falkow, 1997). During bacterial infection, macrophages serve as professional phagocytes and key effectors of the innate and adaptive immune responses. *S. typhimurium* capitalizes on themacrophage's phagocytic nature, and has been shown by confocal microscopy to reside intracellularly within macrophages, where it replicates within specialized vacuoles (Richter *et al.,* 1997).

3.5.3. *Staphylococcus aureus*

Staphylococci are Gram-positive bacteria, with diameters of 0.5 – 1.5 μm and characterized by individual cocci, which divide in more than one plane to form grape-like clusters. To date, there are 32 species and eight sub-species in the genus *Staphylococcus*, many of which preferentially colonise the human body (Kloos and Bannerman, 1994). However *Staphylococcus aureus* and *Staphylococcus epidermidis* are the two most characterized and studied strains. The staphylococci are non-motile, non-spore forming facultative anaerobes that grow by aerobic respiration or by fermentation.

Staphylococcus aureus is a major pathogen of increasing importance due to the rise in antibiotic resistance (Lowy, 1998). It is distinct from the e.g. *S. epidermidis*, and more virulent despite their phylogenic similarities (Projan and Novick, 1997). The species named *aureus*, refers to the fact that colonies (often) have a golden colour when grown on solid media, whilst CoNS form pale, translucent, white colonies (Howard and Kloos, 1987).

4. Material and Methods

4.1. The Study Area

The study was conducted in Garba Guracha town, Kuyu woreda, North Shewa Zone, Oromia region located at 160km North of Addis Ababa (**Fig 1**). Kuyu woreda characterized by three climatic zone: *dega, weyinadega and kola*. According to statistics from of distinct agricultural office, in the study area, the annual rainfall is 600-800 mm and annual temperature is 5^{o}C-30^{o}C.This district is known for its agricultural production. Kuyu woreda is also known for honey production.

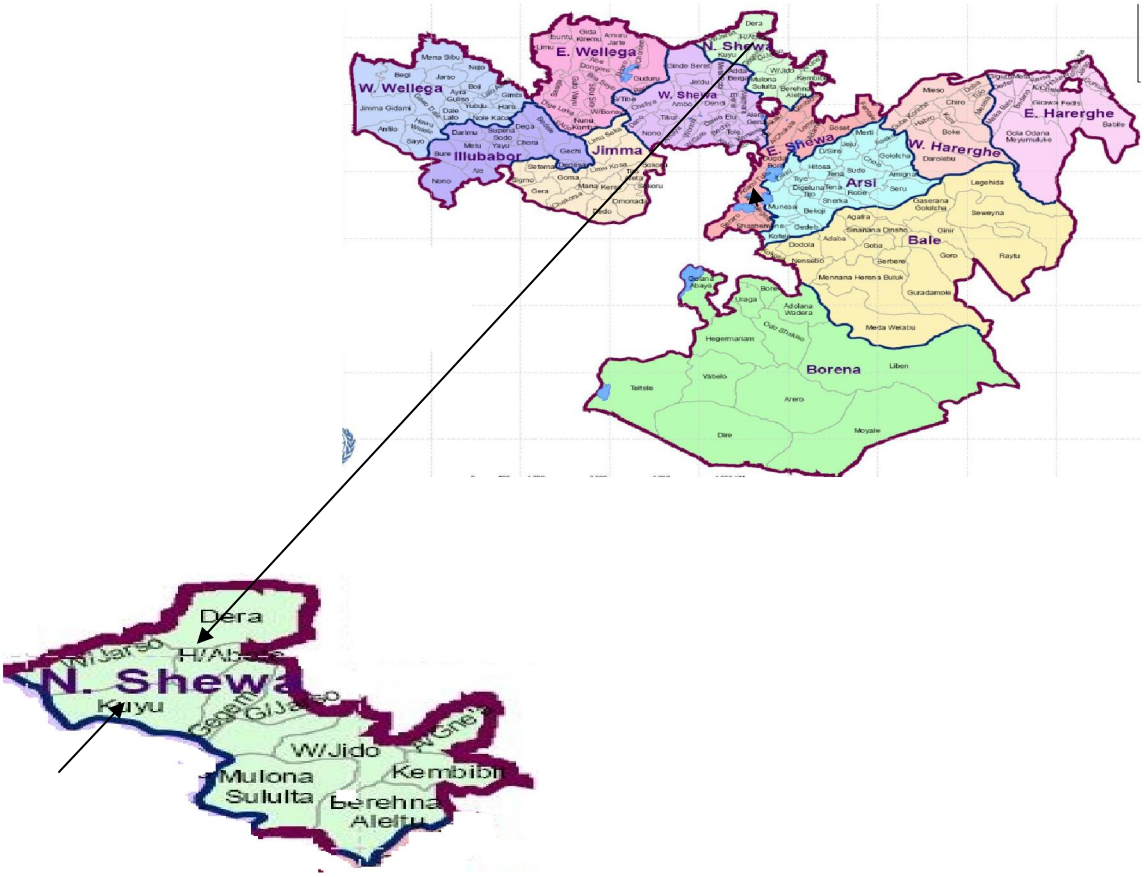

Fig.1 Map of study area (*http://us.yhs 4)*

4.2. Sample Collection

Thirty five (35) honey samples, each of 200g were purchased from North shewa Garba Guracha town from open market. All samples were collected aseptically using sterile glass jars aseptically from December, 2013 to February, 2014 and stored at room temperature (25 ± 2°C) until processed for microbiological and some physicochemical features. Laboratory analysis was conducted at Jimma University College of agriculture, Biology Department and Chemistry Department.

4.3. Physico-Chemical Analysis

4.3.1. Determination of pH

The pH of honey was determined according to the method described by the International Honey Commission (White, 1969). Five grams of each honey sample was diluted with 50ml distilled water to make a 10% solution. The pH was measured using a digital pH meter which was calibrated at room temperature using buffer solutions at pH 4 and pH 7. To ensure accurate pH measurement, the instrument was calibrated every time before use. Results were expressed in micro Siemens per centimeter (μS/cm) (Devillers *et al.,* 2004).

4.3.2. Determination of Ash Contents

Ten gram of each sample was separately weighed into a previously weighed and dried crucible. The crucible with its content was placed in a muffle furnace. The furnace was set to 600 °C and the samples were ignited for 6 hours until they were reduced to ash. The ignited samples were allowed to cool in a desiccator and weighed (AOAC, 1990). The percentage ash content was calculated.

$$\textbf{\%Ash} = \frac{weight\ of\ ash}{sampls} \times 100\%$$

$$\%\text{Ash} = (Weight\ of\ crucible\ +\ ash) - (Weight\ of\ empty\ crucible)/sample\ weight x\ 100$$

4.3.3. Moisture contents

The moisture content of each honey sample was determined by measuring 10g of the sample and placing into a pre- weighed aluminum drying dish. The sample was dried to constant weight in an oven at 105^{0}C for 4 h under vacuum (AOAC, 2000).

$$\text{Moisture (\%)} = \frac{Weight\ of\ fresh\ sample - Weight\ of\ dry\ sample \times 100}{Weight\ of\ fresh\ sample}$$

4.3.4. Determination of Heavy Metals.

Analysis of Zinc (Zn) and Lead (Pb) were conducted using flame atomic absorption spectrophotometer. According to US EPA (1983) Samples were prepared, so that 10g of honey was burned until turned in to ashes, and the rest was calcined 13 h in furnace at 450^{0}c.Residual ash was dissolved in 10 ml of 0.1M HNO_3 Nitric acid plus 1 ml of hydrogen peroxide H_2O_2 was added to a beaker containing the ashes stirred and then heated the mixture on a hotplate to almost complete dryness. Two ml of the 0.1M HNO_3 was added to the contents of the beaker, transferred to volumetric flask and diluted with dionized water to 50 ml. The same method was applied to sterilized distilled water to compare the result was measured using Atomic Absorption Spectrometry (US EPA, 1983).

4.4. Microbial Enumeration.

A 25 g of honey sample was added to 225 ml of sterilized buffered peptone and thoroughly mixed. Six sterile test tubes containing 9 milliliters of sterile distilled water were arranged. A tenfold serial dilution was done to the extinction transferring 1 ml of the sample into the first test tube (10^{-1}) which was well shaken by vortex mixe and 1 milliliter was taken again from the 10^{-1} dilution and transferred to the next test tube (10^{-2}). The dilution continued to 10^{-6}. Each test tube was shaken by vortex mixture vigorously before each transfer to prepared medium (APHA,

1992). From appropriate dilution 0.1ml of aliquot, spread-plated on each prepared presolidified media and incubated at suitable temperature (Table 1). After incubation of the plates, counts of the number of colonies in each plate was done with a hand tally counter (Fawole and Oso, 1986). The mean of the counts were obtained and multiplied by the appropriate dilution factor.

$$\text{CFU/ml}=\frac{(Number\ of\ colonies)(dilution\ factors)}{plated\ volume\ in\ ml}$$

$$\text{Dilution factors}=\frac{1}{dilution}$$

Table .1 Summary of Microbial media and their appropriate incubation time, and duration at defined temperature.

Microbial groups	Media	Length of incubation(h)	Cultivation Method	Temperature(^{0}C)
Aerobic plate count	Plate count bile agar	48	Aerobic	35
Coliform counts	Violet red bile agar	24	Aerobic	37
Molds & yeasts count	Potato dextrose agar	120	Aerobic	25
Lactic acid bacteria	Mann Rogosa sharpe	48-72	Anaerobic	32
Enterobacteriacaea	MacConkey agar	24	Aerobic	35
Salmonella & shigella	Xylose-lysine Deoxycholate agar	24	Aerobic	35

4.4.1. Total Aerobic Plate Counts

For Total aerobic plate count (TAPC), a 0**.1**ml aliquot was spread on pre-solidified Plate Count Agar (PCA) and incubated at 35 ^{0}C for 48 hours (Trytinopulou *et al*.,2002). Then the total aerobic mesophilic counts were enumerated after incubation.

4.4.2. Total fecal /Coliform/Counts

From appropriate serial dilution 0.1ml aliquot was spread on pre-solidified surface of Violet red bile agar (VRBA) and incubated at 35 ^{0}c for 24 hours. Purplish red colonies surrounding by reddish zone were considered as coliforms and counted (Weil *et al*., 2006).

4.4.3. Enterobacteriaceae counts

To counts the number of members of the family Enterobacteriaceae 0.1ml of aliquot was spread-plated on MacConkey Agar and incubated at 35 ^{0}C for 24 hours (Pons Sanchez *et al.,* 2005). The pink red purple colony was counted as member of family Enterobacteriaceae.

4.4.4. Lactic Acid Bacteria Counts

To counts the number of lactic acid bacteria a 0.1ml of aliquot was spread-plated on de Mann Rogosa sharp agar (MRS) and incubated at 35 ^{0}C for 48 hours under anaerobic (Abegaz, 2007).

4.4.5. Yeast and Moulds counts

From appropriate dilutions a 0.1ml aliquot spread-plated on pre-solidified surface of Potato Dextrose Agar (PDA) and incubated at 25^{0}C for 5 days. Smooth (non hairy) colonies without extension at periphery was counted as yeast. Hairy colonies with extension at periphery was counted as moulds (Spencer *et al.,* 2007)

4.5. Microbial Analysis

After enumeration of aerobic mesophilic bacteria, two to three colonies with distinct morphological differences such as color, size and shape were randomly picked from countable plates and aseptically transferred in to a tube containing 5 ml nutrient broth. The inoculated cultures were incubated at 32^0 C for 24 hrs. Cultures were purified by repeated plating and preserved on Nutrient agar slants at 4^0c for a month. Finally, the pure cultures were characterized to family/ genus levels. The characterization of isolates was done based on Johan (2012) bacterial classification manual.

4.5.1. Cell Morphology

In order to assess the cell Morphology of the culture, gram staining, motility test and endospore test were done. The morphological study includes determination cell shape, cell arrangement, presence and absence of endospore and motility.

4.5.2. Gram staining

The smear of pure isolates was prepared on a glass slide and allowed to air –dry and heat-fix.the heat-fix smear was flooded with crystal violet dye for 1 minute and rinsed under tap water for 3 seconds. Then, the slide was flooded with iodine solution for 1 minute and rinsed under tap water for 3 seconds. After rinsing, the smear was decolorized with 96% of ethanol for 10 seconds and the slide containing smear washed gently under tap water for 3 seconds. Thereafter, the smear was counter stained with safranin and air dried. Finally, the stained slides were observed under microscope and the isolates gram reaction determined as either Gram positive or Gram negative cells based on whether the primary dye was retained or not. Gram-positive bacteria stain deep violet to blue and gram-negative bacteria stain pink to red.

4.5.3. Motility Test

Motility medium was prepared using a test tube. Broth culture 24 hrs old of the isolates were inoculated into pre-sterilized semisolid motility medium separately using straight needle and incubated at 35^0C for 24 hrs. If a bacterial isolate was able to grow out of the inoculation line through the medium, the cell is capable of motility. For non motile cells, growth occurs only along the inoculation line but no further extension (Shields and Cathcart, 2012).

4.5.4. Endospore Test

Endospore test was done according to Schaeffer and Fulton (1933). A bacterial smear was air dried and heat-fixed on a glass slide and flooded with malachite green stain solution and steamed for 5 minutes.The slides washed in tap water, counter stain with safranin for 30 seconds, re-rinsed in tap water and blot dried. The slide was rinsed under gently running tap water and counter stained with safranin for 30 seconds. The slide was re-rinsed in tap water and blot dried examined under the oil immersion lens (1,000X) for the presence of endospores. Endospores are bright green and vegetative cells are brownish red to pink.

4.6. Biochemical Test

4.6.1. KOH- test (test for Lipoplysaccharide)

Two drops of 3% KOH solution were placed on a clean microscopic slid. A colony was aseptically picked from the surface of nutrient agar using an inoculating loop and stirred in the KOH solution for 10 seconds to 2 minutes. The inoculating loop was raised slowly from the mass and when the KOH solution became viscous, the thread of slime followed the loop for 0.5 to 2cm or more in gram- negative bacteria. When a watery suspension that did not follow the inoculating loop observed, the reaction was considered negative and the isolate was reported as gram positive bacteria (Gregerson, 1978)

4.6.2. Catalase Test

Catalase Test was carried out By flooding overnight grown culture with a 3% solution of H_2O_2. The formation of bubbles indicate the presence of catalase(Mac faddin,1980)

4.6.3. Oxidation Fermentation Test

Two test tubes of Baird parker's medium were heated in boiling water for 10min to drive off the oxygen, then cooled and inoculated with discrete colony by inserting a straight wire vertically in to cool medium and then one tube was incubated aerobically and the second tube incubated anaerobically by sealing the surface with a layer of sterile liquid paraffin oil to create anaerobic conditions at 35-37°C for 72hr. Tubes were examined daily for color change. The results were considered as "Oxidation" when the aerobic test tubes turned yellow, and anaerobic tubes turned green (for gram negative rod). For gram positive cocci, yellow color in aerobic test tube, purple in anaerobic. The result is "Fermentation" if there are acid production for both gram positive cocci and gram negative i.e. yellow color throughout the medium (Health Protection Agency,2010)

4.7. Isolation *Salmonella and Shigella*

For detection of *Salmonella* spp and *shigella* spp 25 g of honey was added to 225 ml of sterilized buffered peptone water and incubated at 35^0C for 24 h. Then 1ml pre- enriched broth culture was added to 10 ml selenite cysteine broth and incubated at 35 ^{0}C for 24 h. Then after a loop full of suspension from culture broth was streaked on to Xylose-lysine Deoxycholate agar (XLD) followed by incubation at 35^0C for 24h.observation was made for presumptive *Salmonella* (black colony surrounding by red color) and *Shigella* colonies (BAM, 2001).

Triple Sugar Iron Agar (Oxoid)

The butt was stabbed and the slant was streaked and incubated at 37 ^{0}C for 24hrs to detect fermentation of glucose, sucrose and lactose as well as production of H_2S. The presence of alkaline (red) slant and acid (yellow) butt, with or without production of H_2S was considered presumptive for *salmonella* spp.

Lysine Iron Agar (Oxoid)

The butt was stabbed and the slant was streaked and incubated at 37 ^{0}C for 24hrs. Then, the production of an alkaline reaction (purple color) throughout the medium waspresumptive for *salmonella* spp.

Urea Agar(Oxoid)

The slant was streaked and the tube was incubated at 37 ^{0}C for 24hrs to assess the hydrolysis of urea. No color change was considered as negative and thus presumptive for *salmonella* spp.

Simmons Citrate Agar (Oxoid)

The slant was streaked and the tube was incubated at 37 ^{0}C for 24hrs to determine citrate utilization as a sole source of carbon. The presence of growth and color change from green to blue was considered as presumptive for *salmonella* spp.

Sulfide Indole Motility (SIM) Medium (Oxoid)

The SIM medium was stabbed to the bottom and incubated at 37 ^{0}C for 24hrs for the determination of H_2S production, indole production and motility. Production of indole was investigated by adding Kovac's reagent (HCl, 250ml, amyl alcohol, 750ml and paradimethylamino-benzaldehyde 50g/I) to growth in this culture medium. The non –utilization of indole and absence of deep red color at the surface of agar was considered as presumptive for *salmonella* spp.

4.8. Antimicrobial Activity of Honey

Three different species of bacteria were used in this study to explore the effectiveness of honey on the inhibition of growth. The bacteria chosen for this study were both Gram-Positive and Gram- Negative Bacteria. The three bacterial species used in this study, were *Escherichia coli, Salmonella typhimurium* and *Staphylococcus aureus*.

In this investigation Mueller-Hinnton agar and Nutrient Broth were used to culture the test strains. The Mueller-Hinnton agar was used to observe the zone of inhibition around sterile absorbent discs. The size of absorbent discs paper was 6mm. The nutrient broth was used in making liquid cultures from isolated colonies. The liquid culture was also used in the disc diffusion assay, the maximum recovery diluents were used to dilute the honeys to make up the serial dilutions (NCCLS, 1996)

Three test tube containing 9ml each of nutrient broth were inoculated separately with *Escherichia coli ATCC25922, Salmonella typhimuriumATCC13311* and *Staphylococcus aureus ATCC25923*using an inoculation loop. The nutrient broth solutions which were inoculated with *Escherichia coli, Salmonella typhimurium* and *Staphylococcus aureus* were incubated at 37˚C for 48 hours.

Honey was diluted using maximum recovery diluent (MRD), in which six dilutions were prepared. The concentration of each dilution was prepared using weight in grams of honey against the volume in cm^3 of MRD, grams/volume (g/vol.). The solvent was sterilized distilled water by using common bottles, the honey concentrations were prepared using the following measurements given below (Table 2) (Swapna, 2013).

Table .2 Honey Dilutions

Concentration%	Weight (gm) of Honey	Volume (cm3) of MRD
0	0	10
10	1	9
20	2	8
30	3	7
40	4	6
50	5	5

Where:MDR maximum recovery diluent

Each honey dilution was kept at room temperature under dark condition.

The Disc Diffusion Assay method was used to test for antimicrobial activity of honey. All the organisms used in the investigation were standard strains, the inoculated culture plates were incubated at the temperatures of 37^0c for 48 hours (Swapna, 2013)**.**

Thirty five sets of three Mueller-Hinnton agar plates were set out; each agar plate in every set was inoculated separately with the three test strains *Escherichia coli, Salmonella typhimurium* and *Staphylococcus aureus,* by pipetting 0.1ml of each culture directly onto the agar surface of each plate of every set. Using the poure plate technique, the bacterial cultures were then spread across the surface using cotton swap. The plates were left to dry for 15 minutes, whilst sterile absorbent paper discs size 6mm were placed into each honey flask aseptically. The absorbent discs were left in the honey for 15 minutes to absorb the honey. An absorbent disc from honey was placed on every pre-inoculated Mueller-Hinnton agar plate in each set. The plates were at 37˚C for up to 48 hours inoculated before detection of antimicrobial activity.

After the plates were incubated, inhibition of the bacterial strains was determined by the visual confirmation of a zone of inhibition. The 0 percent honey dilution for each honey, which contained only MRD as stated in (Table 2) is a negative control for inhibition. The degree of

inhibition was recorded by measuring the diameter of the zone of inhibition in millimetres (mm) using a transparent ruler. The measurement included the diameter of the absorbent disc.Zone of inhibition of the test samples on each bacteria strain was was expressed as mean ±SD in (mm).

4.9. Statistical Analysis

Each experiment was done in triplicate to get an average result and values were presented as mean ± SD. Data were organized and summarized using simple descriptive statistics and Microsoft office excel. Data analysis was computed using SPSS computer software (version 16).

5. Results

5.1. Socio-Demographic Characteristics

A total 35 people interviewed in this study, a significant number (68.6%) of the respondent were male (Table 3).According to demographic data information; significant number (42.9%) and 31.4% of the respondent were with an age group of 30-39 and 40-50 years respectively.

Table.3. Socio-demographic characteristics of respondents in the study area.

Characteristics		Number of respondent (N=35)	
		Frequency	Percent (%)
Sex	Male	24	68.6
	Female	11	31.4
Age	<20	1	2.8
	20-29	5	14.3
	30-39	15	42.9
	4o-49	11	31.4
	≥50	3	8.6
Religion	Muslim	_	_
	Orthodox	29	82.9
	Protestant	6	17.1
Marital status	Married	29	82.9
	Single	3	8.6
	Divorced	2	5.7
	Widowed:	1	2.8
Residence	Urban	2	5.7
	Rural	33	94.3
Academic status	Illiterate	23	65.7
	Grade 1-4	7	20
	Grade 5 -8	3	8.6
	Grade9-12	2	5.7
	Above Grade 12	_	_
Occupation	Agriculture/farming	31	88.6
	Trade	4	11.4
	Civil servant	_	_
Climatic condition Of honey area?	Dega	3	8.6
	Weyenadega	11	31.4
	Kola	21	60

Within the respect of the educational status, 65.7%, 20%, 8.6%, 5.7%, 0% of the respondent Illiterate ,Grade 1-4 , Grade 5 -8,Grade9-12, Above Grade 12 respectively (**Table 3).** .
All of the respondents lived in Rural area (Table 3). Occupationally 88.6% and11.4% was farmers and Business man respectively. According to demographic data information 60% of honeys were harvested from Kola and 31.4% were harvested from Weyeneadega (Table 3).

5.1.1. Description of Honey Samples

Most (82.9%) of the respondent harvested honey two times per year and all (100%) were harvested more honey in the season of during autumn (Table 4). Most (77.2%) of the respondent stored honey in plastic jar and the other 17.1%, 5.7% stored the same in Kettle and clay pot respectively. The period of storage of honey before bringing it to the open market varied and the respondents stored it for one month (60%), two weeks (31.4%), and two months (8.6%), respectively. Most respondents (74.3%) believed that the shelf life of honey was two years. All the respondents had used honey for medicinal purpose, especially for treatment of asthma (100%), wound healing (85.7%) and diarrhea (100%).

Table .4 Descriptions of Honey Samples of Kuyu woreda, North Ethiopia2013/14

Characteristics		Number of respondent (N=35)	
		Frequency	Percent (%)
No of harvest honey per Year	▪ One	6	17.1
	▪ two	29	82.9
	▪ three	_	_
Season when more honey is harvested	▪ Spring	_	_
	▪ Autumn	35	100
	▪ Winter	_	_
	▪ Summer	_	_
Material used for storage of honey	▪ clay pot	2	5.7
	▪ plastic jar	27	77.2
	▪ "Qanqaloo"	-	_
	▪ kettle	6	17.1
Duration of Storage of honey in container	▪ Two weeks	11	31.4
	▪ One month	21	60
	▪ Two months	3	8.6
	▪ Above three months	_	_
Shelf life of honey	▪ Less than one year	_	_
	▪ Two years	26	74.3
	▪ Three years	9	25.7
	▪ More than three years	_	_
Uses of honey in your locality	▪ Medicinal	33	94.3
	▪ Food	2	5.7
	▪ Source of income	_	_
Is honey cured athsma?	▪ Yes	35	100
	▪ No	_	_
Is honey used for wound healing	▪ Yes	35	100
	▪ No	_	-
Does honey used to cured diarrhea	▪ Yes	35	100
	▪ No	_	_
Is honey cured skin disease?	▪ Yes	30	85.7
	▪ No	5	14.3

5.2. Moisture, pH and Ash contents of honey samples

The mean value of moisture content was 16.78% ± 1.37 ranging between 14.2% - 19% (**Table 5**). The pH ranged from 3.81 - 4.34, with mean value of 4.10±0.13. The minimum and maximum ash (%) of the honey samples were 0.46%-1.23%, respectively, with mean values of 0.84%± 0.21(**Table 5**).

Table.5. Moisture (%), pH and Ash (%) contents of the studied honey samples. The values were means±standard deviation.

Parameters	Mean± SD	Minimum	Maximum
Moisture%	16.78 ± 1.37	14.2%	19%
pH	4.1 ±0.13	3.81	4.34
Ash%	0.84 ± 0.21	0.46%	1.23%

5.2.1. Heavy Metals in Honey

The level of Zinc and Lead in the honey samples the minimum and the maximum values of 0.01 and 0.27(mg/L), for lead and 0.36 and 0.59 for zinc, respectively. The overall mean concentrations of lead and zinc were 0.08±0.04 and 0.45 ±0.06, respectively (Table 6). These values are relatively higher when compared with the amount of both heavy metals in the blank (distilled water) with concentrations of 010 and 0.012 Pb & Zn, respectively.

Table 6 Concentration of some heavy metals in honey samples (n-35), North Ethiopia2013/14.

Values are mean±SD

Lead and Zinc in 35 honey Samples				
Samples	Element	Con (Mg/l) Mean ± S.D	Minimum	Maximum
Distilled water	Zn	0.012		
	Pb	0.010		
Honey	Zn	0.45 ±0.06	0.36	0.59
	Pb	0.08±0.04	0.01	0.27

5.3. Microbial Counts and Dominant isolates.

Aerobic Mesophilic bacteria were isolated from all samples of honey. The number of Aerobic mesophilic bacteria (AMB) ranged between 3.94 log CFU/ml -5.95 log CFU/ml with mean counts of 5.00 ± 0.55 logCFU/ml (Table 7). *Salmonella sp*, *Shigella* spp, *Lactic acid bacteria*, *Enterobacteriaceae* and coliform were not detected. All samples contained below detectable levels of yeasts and moulds, with mean yeasts and moulds counts of 1.85±0.32 log cfu/ml and 1.16 ±0.21 log cfu/ml respectively(Table 7).

Table.7 Mean Microbial Counts (logCFU/ml) of honey samples, North Ethiopia, 2013/14.

	Mean count (log CFU/ml				
Microbial groups	Sample size	Mean±SD	Minimum	Maximum	Cv(%)
AMB	35	5.00±0.55	3.94	5.95	11.41
Yeast	35	1.85±0.32	1.04	2.85	3.12
Moulds	35	1.16±0.21	1.00	1.70	2.62

Where: AMB=Aerobic Mesophilic bacteria.

Of the total 40 isolates, most of the isolates (90%) were Gram positive, which included *Bacillus* spp, *Staphylococci* and *Streptococci* (Table 8). Of (90%) Gram positive, 30% were revealed the presence of endospore and were rod shape (Table 8). Based on colony arrangements, 70% were cocci, of that 50% were cluster cocci (*Staphylococci*) and 20% were straight chain (*Streptococci*). Biochemically, the majorities (80%) of the isolates were showed catalase positive (*Staphylococci*) and 20% were catalase negative(*Streptococci)*. The KOH testes confirmed that the majority of the isolates (90%) were No thread slime i.e. gram positive bacteria (Table 8)

Table 8 Dominant bacteria in honey samples (n-35), Garba guracha town, N/Ethiopia2013/14

No isolate	Morphological characteristics			Biochemical test				Isolates possible identity
No of colony	Shape	Colony	Endospore	Gram rxn	Moti-lity	catalase	O/F test	
2o	cocci	cluster	Negative	positive	none	positive	oxidative	*Staphylococcus* spp
8	cocci	straight	Negative	positive	None	negative	oxidative	*Streptococci* spp
12	rod	single bacillus	Positive	positive	motile	positive	oxidative	*Bacillus* spp

5.4. Antimicrobial Activity of Honey

As shown in Table 9, concentrations of honey up to 50%, had antibacterial activity against the gram negative bacteria *Escherichia coli* and *S. typhimurium* but none of the honey samples showed no antibacterial activity against that of the gram positive *Staphylococcus aureus.* The maximum and the minimum diameter of the zones of Inhibition showed against *S. typhimurium* were 16.11 ±0.70 mm and 9.03 ± 0.94mm respectively with concentrations of honey at 50% and 10% of honey concentration. The maximum and the minimum zones of Inhibition for *Escherichia coli* were (13.60 ± 2.1) and (8.08 ±0.64) respectively. Accordingly, the highest mean diameter of inhibition 16.11+ 0.7 was observed at concentration of 50% (Table 9).

Table .9 Inhibition zone diameter (mm) of different concentration of honey on selected tested strains. North shewa Ethiopia 2013/14.The values are means of replicates ± Standard deviation (Mean± S.D)**.**

Zone Inhibition(mm) against tested strains (Mean±S.D)			
Honey Conc.%	*E. coli* ATCC25922	*S. typhimurium* ATCC13311	*S. aureus* ATCC25923
0	NA	NA	NA
10	8.08 ± 0.64	9.03 ± 0.94	NA
20	9.49 ± 0.80	11.08 ±0.99	NA
30	10.51 ± 1.80	12.91 ±0.90	NA
40	11.93 ± 1.98	14..57 ±0.76	NA
50	13.60 ± 2.1	16.11 ±0.70	NA

Where: NA, Not have activity on *S. aureus*

6. DISCUSSION

Micro-organisms in honey may influence the stability of the product and its hygienic quality (Snowdon and Cliver, 1996). However, due to the natural properties of honey and control measures in the honey industry, honey is a product with minimal types and levels of microbes. Besides, the use of honey as a traditional remedy for microbial infections dates back to ancient times (Molan, 1992). Nevertheless, the microbial safety of honey could vary from place to place based on the hygienic practice exercised during its storage and handling. Accordingly, honey samples collected from open market of Garba Guracha Town, Kuyu woreda, were evaluated for microbiological safety and selected physicochemical characteristics.

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 level, the higher the probability that honey will get fermented upon storage as it may serve as a substrate for the growth of microorganisms. Accordingly, USDA (1985) set the standards for moisture content in honey: $\leq$ 18.6% as good, $\leq$ 20% as reasonably good and >20% as bad. In the current study, the mean moisture contents of the studied honey samples was 16.78% $\pm$ 1.37 which range between 14.2% and 19%. Thus, the mean value obtained for moisture content in this study fell within the ranges recommended as 'good quality" honey ($\leq$ 18.6%) by USDA (1985), which is a good attribute for the studied honey samples. In agreement with our observation, Germany, Belgium Australia, Italy and Spain considered maximum moisture content of honey to be 17.5-18.5 % which is reasonable quality level. But, according to the Commission of Food Codex, moisture content valid for worldwide honey trade is 21% (Ali and Myrnzamy, 2002).

The acidic pH of honey is a good attribute as it promotes healing wound by causing oxygen release from hemoglobin (Finola *et al*., 2007). In addition, this acidic pH of honey also prevents the growth of many species of bacteria. The values obtained for pH in the current study ranged between 3.81 to 4.34, with mean values of 4.10 $\pm$0.13. In addition, on the basis of their pH, the

honey samples could be classified as Honeydew and Honey Blossom. Honeydew honeys tend to have higher pH values ranging from 4.5 to 6.5 because of their higher minerals, while the pH of Blossom honeys varies from 3.3 to 4.6 (Chirife *et al*., 2006). So, the values we obtained for pH in this study could be classified under Bloosom Honey. These observations are in agreement with those reports made by Codex Almentariou Commission (2001) where acceptable ranges of pH of honey were set between 3.2 and 4.5. The results of this study are also in agreement with observation of Hussain (1989) who reported pH values ranged between 3.0 - 5.0 in pure honey. Those variations in pH ranges are mainly due to the variation in level of minerals present in the honey. Likewise, the floral difference may also contribute to the variation in the observed pH ranges.

The ash contents of honey samples in the current study fell within the range of 0.46%-1.23 % and the mean value was 0.84 ±0.21%. Williams *et al* (2009) who worked on different varieties of honey had obtained ash content in the range of 0.020 - 1.028%. The variation may be due to many factors such as soil conditions, atmospheric conditions and physiology of each plant.**.** The percent ash content is a reflection of the total inorganic minerals that are present in a sample (AOAC, 2000). The ash content of the samples investigated in this study was in the acceptable range (0.02 - 1.028%) in reference to earlier report Willams *et al*. (2009).

The mean values of Pb and Zn (mg/l) in the studied honey samples were 0.08±0.04 and 0.45 ±0.0 6, respectively. Heavy metal contamination and trace element composition of honey could also be the result of inappropriate actions during processing and storage steps. As a matter of fact, the acidic property of honey could lead to release of heavy metals from metallic tools and containers (Pisani *et al*., 2008). According to this report, average concentrations of Se, Cu, Cd, Pb, As and Mn in honey are less than 0.5 mg/kg. The lowest level was found for lead, at a concentration of 0.11 mg/kg (Ahmed, 1999). During the current study, emphasis was given to the detection of two of the heavy metals, namely Pb and Zn. The obtained results were in agreement

with the warning that only very little metal pollution need enter honey (< 0.5 mg/l for Pb concentration and <1mg/l for Zn (RDA, 1980).

High concentration of these and related heavy metals have deleterious health effect. Lead is a poisonous metal that can damage nervous connections (especially in young children) and cause blood and brain disorders. Lead poisoning typically results from ingestion of food or water contaminated with lead; but may also occur after accidental ingestion of contaminated soil, dust, or lead based paint **(**Aksenova, 2000; Beljaeva, 2000). Furthermore, long-term exposure to lead or its salts (especially soluble salts or the strong oxidant PbO_2) can cause nephropathy, and colic-like abdominal pains. The effects of lead are the same whether it enters the body through breathing or swallowing. Lead can affect almost every organ and system in the body. The main target for lead toxicity is the nervous system, both in adults and children. Long-term exposure of adults can result in decreased performance in some tests that measure functions of the nervous system (Smolders, 2004).

In our study the counts of aerobic mesophilic bacteria (AMB) varied between 3.94 log CFU/g - 5.95 log CFU/g with mean values of 5.00 ± 0.55 log CFU/g in the 35 samples. According to published data, the total aerobic viable counts for honeys can range from 0 to several thousand per gram. This variation in bacterial counts may be due to the type of sample analyzed (raw, finished or trade), the freshness of the honey, the time of harvest and the analytical techniques used (Snowdon and Cliver, 1996). Nakano and Sakaguchi (1991) tested 70 honey samples from retail outlets in Japan, and recorded a mean aerobic viable count of 83 cfu/g. From 175 samples of commercial honey from different geographical regions of France, Tysset and Rousseau (1981) found a mean viable counts o 227 cfu/g, with values that varied from 3 to 9500 cfu/g.

There are few reports that quantify the levels of moulds and yeasts in honey. Among 50 Italian honeys samples investigated, moulds were found at levels ranging from 1 to 43 cfu/g (Piana *et al*., 1991). Likewise, in 175 honey samples collected from France, moulds and yeasts count varied from 0 to 2500 cfu/g, with the mean count of 90 cfu/g (Tysset and Rousseau , 1981).

Nakano and Sakaguchi (1991) reported mean yeast counts of 9 cfu/g from 70 retail honey samples, which varied from 0 to 300 cfu/g. In our study, yeast and molds were detected at very low counts in the analyzed honey samples with mean yeast and mold counts of 1.85±0.32 log cfu/ml and 1.16 ±0.21 log cfu/ml, respectively**.** Rall *et al.* (2003) encountered an incidence of 64% of mould and yeasts in industrial and domestic production honeys with counts that ranged from nil to $1.5x10^5$ cfu/g. Enumeration of moulds and yeasts provides information on the quality of honeys as well as shelf life and spoilage potential as a high yeast count, increased moisture, moderate temperatures and granulation encourages fermentation (Snowdon and Cliver, 1996).

Detection of bacterial in honey was reported to be associated with bees including *Bacillus, Clostridium, E. coli, Enterobacter, Klebsiella, Proteus*, etc., and many of these have been found in honey. Studies on survival of some *Salmonella* species or other vegetative pathogenic organisms, which are not normally present in honey, had been reported (Snowdon and Cliver, 1996). To the contrary, in each of 35 honey samples of our study *Salmonella sp*, *Shigella* spp, *Lactic acid bacteria*, and *Enterobacteriaceae* were not detected.

Of the total 40 characterized colonies of pure culures, most of the them (90%) were Gram positive, of which about 30% had endospore and Rod shaped indicating the presence of *Bacillus* spp . Based on colony arrangements, 70% were coccoid, of which 50% were clustered and 20% were straight chained which indicated the presence of *Staphylococcus* spp and *Streptococcus* spp, respectively. The presence of the above microorganism in honey samples could be due to poor general sanitation of the seller and/or contamination from the environment either during transportation and/or storage. On the other hand, the presence of *Staphylococci* could be an indicator for poor handling and contact with bare hand. Existence of aerobic bacterial spore count revealed the contamination of honey with spore forming bacteria such as *Bacillus spp.* Some of the bacteria most commonly found in the beehive environment and honey are Actinomyces, *Bacillus, Clostridium, Corynebacterium, Pseudomonas, Micrococcus* and

many species of yeasts and fungi (Snowdon and Cliver, 1996). In honey samples collected from local markets in Romania (Popa *et al.,*2009) reported the presence of *Bacillus* sp.

All honeys are non-sterile with a natural bacterial flora (total viable count ranging between 0 and 5000 CFU/g) mainly composed of Gram-positive sporing bacteria, such as *Bacillus* spp. which accounts for an average of 60% of bacteria recovered, depending on the level of processing of honey (Snowdon and Cliver, 1996). Earlier studies on qualitative examination of the organisms recovered on viable count plates from 12 samples of French honey (Iurlina and Fritz*,* 2005) showed the presence of microorganisms such as *Bacillus, Brevibacterium, Enterobacter*, *Micrococcus* and *Pseudomonas* with the most common isolates being *B. cereus* and *B. pumilus*. On the other hand, Piana *et al*. (1991) reported *B. cereus* spores in 24 of 50 samples tested. Most of the spores belonged to the genus *Bacillus, B. cereus* being the predominant species. Most microbes found in honey are usually not dangerous for the consumer's health. However, normal honey must lack pathogenic micro-organisms that produce enteric illnesses (Tchoumboue *et al*.,2007). In general, microbes of concern commonly found in honey are yeasts and spore-forming bacteria. Honey also support microorganisms like coliforms and those that under certain conditions could cause human illness although coliforms were not detected in the current study. The primary sources of microbial contamination are likely to include pollen, the digestive tracts of honey bees, dust, air, earth and nectar, sources which are very difficult to control. The post harvest sources of microorganisms that influence any food product are also sources of contamination for honey because it is a ready-to-eat food. These include air, traditional way of handling, processing starting from harvesting process to consumption, materials used, storage condition, and cross contamination (Natea and Woyessa, 2014)

Honeys have long been recognized for their antimicrobial activity against bacteria, moulds and yeasts with unique properties that render it bacteriostatic and bactericidal. The high osmotic pressure, low water activity, low pH, low redox potential, hydrogen peroxide and other phytochemical factors might contribute to the antimicrobial nature of honey. Their relative importance's depend on the sensitivity of the species and the level of additional factors in any

honey (Molan, 1992). The disc diffusion method is mainly a qualitative test for detection of the susceptibility of bacteria to antimicrobial substances; however, the MIC reflects the quantity needed for bacterial inhibition. In general, the results of the current study on anti microbial activities of honey show that honey exhibited certain levels of antibacterial activity which generally increased with increase in concentration. The MIC of honey samples was found to be 1g/9ml for both *E. coli* and *S. typhimurium***.** However, the degree of antibacterial activity varied based on the type of bacteria and geographical area of honey. Accordingly, MIC values have been observed to lie between 10% and 20% concentration against *S. typhmurium and E. coli* tested in this analysis. These results show that with concentrations of honey up to 50%, the honey samples have antibacterial activity against the gram negative bacteria *Escherichia coli* and *S. typhimurium* but each 35 honey samples showed no antibacterial activity against of the gram positive *Staphylococcus aureus* up to 50%concentration of honey. The maximum and the minimum zones of Inhibition against *S. typhimurium* were 16.11± 0.70 and 9.03±0.94, respectively. Likewise, at 50% and 10% concentration of honey the maximum and the minimum zones of Inhibition for *Escherichia coli* were 13.60±2.1 and 8.08±0.64, respectively. These results are in agreement with earlier report by Ali ATM *et al* (1991) who observed that honey with concentration of 20 % was sufficient to inhibit the growth of a range of isolates. The expected range of the MIC value was between 10- 50 % as shown by Barret *et al. (*2006). On the other hand , MIC of honey between 5%-10 % has been observed (Al-Waili, 2004).

The sensitivity of the microorganism itself plays an important role in its susceptibility to honey as an antibacterial agent, i.e. isolates of *Staphylococcus aureus* were more resistant than *E. coli* and *S. typhimurium*. Molan (1996) has reported difference in the relative sensitivity of various species to antimicrobials depending on the virulence of the isolated microorganism. In general, the microbial quality and antimicrobial activities of honey appear to vary depending on the intrinsic and extrinsic parameters of the source sample.

7. CONCLUSION

Microbiologically, the honey samples were dominated by aerobic spore formers and few non-spore forming gram positive cocci, including Staphylococci and some streptococci. However, the numbers of aerobic mesophilic bacteria, moulds and yeasts in the honey samples were relatively very low. On the other hand, the presence of staphylococci could be indicators for poor handling and contact with bare hand. Existence of aerobic bacterial spore revealed the contamination of honey with spore forming bacteria such as *Bacillus* in adverse condition

- The level of two of the heavy metals, namely zinc and lead, were within the tolerable range.
- The pH and moisture contents of honey samples were not optimum to support growth of most microorganisms, including lactic acid bacteria and coliforms. Even the counts of yeasts, molds, and aerobic mesophilic bacteria were relatively low despite the presence.
- The antimicrobial activity of honey increases with increase in concentration, except for *Staphylococcus* spp. The higher the concentration of honey the greater its usefulness as an antibacterial agent. It is also evident that the different species of bacteria differ in their susceptibility to antibacterial activity of honey
- The use of honey for would healing purpose is wel understood in the study area

8. RECOMMENDATIONS

Based on the findings of this study the following recommendations are made:-

- Antimicrobial activities of honey against other foodborne pathogens of interest need to be done to have clear picture of activities of honey, in general.
- The *in vitro* study proved that honey has antibacterial activity. This calls for further study on antimicrobial activity of honey samples *in vivo,* using animal models.
- Consideration should also be given to the way that honey is processed if it is intended for sale as an antibacterial product.
- Different spices are known to have antimicrobial activities. The synergistic effect of honey mixed with these different spices need to be evaluated for better application of honey for therapeutic purpose.
- The need for further study on physico-chemical, microbiological and antimicrobial activity of honey collected from different agro- ecological region of Ethiopia.

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APPENDICES

Appendix 1. Questionnaires designed to gather information about honey.

JIMMA UNIVERSITY

POST GRADUATE STUDIES, DEPARTMENT OF BIOLOGY.

QUESTIONNIRE INTERVIEW TO COLLECT DATA ON HONEY SAMPLES; KUYU WOREDA G/GURACHA TOWN

Interview designed for Physio-chemical and anti microbial activities of honey in Garba Guracha, North Shewa Ethiopia.

Part I. Background Information of Respondents

1. Woreda_____
2. Kebele_____
3. Sex Male:_____ Female: _____
4. Age: _____
5. Religion Muslim: _____ Orthodox:_____ Protestant:_____ Other:_____
6. Marital status:
    a. Married: _____ Single: _____ Divorced: _____ Widowed: _____
7. Academic status:

- Illiterate: _____
- Educated: Grade 5 or below: _____
- Grade 5 to 10:_____
- Preparatory: _____
- Diploma: _____
- Bachelor: _____

8. Occupation/Economic activity:

- Agriculture/farming: _____
- Trade: _____

- Civil servant: _____

II. Part two:

1. How many times harvest honey per year?

A. One B. two C. three D. four

2. Climatic condition of your area?

A. Dega B .Weyenadega C.Kola

3. In which season more harvest honey?

A. Spring B. Autumn C. Winter D. Summer

4. After harvesting honey which material used for storage?

A. clay pot B. plastic jar C. Goat skin balloon D. kettle

5. For what period do you store honey in material under question no. 3

A. Two weeks B. One month C. Two month D. Three & above

6. What is the shelf life of honey?

A. less than one year B. two years C. three years D. more than five years

7. The weather condition of this honey harvested

A. Kola B. Weyenadega C. Dega

8. For what purpose Use honey in your locality?

A. medicinal B. food C. source of income

9. If your answer Question No 6 is for **"medicinal value"** honey cure what type of disease in your locality?

_____

_____

_____

_____

_____

10. Mention some vegetation that found in your locality?

_____

_____

_____

_____

_____

11. Which plant you expected that honey bee used to make honey?

_____

_____

_____

_____

12. Local name of honey?

_____

THANK YOU VERY MUCH FOR YOUR UNDESERVED COLLABORATION IN TO THE QUESTIONNARES

Appendix 2 Heavy Metals in Honey studied.

Samples	Pb Conc (mg/L)	Zn Conc (mg/L)
(Blank)Distilled H_2O	0.0105	0.01241
1	0.0788	0.4193
2	0.0685	0.3922
3	0.1102	0.4484
4	0.0341	0.3658
5	0.0411	0.4142
6	0.0652	0.466
7	0.1011	0.4318
8	0.0989	0.3818
9	0.1103	0.4159
10	0.1045	0.5167
11	0.0324	0.4148
12	0.07549	0.488
13	0.0099	0.4012
14	0.0437	0.38
15	0.0991	0.416
16	0.0668	0.5613
17	0.1311	0.4193
18	0.0252	0.5756
19	0.0831	0.4689
20	0.0746	0.4474
21	0.0911	0.5658
22	0.1118	0.419
23	0.1216	0.5922
24	0.0173	0.4459
25	0.0532	0.3658
26	0.2671	0.476
27	0.0785	0.5432
28	0.1018	0.4321
29	0.1021	0.3868
30	0.0361	0.4195
31	0.0793	0.4317
32	0.0268	0.5779
33	0.0666	0.5876

34	0.1063	0.4651
35	0.1198	0.4639
Mean	0.08	0.45

Appendix 3 physico-chemical of honey samples

Samples	Moisture contents %	pH	Ash%
1	17	3.91	0.88
2	18	4.13	0.81
3	17	3.98	0.66
4	18.3	4.04	0.48
5	18.1	4.24	1.01
6	17.6	4.04	0.99
7	16	4.06	1.07
8	18.3	4.22	1.23
9	19	4.34	0.52
10	18.6	4.11	0.6
11	17	4.3	0.66
12	17.2	4.19	1.01
13	17.6	4.27	0.98
14	15.1	3.92	1.02
15	16.2	4.18	0.49
16	15	4.24	0.94
17	19	4.26	0.66
18	15	4.2	0.74
19	15.2	3.97	0.83
20	16	4.25	1.02
21	16	4.09	1.12
22	17.4	4.23	0.58
23	15.5	4.09	1.03
24	14.8	4	1.01
25	14.6	3.88	1.02
26	18	4.05	0.67
27	16.5	4.2	0.46

28	16	4.15	0.48
29	18	4.01	1.04
30	19	4.04	0.86
31	14.2	3.94	0.79
32	15	4.03	0.92
33	17.4	4.07	0.98
34	17	4.28	0.9
35	16.6	3.81	1
Mean	16.78	4.10	0.84

Appendix 4.The major honey plant in kuyu woreda distinct

No	Local name	*Scientific name*
1	*Laaftoo*	*Acacia abyssinica*
2	*Waddeessa*	*Cordiana Africana*
3	*Nuugii*	*Guizotia Abyssinica*
4	*Bosobila*	*Ocimum basilicum*
5	*Roosa*	*Rosa abyssinica*
6	*Mishingaa*	*Sorghum bicolor*
7	*Boqollo*	*Zea mays*
8	*Baaqela*	*Vicia faba*
9	*Atara*	*Pisum sativa*
10	*Bargamoo*	*Eucalayptus globulus*
11	*Bakkanisa*	*Croton macrostachys*
12	*Siddisa*	*Trifoliumsteudneri/accaule*
13	*Eebicha*	*Vernonia spp*
14	*Hagamsa*	*Carissa edulis*
15	*Baddeessaa*	*Syzygium guineense*
16	*Paappayyaa*	*Carica papaya*
17	*Shukkurtii*	*Allium cepa*
18	*Talbaa*	*Linum vsitatissiumum*
19	*Saardoo*	*Eleusine floccifolia*
20	*Barbarree*	*Capsicum annuum*
21	*Missira*	*Lens culiaris*
22	*Ejersa*	*Olea Africana*
23	*Gesho*	*Rhamnus prinoides*
24	*Dhumuga*	*Justitia schimperina*
25	*Koshim*	*Dovalis abssinicus*

APPENDIX 5 Table 8 Dominant bacteria in honey samples (n-35), Garba guracha town, N/Ethiopia2013/14

Morphological and Biochemical tests			NO of colonies	Percent %
Morphological features	Shape	Cocci	28	70
		Rods	12	30
	Colony arrangements	Single bacillus	8	20
		Diplo bacillus	4	10
		Clusters	20	50
		Straight chains	8	20
	Endospore test	Positive	12	30
		Negative	28	70
	Gram reactions	Positive	36	90
		Negative	4	10
	Motility test	Positive	18	40
		Negative	22	60
Biochemical test	Catalase test	Positive	32	80
		Negative	8	20
	KOH test	NO Slime(gram positive bacteria)	36	90
		Thread of slime(gram negative bacteria	4	10
	O/F test	Oxidative	37	92.5
		Fermentative	3	7.5