EVALUATING THE ANTIBACTERIAL EFFICACY OF HONEY AND LEMON JUICE AGAINST BACTERIA ISOLATES FROM RESPIRATORY TRACT INFECTIONS

BY

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ABSTRACT

The antibacterial effect of honey and lemon were investigated. Their use separately and in combination were compared with that of standard antibiotics against *Klebsiellapneumoniae*, *Staphylococcusaureus*, *Streptococcus pneumoniae*, *Pseudomonas aeruginosa*, *Haemophilusinfluenzae* and *Streptococcus pyogenes*isolates from sputum, ear swab, nasal secretion and throat swab samples of patients from Ahmadu Bello University Teaching Hospital (ABUTH), Zaria, and Ahmadu Bello University Health Services (ABUHS) Zaria, Nigeria. Agar diffusion and broth dilution methods were employed to assess degree of susceptibility of the isolates to honey and/or lemon, and the standard antibiotic formulations. Minimum Inhibitory Concentrations and Minimum Bactericidal Concentrations were evaluated. Rate of Kill was carried out to know the death/survival rate of the bacterial isolates after exposure to the agents. Test for synergy was also carried out to know if there is synergistic effect between the honey and the lemon juice. The zones of inhibition (mm) of 13-32 (Ceftriaxone), 07-29 (Gentamicin), 07-35 (Amoxicillin-Clavulanic acid), 14-33 (Levofloxacin), 07-30 (Azithromycin), 10-34 (Honey), 12-29 (Lemon), and 18-29 (Honey/Lemon mixture) were obtained. Minimum Inhibitory Concentrations range between 1.95-125 µg/ml (Ceftriaxone), 1.56-100 µg/ml (Gentamicin), 3.91-125 µg/ml (Amoxicillin-Clavulanic acid), 0.98-62.5 µg/ml (Levofloxacin), 0.78-100 µg/ml (Azithromycin), 20-75 µg/ml (Honey), 15.0 - 47.5 µg/ml (Lemon), and 15-50 µg/ml (Honey/Lemon mixture). Excellent antibacterial activity was observed with lemon, honey and lemon mixture, Levofloxacin, Ceftriaxone and Gentamicin, while honey, Amoxicillin-Clavulanic acid and Azithromycinshowed less antibacterial activity. Better killing of the bacteria isolates on exposure to honey and lemon was observed with Lemon and Honey/Lemon mixture than the Honey alone. There is additive effect, but no antagonistic effectin the mixture of honey and lemon juice. Generally, their activity is within the additive level (>0.5 to ≤1) of Fractional

Inhibitory Concentration index. This study justifies the use of Honey and Lemon separately and in mixture as an alternative medicine by the populace in the treatment of respiratory tract infections.

Key words: Honey, Lemon, Standard antibiotics, Synergistic effect, Antibacterial activity, zone of inhibition.

CHAPTER ONE

1.0 INTRODUCTION

Honey is a sweet food made by bees using nectar from flowers. The variety produced by honey bees (the genus *Apis*) is the one most commonly referred to and is the type of honey collected by beekeepers and consumed by humans. The various species of *Apis* include; *Apis andreniformis*, *Apis florea*, *Apis dorsata*, *Apis cerana*, *Apis koschevnikovi*, *Apis mellifera*, *Apis nigrocincta* (Silveira *et al.,* 2002).

Honey bees convert nectar into honey by a process of regurgitation and store it as primary food source in wax honeycombs inside the beehive. The sweet taste of honey is as a result of the monosaccharides; fructose and glucose and has approximately the same relative sweetness as that of granulated sugar (National Honey Board, 2010). It has attractive chemical properties for baking, and a distinctive flavor that leads some people to prefer it over sugar and other sweeteners. Most microorganisms do not grow in honey because of its low water activity of 0.6 (Molan, 1992c).

Hydrogen peroxide (H_2O_2), methylglyoxal (MGO), bee defensin, pH and osmotic effect of honey are known to be responsible for the antimicrobial effects (Mandal, 2011).

Lemon fruit is an inexpensive, easily available citrus fruit, popular for its culinary and medicinal uses (NPCS, 2012). The Lemon fruit juice consists of about 5% citric acid that gives a sour (tarty) taste to the lemon. It is a rich source of vitamin C (Andrew, 2010; NPCS, 2012).

Lemon has been classified scientifically into:

Kingdom: *Plantae*;

Order: *Sapindales*;

Family: *Rutaceae*;

Genus: *Citrus*;

Species: *limon*; and

Binomial name: *Citrus limon*.

The different varieties of lemon are Bush lemon, Eureka, Lisbon, Meyer, Ponderosa, and Variegated pink. However, the species used in this work was the Eureka species as identified in the Herbarium section of Biological Science, Ahmadu Bello University, Zaria.

Eureka fruit has a markedly ribbed surface. The fruit color is yellow at maturity. It is a sour lemon variety and usually has fewer seeds. It is one of the two most popular sour lemons in the world (Lisbon lemon is the other one) (NPCS, 2012). Lisbon lemons contain more seeds than Eureka lemons which are nearly seedless. The pulp of a Eureka lemon is greenish-yellow. There are two kinds of Eureka lemon fruits: the pink Eureka variegated lemon fruit and the plain yellow.





Plate I_a: Eureka Lemon Fruits

Plate I_b: Eureka Lemon Fruits.

(Moon Growers, 2010).

1.1 Background of the Study

The respiratory tract extends from the larynx to the nostrils and comprises of oropharynx and the nasopharynx together with the communicating cavities, the sinuses, the middle ear and extends to the lungs. The respiratory tract is the part of the anatomy involved with the process of respiration (Jeremy *et al*., 2006). The respiratory tract is divided into the upper and the lower respiratory tract. The upper respiratory tract is generally considered to be the airway above the glottis or vocal cords. This includes the nose, sinuses, pharynx, and larynx (Sabyasachi, 2008). Whereas, the lower respiratory tract consists of the trachea (wind pipe), bronchial tubes, the bronchioles, and the lungs (Venkat, 2013). Infections involving this tract are referred to as respiratory tract infections (Eccles *et al*., 2007). Several parts of the respiratory tract may be affected by the infection giving rise to several conditions including pharyngitis, tonsillitis, and resulting to a "sore throat", nasopharyngitis, otitis media, sinusitis, epiglottitis, bronchitis, pneumonia, etc. Infection of the respiratory tract is one of the most common illness in the general population and results in significant morbidity, which may account for missed days of work and school, it also contribute to mortality.

The respiratory tract infections range from pneumonia (*caused by Klebsiella pneumoniae*, *Haemophilus influenzae*, *Streptococcus pneumoniae*), ear infection-(otitis media), conjunctivitis, epiglottis (caused by *Haemophilus influenza* and *Pseudomonas aeruginosa*), tonsillitis, pharyngitis, laryngitis, sinusitis, and common cold (caused by *Staphylococcus aureus*, *Streptococcus pyogene*, *Haemophilus influenzae*, and/or *Pseudomonas aeruginosa*) which account for number of death in patients especially the immune-compromised, despite improvement in antimicrobial therapy (Stephen *et al.,* 2004*)*. Up to 15% of acute pharyngitis cases may be caused by bacteria, commonly *group A Streptococcus* in *Streptococcal pharyngitis* (Strept-Throat) (Jeremy *et al.,* 2006).

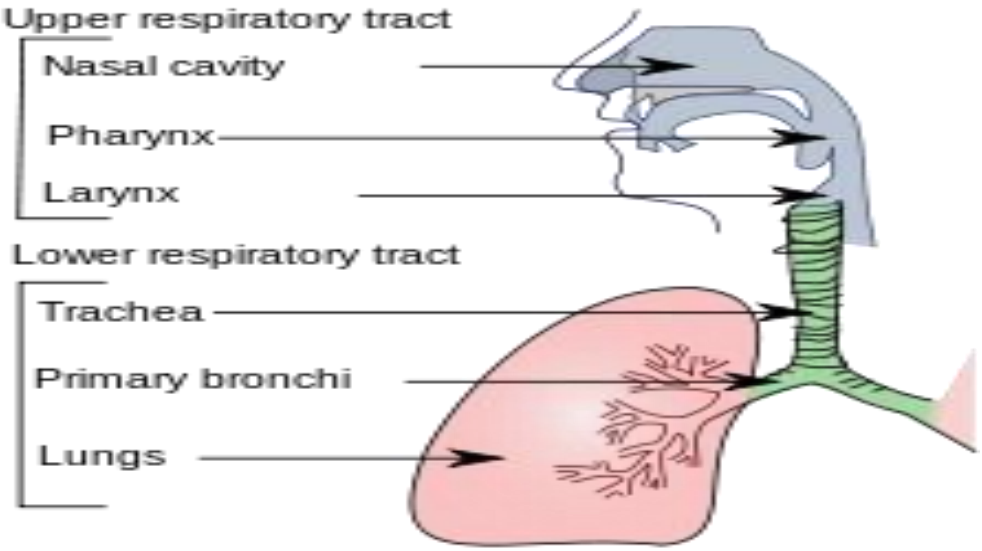


Figure 1.1: The Respiratory Tract (Jalanpalma, 2010).

1.2 Statement of Research Problem

Respiratory tract infection is the most common infectious illness in the general population. It is the leading reasons for people missing work, meeting, school, etc.

Solutions has been found for many infectious illness, but common cold and other respiratory tract infections has been (and is still) a problem to man.

Antimicrobial resistance is a natural phenomenon which started since the introduction of antibiotics. However it becomes a threat to the general population when it is abused, mis-handled, misuse, or due to over-use of the available antibiotics.

The use of honey and lemon by mankind as food and medicine has a long history. Even primitive men knew the importance as food and medicine. However, their knowledge of the efficacy of these natural products is not vast.

1.3 Justification

Considering the few groups of antibiotics in use, and having no additional group(s) for the past 20 years (with only modifications of the ones in use). Therefore, it is necessary to look for alternative remedy for RTIs. There is also need for research (investigations) to be conducted in this area and developing more measures before resistance will be developed against virtually all the existing groups of antibiotics.

Until recently, Research and Development (R&D) efforts have provided new drugs in time to treat bacteria that became resistant to older antibiotics. The supply of new replacement antimicrobial agents has slowed dramatically and we face the prospect of a future where we have far fewer options in the treatment of infectious disease (Walsh, 2013). The potential crisis at hand is the result of a marked decrease in industry R&D, and the increasing prevalence of resistant bacteria. Infectious disease physicians are alarmed by the prospect that effective antibiotics may not be available to treat seriously ill patients in the near future (Walsh, 2013). Poor financial investment in antibiotic research has exacerbated the situation (Walsh, 2013).

There are a lot of natural products in our environment (fruits, leaves, roots, stem barks, etc.) which we can explore to combat many emerging and re-emerging

infectious diseases; of which we would not know if they are not tried/tested. For instance, recent research has shown that a modified peptide found in honeybees was found to be effective against 37 species of bacteria (Chemical and Engineering News, 2012). This therefore makes honey a promising antibacterial agent.

1.4 Aim of the Study

To evaluate the antibacterial activities of lemon juice and/or honey on some bacteria species identified as responsible for Respiratory Tract Infections.

1.5 Specific Objectives

Isolation and identification of some bacteria associated with respiratory tract infections.

Preparation of purified extracts of honey and lemon juice.

Determination of the antibacterial activities of honey, lemon juice and honey/lemon mixture at varied concentrations on the bacteria isolated by agar diffusion and broth dilution techniques.

Determination of the susceptibility pattern of the bacteria isolated to some reference antibiotics.

Evaluation of the rate of kill of the bacteria isolated by honey and lemon juice.

1.6 Research Hypothesis

H_0:

Honey does not have antibacterial activity against isolates from respiratory tract infections.

Lemon juice does not have antibacterial activity against isolates from respiratory tract infections.

Honey and lemon juice when used in combination do not produce synergistic effect against bacteria isolates associated with Respiratory Tract Infections (RTIs).

H_1:

Honey has antibacterial activity against isolates from respiratory tract infections.

Lemon has antibacterial activity against isolates from respiratory tract infections.

Honey and lemon juice when used in combination produce synergistic effect against bacteria isolates associated with Respiratory Tract Infections (RTIs).

1.7 Constraints

Infrastructural conditions such as electricity supply, instruments/equipment.

The number of isolates that were obtained from the samples over the period of the study.

1.8 Limitation

It is an in-vitro study: Antibacterial activity of honey and/or lemon juice was tested on bacterial isolates of RTI (and compared with some reference/conventional antibiotics).

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Honey

2.1.1 Origin of Honey

Honey collection is an ancient activity. Crane (1983) reported that, honey collection began for at least 10,000 years ago. She depicts this with a cave painting in Valencia, Spain. The painting is a Mesolithic rock showing two female honey-hunters collecting honey and

honeycomb from a wild bee nest. The two women are depicted in the nude, carrying basket and using a long wobbly ladder to reach the wild nest. Ancient Egyptians appreciated the value of honey and even went as far as domesticating bees and building apiaries. Mexico is also an important producer of honey, providing more than 4% of the world's supply. Much of this (about one-third) comes from the Yucatán Peninsula. Honey production began there when the *Apis mellifera* and the *A. mellifera ligustica* were introduced there early in the 20th century (Food and Agriculture Organization of the United Nations, 2008). Most of Mexico's Yucatán producers are small, family operations that use original traditional techniques, moving hives to take advantage of the various tropical and subtropical flowers (Lavin and Mariely, 2008).

2.1.2 Properties of Honey

The physical properties of honey vary, depending on water content, the type of flora used to produce it (pasturage), temperature, and the proportion of the specific sugars it contains. Fresh honey is a supersaturated liquid, containing more sugar than the water can typically dissolve at ambient temperatures. At room temperature, honey is a super-cooled liquid, in which the glucose will precipitate into solid granules. This forms a semisolid solution of precipitated glucose crystals in a solution of fructose and other ingredients. Ripe honey, as removed from the hive by a beekeeper, has a long shelf life, and will not ferment if properly sealed (National Honey Board, 2010).

The melting point of crystallized honey is between 40 and 50 °C, depending on its composition (Root & Root, 2005). Below 5 °C, the honey will not crystallize and, thus, the original texture and flavor can be preserved indefinitely (Tomasik, 2004). As the temperatures become colder, the viscosity of honey increases. Like most viscous liquids, the honey will become thick and sluggish with decreasing

temperature. At −20 °C, honey may appear or even feel solid, but it will continue to flow at very slow rates. Honey has a glass transition between -42 and -51 °C. Below this temperature, honey enters a glassy state and will become an amorphous solid (noncrystalline) (Kantor *et al.*, 1999; Vidal and Israeloff, 2000).

Honey has the ability to absorb moisture directly from the air, a phenomenon called hygroscopy. The amount of water the honey will absorb is dependent on the relative humidity of the air. This hygroscopic nature requires that honey be stored in sealed containers to prevent fermentation, which usually begins if the moisture content rises above 25 %. Honey will tend to absorb more water in this manner than the individual sugars would allow on their own, which may be due to other ingredients it contains (Root & Root, 2005).

Like all sugar compounds, honey will caramelize if heated too much, becoming darker in color and eventually burning. The fructose content of honey makes it to caramelize at lower temperatures than the glucose (Hans-Dieter, 2004). Honey also contains acids, which act as catalysts, decreasing the caramelization temperature even more (Zdzislaw, 2007). Of these acids, the amino acids, which occur in very small amounts, play an important role in the darkening of honey. The temperature at which caramelization begins varies, depending on the composition, but is typically between 70 °C and 110 °C. The average pH of honey is 3.9, but can range from 3.4 to 6.1. Honey contains many kinds of acids, both organic and amino, depending on the type of honey. These acids may be aromatic or aliphatic. The aliphatic acids contribute greatly to the flavor of honey by interacting with the flavors of other ingredients. Gluconic acid, for instance, is a flavor enhancer. The aromatic acids, such as malic acid, come mostly from the flowers, adding to the aroma and

taste of the honey. Organic acids comprise most of the acids in honey, accounting for 0.17–1.17 %. Gluconic acid being present in the highest proportion. Other organic acids are minor, consisting of formic, acetic, butyric, citric, lactic, malic, pyroglutamic, propionic, valeric, capronic, palmitic, and succinic, among many others (Alistair *et al*., 1995).

2.1.2.1 Antibacterial Properties of Honey

The antibacterial mechanisms known to date are Hydrogen peroxide (H_2O_2), methylglyoxal (MGO), bee defensin-1, the osmotic effect and the pH (Wahdan, 1998; Mandal, 2011).

The antibacterial activity of honey is based partly on its osmotic effects in that the bacteria that cause infections are unable to survive in honey because they become dehydrated. In addition, it was noted that the presence of hydrogen peroxide generated by the enzymatic activity of glucose oxidase in dilute honey contribute to the antibacterial activity. As hydrogen peroxide decomposes, it generates highly reactive free radicals that react and kill bacteria (Mandal, 2011).

Although honey has been used as a medicine since ancient times in many cultures, in its ancient usage there was no recognition of its antibacterial properties. It was just known to be an effective remedy. This is not surprising, considering that it is of recent that it has become known that many ailments as a result of infections by microorganisms can be treated with honey. Now it can be recognized that the effectiveness of honey in many of its medical uses is probably due to its antibacterial activity. Key factors to consider with respect to this are:

Osmotic effect: honey is a saturated solution of sugars, 84% being a mixture of fructose and glucose.

Acidity: honey is characteristically quite acidic, its pH being between 3.4 and 6.1, which is low enough to be inhibitory to many bacteria.

Hydrogen peroxide: the major antibacterial activity in honey has been found to be due to hydrogen peroxide produced enzymatically in the honey.

2.1.3 Nutritional Content of Honey

Honey has been classified in terms of nutritional values as shown below:

Honey Nutritional Value for 100g (3.5 oz)

Energy	1,272kj (308kcal)
Carbohydrate	82.4g
[-Sugars	82.12g]
[-Dietary fiber	0.20g]
Fat	0.00g
Protein	0.30g
Water	17.1g
Riboflavin (vitamin B2)	0.04mg (3%)
Niacin (vitamin B3)	0.12mg (1%)
Panthothenic acid (B5)	0.07mg (1%)
Vitamin B6	0.02mg (2%)
Folate (vitamin B9)	2.00mg (1%)
Vitamin C	0.50mg (1%)

Calcium	6.00mg (1%)
Iron	0.42mg (3%)
Magnesium	2.00mg (1%)
Phosphorus	4.00mg (1%)
Potassium	52.0mg (1%)
Sodium	4.00mg (0%)
Zinc	0.22mg (2%)

Shown is for 100g, roughly 5 table spoon full. Percentages are relative to US recommendations for adults.

Source: USDA (2012).

2.1.4 Uses of Honey

2.1.4.1 Medicinal Uses of Honey

Scientists and doctors are rediscovering the effectiveness of honey as a wound treatment. Clinical observations and experimental studies have established that honey has effective antibacterial and anti-inflammatory properties. It painlessly removes pus, scabs and dead tissues from wounds and stimulates new tissue growth (Molan, 1992).

Historically, honey has been used by humans to treat a variety of ailments, from gastric disturbances to ulcers, wounds and burns, through ingestion or topical application (Drgrotte, 2012). Traditional use of honey has included honey mixed with lemon for the treatment of sore throats, common cold, and catarrh essentially as soothing relieve.

In a study in New Zealand, Manuka honey was shown to be effective against *Helicobacter pylori* (Al Somai *et al*., 1994). The presence of potassium, sodium, calcium, and magnesium means that honey is capable of neutralizing acid in the body and thus maintaining the acid-alkaline balance (McCarthy, 1995). Honey can be used as preservative.

2.1.4.2 Other Uses of Honey

Sweetener: Honey is slightly sweeter than sugar, so less can be used to achieve the same sweetness intensity. It is used as sweetener in some commercial beverages.

Flavor: Honey does not only impart a unique flavor to any dish, but it also balances and enhances the flavor profiles of other ingredients used in a recipe. It is used in cooking, baking, as a spread on bread, and as an addition to various beverages, such as tea.

Humectant: Honey provides and retains moisture to a variety of dishes and can even extend the shelf life of baked foods.

Energy Booster: honey is a great natural source of carbohydrates which provide strength and energy to our bodies, honey is known for its effectiveness in instantly boosting the performance, endurance and reduces muscle fatigue of athletes. Its natural sugars play an important role in preventing fatigue during exercise. The glucose in honey is absorbed by the body quickly and gives an immediate energy boost, while the fructose is absorbed more slowly providing sustained energy.

2.2 Lemon

2.2.1 Origin of Lemon

The first substantial lemon cultivation in Europe began in Genoa in the middle of the 15th Century. It was later introduced to the Americas in 1493 when Christopher Columbus brought lemon seeds to Hispariola along his voyages (NPCS, 2012). Spanish conquest throughout the then New World helped spread lemon seeds. Most of the Western World's entire supply of good quality lemon is grown in Italy and California. Indeed, these two places contribute the most to the world's supply of lemons (Williams, 2012).

From Europe, lemon was introduced to Persia (because then they were not widely cultivated) and then to Iraq, and Egypt around AD 700. The lemon was first recorded in literature in a 10th Century Arabic treatise on farming, and was also used as an ornamental plant in many Islamic gardens. It was distributed widely throughout the Arab world and the Mediterranean region between AD 1000 and AD 1150. The genetic origin of the lemon however was reported to be hybrid between sour orange and Citron (Gulsen, 2001).

2.2.2 Varieties of Lemon

There are different varieties of lemon. This includes:

The Bonnie Brae: is oblong, smooth, thin skinned and seedless; mostly grown in San Diego County (Carque, 2006).

The Bush lemon tree: a naturalized lemon, grows wild in subtropical Australia. The Bush Lemon Tree (also known as the Rough Lemon Tree), is a comparatively more thorny character. Its lemons have a tarter, more unique flavor than those of the Meyer variety, and they are typically thicker skinned and seed-filled. It is very hardy, and has a thick skin with a true lemon flavor; the zest is good for cooking. It grows to about 4m in a sunny position. The Rough Lemon Tree's strong, fruitful nature allows it to sprout new plants easily (Williams, 2012).

The Eureka: grows year-round and abundantly. This is the common supermarket lemon. Eurekas are a variety of *Citrus limon* that was developed in California in 1958. Thomas A Garey, a prominent Los Angeles nurseryman gave the tree the name Garey Eureka (NPCS, 2012). They are known for their hardy nature and large production. Generally speaking the Eureka lemon trees grow to around 5 m (16ft) tall, which makes it one of the larger lemon trees. One advantage of using a Eureka lemon tree is that they grow quite quickly. They tend to be a larger tree than Meyer lemons, however there are many new dwarf varieties

available which only grow to around 2 m tall. Like most citrus, Eureka lemons prefer Temperate, Warm temperate, Mediterranean (cool temperate, with less production) climates. Eureka lemons prefer mild winters and warm summers and avoid areas with heavy frosts (as it burns the new growth). They are actually a very hardy tree and can withstand dry periods, however, during very dry times, trees will stress and drop fruit. The Eureka Tree steadily produces lemon clusters during the spring and summer months, and these medium-skinned lemons are often nicknamed “common supermarket lemons” for their marketability (Williams, 2012).

The Femminello St. Teresa, or Sorrento: This is native to Italy (Los Angeles times, 2011). This fruit's zest is high in lemon oils. It is the variety traditionally used in the making of limoncello (Los Angeles times, 2011).

The Lisbon: The Lisbon Tree is notable for its lemons of comparatively higher juice and acidity levels. These trees are particularly thorny, especially when young, and also extremely productive. The Lisbon Tree bares many similarities to the Eureka Tree in appearance and productive nature, though unlike the Eureka lemon (which originated in California), the Lisbon Tree originated in Portugal and was brought to the United States by way of Massachusetts. In addition, a lemon from the Lisbon tree can be expected to be more seed-filled than its Eureka counterpart (Williams, 2012).

The Meyer lemon: This variety is a cross between a lemon and possibly an orange or a mandarin, and was named after Frank N. Meyer, who first discovered it in 1908 (NPCS, 2012). Meyer Trees are a popular choice amongst those in the market for a visually appealing evergreen to use as decoration indoors or outdoors. Its leaves are characteristically lush and glossy, and decidedly the most aromatic of any lemon tree. The trunk of a Meyer lemon tree is nearly thorn-free. The fruit of a Meyer Tree is less acidic and

less tart than regular lemons; in fact, Meyer lemons are typically used as lemon substitutes in the market-place. Meyer lemons have a much thinner rind, and often mature to a yellow-orange color. Therefore, the lemons of a Meyer Tree are physically similar to oranges in shape, size, color of peel and pulp. Meyer lemons are slightly more frost-tolerant than other lemons (Williams, 2012).

The Ponderosa lemon: This variety is more cold-sensitive than true lemons; the fruits are thick-skinned, seed-filled, yellow (which are actually a cross between a citron and a lemon) and very large (NPCS, 2012). The Ponderosa Tree is unique because of its extremely thorny trunk. The Ponderosa Tree is infamously tricky to cultivate fruit from, as its cold-sensitive nature restricts it to grow only in consistently warm climates (Williams, 2012).

The Villafranca: as its name would suggest, hails from Sicily; it spread across Europe before finally arriving in the United States. The tree is characterized by its thick leaves and abundance of thorns, which tend to diminish as the tree ages. The Villafranca is a less popular tree for growing marketable lemons, though it has managed to find popularity in Israel (Williams, 2012).

2.2.3 Properties of Lemon

Lemon is propagated from stored seeds in the spring time. Plants grow optimally on well drained soils with good exposure to incumbent sunlight. The lemon is both a small evergreen tree, and the trees oval yellow fruit (when ripe) (NPCS, 2012).

Commercially speaking, Eureka variety is prolific plant and gives a high yield. It starts bearing fruits early from late spring to the end of summer in a given year. The other preferred lemon variety called the Lisbon bears a single large crop in a year; this is borne either in the spring or in the winter months. Lemon trees of good quality have been known to bear upward of three thousand lemons in a single year. One reason for the high yield of

lemon is that lemon trees tend to bloom and ripen their fruits every month of the year. The period from January to May is the time when the highest numbers of fruits are borne on the plants (Herbs, 2000).

Lemon juice contains high levels of Vitamin C. On average, lemon fruits contain about twice as much Vitamin C as oranges. Physicians first discovered these characteristics of lemons in the Seventh Century, when they found that consuming lemon juice everyday prevents outbreaks of scurvy. However, the high levels of Vitamin C in lemon juice are lost if the juice is left exposed to the air for a long period of time (Jeremy, 2009; Andrew, 2010).

To get the best ripeness and juice from lemons, one should choose lemons having a deep yellow-color. Ideally, lemons must feel firm when pressed, but not hard or soft. Fruits that have been bruised must be avoided as *mould* tends to affect lemon fruits that have been mechanically injured in some way. Also, lemons left unrefrigerated for long periods of time are susceptible to *mould*. Shriveled or hard skinned fruits must also be avoided, especially those that are too soft or spongy when held. Such lemons may be dried inside or old, or they could be mechanically injured or be rotten in the core.

The average lemon contains approximately 3 tablespoons of juice. Allowing lemons to come to room temperature before squeezing (or heating briefly in microwave) makes the juice easier to extract. Citric acid makes up about five to six percent of the juice and tissues of lemons (and limes). At this percentage with its low pH, it breaks down the cell membrane of bacteria, similar to the effects of heating. This means that foods to which lemon and lime juice are added do not need to be heated as much to obtain the same antibacterial effects (Sherman and Billing, 1999).

2.2.4 Nutritional Content of Lemon

Lemon has been classified in terms of nutritional values as shown below:

Lemon (raw, without peel)-Nutritional value per 100g (3.5oz):

Energy	121kj (29kcal)
Carbohydrates	9.32g
-Sugars	2.50g
-Dietary fiber	2.80g
Fat	0.30g
Protein	1.10g
Thiamine (Vit. B1)	0.04mg (3%)
Riboflavin (Vit. B2)	0.02mg (1%)
Niacin (Vit. B3)	0.10mg (1%)
Pantothenic acid (B5)	0.19mg (4%)
Vitamin B6	0.08mg (6%)
Folate (Vit. B9)	11.0µg (3%)
Vitamin C	53.0mg (88%)
Calcium	26.0mg (3%)
Iron	0.60mg (5%)
Magnesium	8.00mg (2%)
Phosphorus	16.0mg (2%)
Potassium	13.8mg (3%)
Zinc	0.06mg (1%)

Percentages are relative to US recommendations for adults (NPCS, 2012)

2.2.5 Uses of Lemon

2.2.5.1 Medicinal uses

Lemon was mainly used as ornament and medicine. In 1747, James Lind's experiments on Seamen suffering from Scurvy involved adding Vitamin C to their diets with lemon juice (Andrew, 2010; NPCS, 2012).

Lemon is an important medicinal plant of the family Rutaceae. It is cultivated mainly for its alkaloids, which are having anticancer activities and the antibacterial potential in crude extracts of different parts (viz., leaves, stem, root and flower) of Lemon against clinically significant bacterial strains has been reported (Kawaii *et al*., 2000).

Citrus flavonoids have a large spectrum of biological activity including antibacterial, antifungal, antidiabetic, anticancer and antiviral activities (Burt, 2004). Flavonoids are generally present in glycosylated forms in plants, and the sugar moiety is an important factor determining their bioavailability.

Lemon is classified as an acidic fruit. The juice of lemons is excellent and effective remedy to treat disorders of the throat and persistent catarrh. Moreover, Vitamin C content of lemon can actively aid in suppressing the onset of cold. Schoffro, (2012) reported that lemons are rich in vitamin C and flavonoids that work in conjunction for a serious punch against infection. Therefore, drinking lemon juice is very good for treating common colds and flu. Lemon juice used as a gargle or oral wash also helps bring relief from sore throats, tonsillitis, and can help alleviate gingivitis, as well as canker sores in a person.

The juice isn't the only medicinal part of the lemon. Citrus peel (per unit weight) has considerably higher quantities of medicinal phytochemicals than the juice. The peel contains higher concentrations of citric acid and active anti-cancer compounds than the juice or the

pulp (Hakim and Harris, 2001). They also discovered that the peel showed strong potential for significantly reducing risk of non-melanoma skin cancers. Schoffro, (2012) reported that lemon contain 22 anti-cancer compounds, including limonene—a naturally-occurring oil that slows or halts the growth of cancer tumors in animals. Lemons also contain a substance called flavonol glycosides which stop cell division in cancer cells.

Bowel-Cleansing: The bitter taste of lemon gives these fruit the ability to increase peristalsis–a pumping-motion in the bowels–which helps to eliminate waste from the bowels and improve regularity (Schoffro, 2012).

Regular consumption results in improved appetite which also eases the acidity in the stomach, helping a person deal better with ulcers, arthritis, physical symptoms of gout, as well as chronic rheumatism.

The use of herbal remedies, including the herb lemon, classified as *Citrus limon*, are popular as an alternative to standard Western allopathic medicine for a variety of problems, including nerve tonic, blood cleanser as well as used in the fight against cancer (Prabuseenivasan *et al*., 2006).

2.2.5.2 Other uses of lemon

The distinctive sour taste of lemon makes it a key ingredient in many dishes across the world. The fruit is used for culinary and non-culinary purposes throughout the world-primarily for its juice, though the pulp and rind (zest) are also used, mainly in cooking and baking. It is used in a variety of food recipes such as lemon cakes, lemon chickens, etc.

Lemon juice is about 5% to 6% (approximately 0.3M) citric acid, which gives lemons a sour taste, and a pH of 2-3. This makes lemon juice an inexpensive, readily available acid for use in educational Science experiments (Rogers, 2011; NPCS, 2012).

Lemon has been used in the preparation of many lemon-flavored drinks and candies, including lemonade (famous European recipe), Sherbet lemon, and lemon plus (NPCS, 2012).

Squeezing a little bit of lemon juice on cut fruit and vegetables like apples, pears, avocados and potatoes can keep them from turning brown (i.e. it can be used as preservative) (Rogers, 2011).

It has been used by many as emergency deodorant, odor destroyer and insect deterrent (Rogers, 2011).

Lemon juice may also be used to soften water. The lemon juice in water makes for an excellent rinse. When using it after shampooing, hair-lemon juice is a natural hair color lightener. E.g. it is used in cleaning kitchen utensils and freshening houses; it is also used to brighten one's laundry, and improve one's hair, nails and skin (Shine, 2008; Rogers, 2011).

2.3 Respiratory Tract Infections

Respiratory infectious diseases are mainly caused by viruses or bacteria that often interact with one another. However, since attachment of a pathogen to mucosal surfaces is the first step towards respiratory disease, and viral infection alters the defense of the host epithelium in general (Vareille *et al*., 2011), it has been postulated that viral presence may render the epithelium more susceptible to bacterial colonization (Bogaert *et al*., 2004). Studies on mouse have shown that viral predisposition to bacterial attachment not only occurs in case of a simultaneous infection, but also up to a week after initial viral infection (Avadhanula *et al*., 2006; Hament *et al*., 2005; Stark *et al*., 2006).

The epithelial layer of the respiratory tract mucosa is the first line of defense against a bacterial invader: loss of barrier function could therefore lead to entry of pathogens. Viruses generally replicate intracellularly and can subsequently disarrange cellular processes or kill infected cells through metabolic exhaustion or direct lysis. Induced cell death may in turn lead to denudation of the epithelial layer (Suzuki and Bakaletz, 1994), exposing the basement membrane. *Streptococcus pneumoniae* was found to bind strongly to fibronectin, which is prominently exposed at the basement membrane after denudation of epithelium (van der Flier *et al*., 1995). Similarly, *Staphylococcus aureus* (Heilmann, 2011) and *Moraxella catarrhalis* (Tan *et al*., 2005) have been shown to bind to extracellular matrix proteins, suggesting that these species might also benefit from virus-induced damage to epithelium.

However, respiratory viruses may also induce changes in immune function favourable to bacterial invasion: fewer Natural Killer (NK) cells may be recruited into the tissue and their functionality may be suboptimal as a consequence of viral-infection. Virus-induced IFN-α and IFN-β may impair recruitment and functionality of neutrophils (Stark *et al*., 2006), and enhanced opoptosis of neutrophils recruited to combat the viral invader, thereby increasing susceptibility to bacterial superinfection. Furthermore, IFN-γ seems to negatively affect the activity of macrophages (Figure 2.0). Viral infected monocytes appear less effective in ingesting and killing bacteria, predisposing them to bacterial overgrowth and invasion.

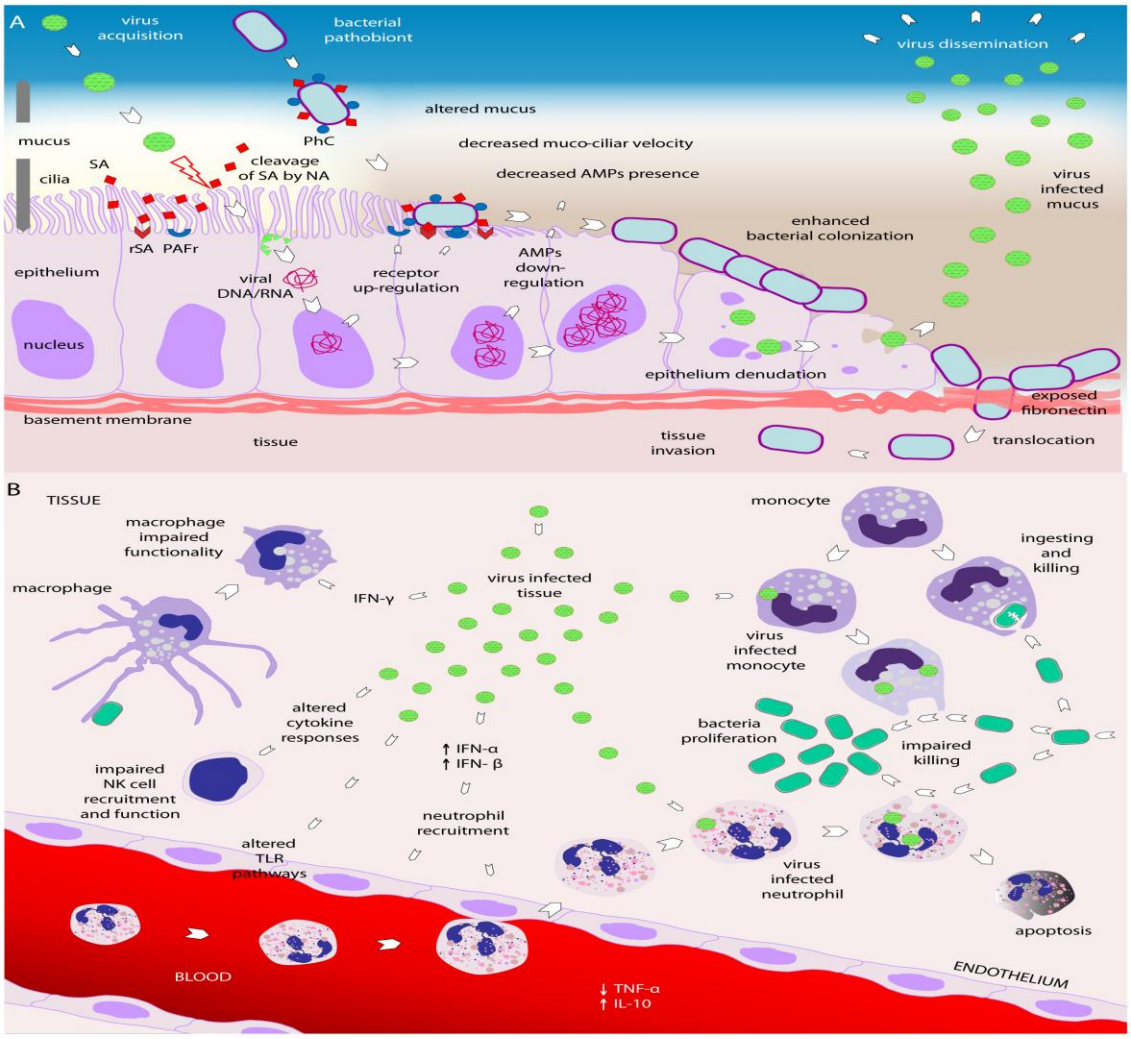


Figure 2.1: Viral–bacterial interactions (Astrid *et al*., 2013)

Respiratory tract infection refers to any infectious diseases involving the respiratory tract. Infections of the respiratory tract are among the commonest types of infections, and account for much consultation in general practice and a high percentage of acute hospital admissions (Jeremy *et al.,* 2006). It causes at least one-half of all symptomatic illnesses in a community, exacting great tolls that can be measured as morbidity, absenteeism from school and works, health care costs, and overuse of antibiotics leading to the emergence of drug-resistant bacteria. The most widespread respiratory tract infection is the common cold. The common cold accounts for up to three-quarters of all illnesses in young infants and up to one-half of illness in adults (Charles, 2011). The importance of otitis media in primary care

also cannot be overemphasized, especially in pediatrics practice; By age 5, between 75% to 95% of children have had at least one episode of otitis media (Charles, 2011). *Pseudomonas aeruginosa* and *Staphylococcus aureus* are common causes of otitis externa. Group A *Streptococci* and various gram-negative rods sometimes cause otitis media, and aerobic bacteria are involved in up to 25% of cases (Charles, 2011).

Respiratory tract diseases are diseases that affect the air passages, including the nasal passages, the bronchi and the lungs. They range from acute infections, such as pneumonia and bronchitis, to chronic conditions such as asthma and chronic obstructive pulmonary disease.

Typical infections of the upper respiratory tract include tonsillitis, pharyngitis, laryngitis, sinusitis, otitis media, certain types of influenza, and the common cold (Eccles *et al*., 2007). Symptoms of upper respiratory tract infections include cough, sore throat, runny nose, nasal congestion, headache, low grade fever, facial pressure and sneezing.

However, lower respiratory tract infections are generally more serious than upper respiratory infections. The lower respiratory tract infections are the leading cause of death among all infectious diseases (Robert *et al*., 2004). The two most common lower respiratory tract infections are bronchitis and pneumonia. Influenza affects both the upper and lower respiratory tracts, but more dangerous strains such as the highly pernicious H5N1 tend to bind to receptors deep in the lungs (van Riel *et al*., 2006).

Cherry *et al.,* (2008) reported that in United States, RTIs are the most common infectious illness in the general population and the leading reasons for people missing work and school. It is also the leading diagnosis in the office setting.

2.3.1 Transmission of Respiratory Tract Infections

Transmission of RTIs is via respiratory droplets or by virus-contaminated hands, which are complicated by bacteria. Infections can also be spread through indirect contact. For example, if you have a cold and you touch your nose or eyes before touching an object or surface, the virus may be passed to someone else when they touch that object or surface. Upper respiratory tract (nose, throat, sinuses) mucosa inflammation causes increased secretions, rhinorrhea and results in sneezing and coughing, facilitating the spread. Acute respiratory tract infections include rhinitis, pharyngitis/tonsillitis and laryngitis often referred to as a common cold, and their complications: sinusitis, ear infection and bronchitis (Mika *et al*., 1998). Symptoms of RTI's commonly include; cough, sore throat, runny nose, nasal congestion, headache, low grade fever, facial pressure and sneezing. Onset of the symptoms usually begins 1-3 days after the exposure to a microbial pathogen. The illness usually lasts 7-10 days. Group A beta hemolytic *Streptococcal* pharyngitis/tonsillitis (Strep throat) typically presents with a sudden onset of sore throat, pain with swallowing and fever (Bisno, 2001). Strep throat does not usually cause runny nose, voice changes or cough. Pain and pressure of the ear caused by viral conjunctivitis are often associated with respiratory tract infections (Jeremy *et al.,* 2006). Influenza (the flu) is a more severe systemic illness which typically involves the respiratory tract.

2.3.2 Treatment of Respiratory Tract Infections

Antibiotics are not recommended for most RTIs because they are only effective if the infection is caused by bacteria. Acute pharyngitis presents a diagnostic and therapeutic dilemma. The majority of sore throat are caused by a variety of viruses; fewer than 20% *are* bacterial and hence potential responsive to antibiotic therapy; *Streptococcus pyogene*s, the most important bacterial pathogen and this responds to oral penicillin. However, up to 10

days' treatment is required for its eradication from the throat. This requirement causes problems with compliance as symptomatic improvement generally occurs within 2-3 days (Stephen *et al.,* 2004).

Although viral infections are important causes of both otitis media and sinusitis, they are generally self-limiting. However, bacterial infections may complicate viral illnesses, and are also primary causes of ear and sinus infections. Actually, treatment depends on the underlying cause. There are currently no medications or herbal remedies which have been conclusively demonstrated to shorten the duration of illness completely (Douglas *et al.,* 2007).

Treatment comprises symptomatic support usually via analgesics for headache, sore throat and muscle aches (Eccles *et al.,* 2007). There is no evidence to the age-old advice to rest when you are sick with respiratory illness. Moderate exercise in sedentary subjects with RTI has been shown to have no effect on the overall severity and duration of the illness. Based on these findings, it was concluded that, previously sedentary people who have acquired RTI and who have initiated an exercise program may continue to exercise (Mika *et al.,* 1998). Getting enough sleep; however, is advisable since even mild sleep deprivation has been shown to be associated with increased susceptibility to infection (Bisno, 2001).

Non-compliance to antibiotic usage will lead to rise in drug resistance by bacteria which are now a growing problem in the world. According to Taverner and Latte (2009), single oral dose of nasal decongestant in common cold is modestly effective for the short term relief of congestion in adults; however, there is an insufficient data on the use of decongestants in children. Therefore, decongestants are not recommended for use in children under 12 years of age with the common cold. Oral decongestant is also contra-

indicated in patients with hypertension, coronary artery disease, and history of bleeding strokes (Reveiz *et al.,* 2007).

The use of vitamin C in the inhibition and treatment of upper respiratory infections has been suggested since the initial isolation of vitamin C in the 1930s. Some evidence exists to indicate that it could be justified in persons exposed to brief period of severe physical exercise and/or cold environments. The benefit versus risk of nasal irrigation is currently unclear and therefore is not much recommended.

2.4 Description, Significance and Health Implication of the Tested Bacteria Isolates

2.4.1 *Klebsiella pneumoniae*

Scientific Classification:

Domain: Bacteria

Phylum: Proteobacteria

Class: Gammaproteobacteria

Order: Enterobacteriales

Family: Enterobacteriaceae

Genus: *Klebsiella*

Species: *K. pneumoniae*

Binomial name: *Klebsiella pneumoniae*

Klebsiella pneumoniae is a Gram-negative, non-motile, encapsulated, lactose fermenting, facultative anaerobic, rod shaped bacterium and measures 2 µm by 0.5 µm.

Although found in the normal flora of the mouth, skin, and intestines, it can cause destructive changes to human lungs if aspirated (Ryan and Ray, 2004).

Members of the *Klebsiella* genus typically express two (2) types of antigens on their cell surface. The first, O antigen, is a component of the lipopolysaccharide (LPS), of which nine (9) varieties exist. The second is K antigen, a capsular polysaccharide with more than 80 varieties (Podschun and Ullmann, 1998). Both contribute to pathogenicity and form the basis for serogrouping.

Many hospital cases around the world have been linked to *K. pneumoniae*. *Klebsiella pneumoniae* is commonly found in the gastrointestinal tract and hands of hospital personnel (Podschun and Ullmann, 1998). The reason for its pathogenicity is the thick capsule layer surrounding the bacterium. It is 160 nm thick of fine fibers that protrudes out from the outer membrane at right angles (Lawlor, 2005). Another site on the human body that this bacteria can be found is the nasopharynx. Its habitat is not limited to humans but is ubiquitous to the ecological environment. This includes surface water, sewage, and soil (Brisse *et al.,* 2001).

To get a *Klebsiella* infection, a person must be exposed to the bacteria. For example, *Klebsiella* must enter the respiratory (breathing) tract to cause pneumonia, or the blood to cause a bloodstream infection.

Infections most often afflict those with compromised immune systems. An opportunistic pathogen, *Klebsiella pneumoniae* is pervasive in hospital settings and difficult to combat. The most significant source of patient infection is contact with feces and contaminated instruments. Even with antimicrobial therapy, it has a high death rate of about 50 percent, and nearly 100 percent for persons with alcoholism and bacteremia Center for Disease Control (CDC), (2012).

2.4.1.1 Significance and Health Implications of Klebsiella pneumoniae

In recent years, *Klebsiellae* have become important pathogens in nosocomial infections. It is clinically the most significant member of the *Klebsiella* genus of Enterobacteriaceae.

Klebsiella pneumoniae is a very common pathogen that is encountered by many health care providers. Strong correlation has been established between the demographic and geographic distribution among world populations and the incidents of community-acquired infections caused by *K. penumoniae*. *Klebsiella pneumoniae* has been considered a respiratory pathogen that causes pneumonia, the symptoms include: toxic presentation with sudden onset, high fever, and hemoptysis (Wen-Chien *et al*., 2002).

Klebsiella pneumoniae is one of the top organisms causing infections in hospitalized patients. These organisms can cause serious bloodstream infections and other intrusive infections that can potentially lead to fatality. A study in a Jamaican hospital was conducted that focused on multidrug resistant (MDR) strains of *K. pneumoniae* over a 5 year period. These MDR strains include resistance to broad spectrum cephalosporins and other extensively used antibiotics. This study led to the conclusion that *K. pneumoniae* showed endemic persistence of selected clones, as opposed to pandemic persistence. This led to the hypothesis that the transmission of the organism was largely due to patient-to-patient contact and to health care worker-to-patient contact in the hospital, or, less commonly, by contamination of the environment. The bacteria are not spread through the air (Nicole *et al.,* 2010).

Infection with carbapenem-resistant Enterobacteriaceae (CRE) or carbapenemase-producing Enterobacteriaceae is emerging as an important challenge in health-care settings (Limbago *et al.,* 2011). One of many carbapenem-resistant Enterobacteriaceae (CRE) is

Carbapenem-Resistant *Klebsiella pneumoniae* (CRKP). Over the past 10 years, a progressive increase in CRKP has been seen worldwide; however, this new emerging nosocomial pathogen is probably best known for an outbreak in Israel that began around 2006 within the healthcare system there (Berrie, 2007). In the USA, it was first described in North Carolina in 1996 (Yigit *et al.,* 2001); since then CRKP has been identified in 41 states (Washington post, 2012); and is recovered routinely in certain hospitals in New York and New Jersey. It is now the most common CRE species encountered within the United States.

Carbapenem-Resistant *Klebsiella pneumoniae* is resistant to almost all available antimicrobial agents, and infections with CRKP have caused high rates of morbidity and mortality, particularly among persons with prolonged hospitalization and those who are critically ill and exposed to invasive devices (e.g., ventilators or central venous catheters). The concern is that carbapenem is often used as a drug of last resort when battling resistant bacterial strains. The worry is that new slight mutations could result in infections for which there is very little, if anything, healthcare professionals can do to treat patients with resistant organisms. Patients with unrecognized CRKP colonization have been reservoirs for transmission during nosocomial outbreaks (Washington post, 2012).

Klebsiella pneumoniae is an important cause of human infections. Infections or diseases are usually nosocomial or hospital-acquired. In 1998, *K. pneumoniae* and *K. oxytoca* accounted for 8% of nosocomial bacterial infections in the United States and in Europe. The associated infections include urinary tract infections, pneumonia, septicemia, and soft tissue infections (Podschun and Ullmann, 1998). The diseases caused by *K. pneumoniae* can result in death for patients who are immunodeficient. Treatment is done by antibiotics such as clinafloxacin (Sylvain *et al.,* 1999). But, there are an increasing number of antibiotic-resistance strains. Ciprofloxacin is an antibiotic that is becoming less effective (Brissel, 2000).

2.4.2 *Streptococcus pyogenes*

Scientific Classification:

Kingdom:	Bacteria
Phylum:	Firmicutes
Class:	Bacilli
Order:	Lactobacillales
Family:	Streptococcaceae
Genus:	*Streptococcus*
Species:	*pyogenes*
Binomial name:	*Streptococcus pyogenes*

Streptococcus pyogenes is a Gram-positive facultative anaerobic bacterium. It is not motile, and does not produce spores. It occurs as long chains of cocci, and occasionally in pairs. *Streptococcus pyogenes* is classified as Group A streptococcus. Group A streptococci typically have a capsule composed of hyaluronic acid and are beta-hemolytic, which is true for *Streptococcus pyogenes* (Todar, 2005). Beta-hemolytic streptococci produce a toxin that forms a clear zone of hemolysis on blood agar, demonstrating its ability to destroy red blood cells. This hemolysis is attributed to toxins formed by Group A streptococci called streptolysins. Streptolysins can destroy not only red blood cells, but also the white blood cells responsible for fighting off bacteria and disease, as well as other body cells (Tortora *et al.*, 2007).

Streptococcus pyogenes, also known as the flesh eating bacteria, is the most pathogenic bacterium in the whole genus (Todar, 2002). The name pyogenes comes from the word pyogenic, which is a classification for the streptococci that are associated with pus formation.

2.4.2.1 Significance and Health Implications of Streptococcus pyogenes

Streptococcus pyogenes has several virulence factors that enable it to attach to host tissues, evade the immune response, and spread by penetrating host tissue layers (Patterson, 1996). A carbohydrate-based bacterial capsule composed of hyaluronic acid surrounds the bacterium, protecting it from phagocytosis by neutrophils (Ryan and Ray, 2004). In addition, the capsule and several factors embedded in the cell wall, including M protein, lipoteichoic acid, and protein F (SfbI) facilitate attachment to various host cells (Bisno *et al*., 2003).

Streptococcus pyogenes is an unusually successful pathogen because of many different properties. First of all, the organism is able to adhere to the cells of its host with strong adhering mechanisms, which is important because the organism would be easily removed by mucus or salivary fluid (Cunningham, 2000). The more adhesions there are, the stronger the adherence will be. Not only does *Streptococcus pyogenes* adhere to its host cells, but it also invades them (Cunningham, 2000). Laboratories have shown that "group A streptococci have the potential to invade human epithelial cells at frequencies equal to or greater than classical intracellular bacterial pathogens." (Cunningham, 2000). There are two proteins necessary for invasion, which are the M protein and the SfbI, which is a fibronectin-binding protein.

Streptococcus pyogenes produces three types of exotoxins. These toxins are responsible for causing fever and scarlet fever rashes; they also increase the risk of endotoxic shock and depress antibody synthesis (Sharma, 2006).

Infections due to certain strains of *S. pyogenes* can be associated with the release of bacterial toxins. Throat infections associated with release of certain toxins lead to scarlet

fever. Other toxigenic *S. pyogenes* infections may lead to streptococcal toxic shock syndrome, which can be life-threatening (Ryan and Ray, 2004).

Streptococcus pyogenes usually begins infection on the surface of the skin or in the throat. From there, the bacterium begins to spread into deeper areas of the skin, which can potentially lead to life-threatening diseases (Facklam, 2002).

It is estimated that there are more than 700 million infections (caused by *Streptococcus pyogenes*) worldwide each year and over 650,000 cases of severe, invasive infections that have a mortality rate of 25% (Aziz *et al.,* 2010). Early recognition and treatment are critical; diagnostic failure can result in sepsis and death (Jim, 2012).

Streptococcus pyogenes is the most common bacterial cause of sore throat (Duckworth, 2006). A painful, red throat with white patches on your tonsils is characteristic of pharyngitis, otherwise known as 'strep throat'. It is usually accompanied by swollen lymph nodes, fever, and headache. Occasionally it is also accompanied by nausea, vomiting, and abdominal pain (National Institute of Allergy and Infectious Diseases, 2005).

Necrotizing fasciitis is a rare, but very serious infection caused by *Streptococcus pyogenes* that is popularly termed "flesh eating bacteria". The bacteria typically enters the body through a minor trauma or surgical wound in persons of compromised immune systems and causes infection just below the skin that spreads to deeper tissues. Necrotizing fasciitis is an extremely quick moving infection that is characterized by the rapid destruction of tissue. It is fatal in 30-40% of cases (Sharma, 2006). For persons with necrotizing fasciitis, surgery often is needed to remove damaged tissue (Centres for Disease Control and Prevention, 2005). Strains of *S. pyogenes* resistant to macrolide antibiotics have emerged; however, all strains remain uniformly sensitive to penicillin (Albrich *et al*., 2004).

Streptococcal toxic shock syndrome is another very serious disease caused by *Streptococcus pyogenes*. It is not the same as the toxic shock syndrome associated with tampons, which can be attributed to *Staphylococcus aureus*. Streptococcal toxic shock commonly occurs in healthy individuals following an initial infection of *Streptococcus pyogenes*. Symptoms include significant pain, swelling, and redness of infected area, fever, dizziness, difficulty breathing, dangerously low blood pressure, and a weak, rapid pulse (Nemours Foundation, 2005). Medical care is crucial as more than 50% of cases of streptococcal toxic shock develop into necrotizing fasciitis or myositis, and there is a 30-70% mortality rate (Stevens, 1995).

Diseases caused by *Streptococcus pyogenes* usually respond well to antibiotic treatment. The American Heart Association and the Infectious Diseases Society of America currently recommend penicillin as the drug of choice for treatment (Sharma, 2006). Those patients with penicillin allergy may be given erythromycin or a cephalosporin (e.g. ceftriaxone), which are effective against most streptococci although some erythromycin resistance is emerging. In very severe *Streptococcus pyogenes* infections such as necrotising fasciitis, clindamycin may be added to penicillin as very large numbers of bacteria may overwhelm penicillin's mechanism of action.

Streptococcus pyogenes is most often spread through contact with mucus or wounds of infected individuals. Due to this, thorough hand washing is a good way to reduce the risk of becoming infected. The Center for Disease Control recommends that persons with streptococcal infections stay at home from work, school, or day care until they have been taking antibiotics for at least 24 hours to reduce the risk of transmitting the infection Center for Disease Control (CDC), (2005).

2.4.3 *Streptococcus pneumoniae*

Scientific Classification:

Domain: Bacteria

Phylum: Firmicutes

Class: Bacilli

Order: Lactobacillales

Family: Streptococcaceae

Genus: *Streptococcus*

Species: *S. pneumoniae*

Binomial name: *Streptococcus pneumoniae*

Streptococcus pneumoniae, or pneumococcus, is a Gram-positive, alpha-hemolytic, aerotolerant anaerobic member of the genus *Streptococcus* (Ryan and Ray, 2004). *Streptococcus pneumoniae* is a Gram-positive, catalase-negative cocci that has remained an extremely important human bacterial pathogen since its initial recognition in the late 1800s. *Streptococcus pneumoniae* can be differentiated from *Streptococcus viridans*, some of which are also alpha-hemolytic, using an optochin test, as *S. pneumoniae* is optochin-sensitive. *Streptococcus pneumoniae* can also be distinguished based on its sensitivity to lysis by bile

(the so-called "bile solubility test"). *Streptococcus pneumoniae* is, in general, optochin sensitive, although optochin resistance has been observed (Pikis *et al*., 2001).

2.4.3.1 Significance and Health Implications of Streptococcus pneumoniae

A significant human pathogenic bacterium, *S. pneumoniae* was recognized as a major cause of pneumonia in the late 19th century, and is the subject of many humoral immunity studies. Despite the name, the organism causes many types of pneumococcal infections other than pneumonia. These invasive pneumococcal diseases include acute sinusitis, otitis media, meningitis, bacteremia, sepsis, osteomyelitis, septic arthritis, endocarditis, peritonitis, pericarditis, cellulitis, and brain abscess (Siemieniuk *et al*., 2011). It is also one of the top two isolates found in ear infection, otitis media (Dagan, 2000). Pneumococcal pneumonia is more common in the very young and the very old (Ryan and Ray, 2004).

Worldwide, *S*. *pneumoniae* remains the most common cause of community-acquired pneumonia (CAP), bacterial meningitis, bacteremia, and otitis media. *Streptococcus pneumoniae* infection is also an important cause of sinusitis, septic arthritis, osteomyelitis, peritonitis, and endocarditis and an infrequent cause of other less common diseases. Worldwide in 2000, 14.5 million estimated episodes of invasive pneumococcal disease were reported in children younger than 5 years of age, which correlates to an estimated more than 800,000 deaths (11% of all deaths in this age group) (Ryan and Ray, 2004).

Surveillance data following introduction and widespread uptake of PCV7 immunization however, showed an astounding reduction in invasive disease of 100% in

children younger than 5 years in the United States (94% in all ages) when considering disease caused by serotypes contained in pneumococcal conjugate vaccine 7 (PCV7) (Dawn, 2012). Many subsequent studies have shown increased rates of invasive and noninvasive disease caused by serotypes not covered by the vaccine, including serotypes 15, 19A, and 33F. For these reasons, work on the development of a vaccine containing additional serotypes continued, and pneumococcal conjugate vaccine 13 (PCV13) was approved by the US Food and Drug Administration (FDA) (2010). Ongoing surveillance will help determine the effects of widespread routine immunization with PCV13 and its expanded serotype coverage on pneumococcal disease in children and adults.

2.4.4 *Haemophilus influenzae*

Scientific Classification:

Domain: Bacteria

Phylum: Proteobacteria

Class: Gammaproteobacteria

Order: Pasteurellales

Family: Pasteurellaceae

Genus: *Haemophilus*

Species: *H. influenzae*

Binomial name: *Haemophilus influenzae*

Haemophilus influenzae, formerly called Pfeiffer's bacillus or *Bacillus influenzae*, is a Gram-negative, coccobacilli bacterium. A member of the *Pasteurellaceae* family, it is generally aerobic, but can grow as a facultative anaerobe (Kuhnert and Christensen, 2008).

First isolated in 1892 by Robert Pfeiffer from the sputum of patients with pandemic influenza infection. In 1920, the organism was named *Haemophilus influenzae* (from the Greek *haemophilus,* meaning "blood-loving") to reflect the fastidious growth requirement of the organism, as well as its apparent association with influenza. *Haemophilus influenzae* was mistakenly considered to be the cause of influenza until 1933, when the viral etiology of the flu became apparent; the bacterium is colloquially known as *bacterial influenza*. In 1933, the discovery of the viral etiology of influenza eventually refuted this erroneous association. Nevertheless, subsequent findings revealed that *H. influenzae* was responsible for a wide spectrum of clinical diseases (Kuhnert and Christensen, 2008).

Haemophilus "loves heme", more specifically it requires a precursor of heme in order to grow. Nutritionally, *Haemophilus influenzae* prefers a complex medium and requires preformed growth factors that are present in blood, specifically X factor (i.e., hemin) and V factor (NAD or NADP) (Todar, 2012). In the laboratory, it is usually grown on chocolate, blood agar which is prepared by adding blood to an agar base at 80^{o}C. The heat releases X and V factors from the RBCs and turns the medium a chocolate brown color. The bacterium grows best at 35-37^{o}C and has an optimal pH of 7.6. *Haemophilus influenzae* is generally grown in the laboratory under aerobic conditions or under slight CO_2 tension (5% CO_2) (Todar, 2012).

In the 1930s, Margaret Pittman defined 2 major categories of *H influenzae:* the unencapsulated strains and the encapsulated strains (which are further classified into serotypes, with the *Haemophilus influenzae* serotype b being the most pathogenic for humans, responsible for respiratory infections, ocular infection, sepsis and meningitis). Non encapsulated organisms from sputum are pleomorphic and often

exhibit long threads and filaments (Todar, 2012). The unencapsulated strains were chiefly responsible for infections at mucosal surfaces, including otitis media, conjunctivitis, bronchitis, and sinusitis. In contrast, one of the 6 antigenically distinct encapsulated strains, strain type b, was associated with invasive diseases (e.g., septicemia, meningitis, cellulitis, septic arthritis, epiglottitis, pneumonia, etc) (Slack *et al*., 1998).

Haemophilus influenzae is a bacterium that can cause a severe infection, occurring mostly in infants and children younger than five years of age. It can cause lifelong disability and can be deadly. In spite of its name, *Haemophilus influenzae* bacteria do not cause influenza (the "flu") (European Centre for Disease Prevention and Control, 2013b).

2.4.4.1 Significance and Health Implications of Haemophilus influenzae

Haemophilus influenzae is highly adapted to its human host. It is present in the nasopharynx of approximately 75 percent of healthy children and adults (Todar, 2012). Todar (2012) also reported that it is usually the unencapsulated strains that are harbored as normal flora, but a minority of healthy individuals (3-7 percent) intermittently harbor *H. influenzae* type b (Hib) encapsulated strains in the upper respiratory tract. Pharyngeal carriage of Hib is important in the transmission of the bacterium. Even with adequate and prompt antibiotic treatment, mortality can reach up to 10% of cases (European Centre for Disease Prevention and Control, 2013a). Vaccine prophilaxis is therefore of paramount importance, in order to protect children.

There are six identifiable types of *Haemophilus influenzae* bacteria (a through f) and other non-identifiable types (called nontypeable). The one most people are familiar with is *Haemophilus influenzae* type b, or Hib. Hib disease is however vaccine-preventable.

2.4.4.2 Haemophilus influenzae presentation:

Hib bacteria are carried in the nose and throat without showing any signs of infection. Hib is spread through coughing, sneezing or close contact with an infected person (Colin, 2010).

Before Hib vaccine was introduced, about four in every 100 pre-school children carried the Hib organism; after the vaccine was introduced, carriage rates fell below the level of detection (Mc Vernon *et al*., 2004).

Hib infections are uncommon in patients older than 6 years. However, the frequency of Hib infections is increased in patients with asplenia, splenectomy, sickle cell disease, malignancies, and congenital or acquired immunodeficiencies (Colin, 2010).

The most common presentation (60% of all cases) of invasive *H. influenzae* type b (Hib) disease is meningitis, frequently accompanied by bacteraemia. Hib meningitis primarily affects children younger than 2 years, with a peak frequency rate occurring in infants aged 6-9 months (Colin, 2010).

Fifteen per cent of cases present with epiglottitis. Epiglottitis most commonly occurs in children aged 2-7 years but can also occur in adults (Colin, 2010).

Bacteraemia without any other concomitant infection occurs in 10% of cases. The remainder is made up of cases of septic arthritis, osteomyelitis, cellulitis, pneumonia and pericarditis (Colin, 2010).

Hib pneumonia typically occurs in children aged 4 months to 4 years (Colin, 2010).

Hib causes septic arthritis and cellulitis in children younger than 2 years. Hib septic arthritis also occurs in adults (Colin, 2010).

Neonatal infection:

Often due to non-typeable *H. influenzae*, which colonises the maternal genital tract (Colin, 2010).

Infection is associated with premature birth, premature rupture of membranes, low birth-weight, and maternal chorioamnionitis (Colin, 2010).

Presentations include meningitis, pneumonia, respiratory distress, scalp abscess, conjunctivitis, and vesicular eruption (Colin, 2010).

Vaccination with Hib conjugate vaccine is effective in preventing Hib infection, but does not prevent infection with the unencapsulated strains, which are termed nontypable_NTHi strains (Slack *et al*., 1998).

The most virulent strain is *H. influenzae* type b (Hib), which accounts for more than 95% of *H. influenzae* infections in children and half of infections in adults. Hib may cause bacteraemia, meningitis, cellulitis, epiglottitis, septic arthritis, pneumonia, pleural or gall-bladder empyema, endophthalmitis, urinary tract infection, abscesses, cervical adenitis, glossitis, osteomyelitis and endocarditis (Devarajan, 2009).

Non-encapsulated and non-typeable, *H. influenzae* strains cause mucosal infections, including exacerbations of chronic bronchitis (Leanord and Williams, 2002), otitis media, conjunctivitis, sinusitis, bronchitis and pneumonia.

The most common severe types of *Haemophilus influenzae* disease are:

Pneumonia (lung infection),

Bacteremia (bloodstream infection), and

Meningitis (infection of the covering of the brain and spinal cord).

However, for the treatment, in severe cases, cefotaxime and ceftriaxone delivered directly into the bloodstream are the elected antibiotics, and, for the less severe cases, an association of ampicillin and sulbactam, cephalosporins of the second and third generation, or fluoroquinolones are preferred. Fluoroquinolone-resistant *Haemophilus influenzae* has been observed (Chang *et al*., 2010). Macrolide antibiotics (e.g., clarithromycin) may be used in patients with a history of allergy to beta-lactam antibiotics. However, Macrolide resistance has also been observed (Chang *et al*., 2010).

2.4.5 *Pseudomonas aeruginosa*

Scientific Classification:

Domain:	Bacteria
Phylum:	Proteobacteria
Class:	Gamma proteobacteria
Order:	Pseudomonadaceae
Genus:	*Pseudomonas*
Species	*aeruginosa*
Binomial name:	*Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a Gram-negative, rod-shaped, asporogenous, and monoflagellated bacterium that has an incredible nutritional versatility. It is a rod about 1-5 µm long and 0.5-1.0 µm wide. *Pseudomonas aeruginosa* can catabolize a wide range of

organic molecules, including organic compounds such as benzoate. This, then, makes *P. aeruginosa* a very ubiquitous microorganism, for it has been found in environments such as soil, water, humans, animals, plants, sewage, and hospitals (Lederberg *et al.,* 2000).

Pseudomonas aeruginosa is a facultative aerobe; its preferred metabolism is respiration. It gains energy by transferring electrons from glucose, a reduced substrate, to oxygen, the final electron acceptor (Rabaey and Verstraete, 2005). When *P. aeruginosa* is in anaerobic conditions, however, it uses nitrate as a terminal electron acceptor (Valls *et al*., 2004). *Pseudomonas aeruginosa* was first described as a distinct bacterial species at the end of the nineteenth century, after the development of sterile culture media by Pasteur. In 1882, the first scientific study on *P. aeruginosa*, entitled "On the blue and green coloration of bandages," showed *P. aeruginosa*'s characteristic pigmentation: *Pseudomonas aeruginosa* produced water-soluble pigments, which, on exposure to ultraviolet light, fluoresced blue-green light. This was later attributed to pyocyanine, a derivative of phenazine, and it also reflected the organism's old names: *Bacillus pyocyaneus*, *Bakterium aeruginosa*, *Pseudomonas polycolor*, and *Pseudomonas pyocyaneus* (Botzenhardt and Doring, 1993).

Since *P. aeruginosa* can live in both inanimate and human environments, it has been characterized as a "ubiquitous" microorganism. This versatility is made possible by a large number of enzymes that allow *P. aeruginosa* to use a diversity of substances as nutrients. Most impressively, *P. aeruginosa* can switch from growing on nonmucoid to mucoid environments, which comes with a large synthesis of alginate. In inanimate environment, *P. aeruginosa* is usually detected in water-reservoirs polluted by animals and humans, such as sewage and sinks inside and outside of hospitals. It is also found in swimming pools and whirlpools because the warm temperatures are favorable to its growth (Botzenhardt and Doring, 1993).

2.4.5.1 Significance and Health Implications of Pseudomonas aeruginosa

Pseudomonas aeruginosa is an opportunistic human pathogen. It is "opportunistic" because it seldom infects healthy individuals. Instead, it often colonizes immunocompromised patients. It is usually linked with patients whose immune system is compromised by diseases or trauma, causing serious infection when their normal defences are weakened; like those with cystic fibrosis, cancer, or AIDS (Botzenhardt and Doring, 1993).

This means that it represents a genuine threat to the most vulnerable hospital patients, most commonly intensive care patients, those with depleted immunity. The severity and type of the illness it causes depends on its route into the body:

If it enters lung tissue, for example in a cystic fibrosis patient, it can cause a form of pneumonia.

Infection of a skin wound or burn can lead to extensive tissue damage or even septic shock.

If the bacterium infects the gastro-intestinal system of a vulnerable patient, then a condition called "necrotising enterocolitis" can follow, which again causes severe tissue damage. This is the form most associated with premature babies.

It has the ability to survive for up to several days on surfaces, further increasing the risk of it being passed on to patients (Botzenhardt and Doring, 1993).

It is such a potent pathogen that firstly, it attacks up two thirds of the critically-ill hospitalized patients, and this usually portends more invasive diseases. Secondly, *P.aeruginosa* is a leading Gram-negative opportunistic pathogen at most medical centers, carrying a 40-60% mortality rate. Thirdly, it complicates 90% of cystic fibrosis deaths; and lastly, it is always listed as one of the top three most

frequent Gram-negative pathogens and is linked to the worst visual diseases (Fick, 1993).

Pseudomonas aeruginosa secrets many virulent factors to colonize the cells of its host. For example, exotoxin A, the most toxic protein produced by *P. aeruginosa*, catalyzes the ADP-ribosylation to form ADP-ribosyl-EF-2, which inhibits the protein synthesis of the host's cells. Moreover, elastase, an extracellular zinc protease, attacks eukaryotic proteins such as collagen and elastin and destroys the structural proteins of the cell. It also breaks down human immunoglobin and serum alpha proteins (Lederberg *et al*., 2000).

Pseudomonas aeruginosa can be transmitted to a host via fomites, vectors, and hospital workers who are potential carriers for multiple-antibiotic-resistant strains of the pathogen. Furthermore, any *P. aeruginosa* already present on a burn victim's skin before the injury can transform from an innocuous organism on the surface of the skin to a source of infection in the bloodstream and body tissues of the same individual (Katie and Ashley, 2000). However, the usual route of transmission is through contaminated hands or medical equipment such as catheters and feeding tubes. Therefore, infection control measures, such as regular hand washing and decontamination of equipment are the most effective tactics to prevent its spread.

Pseudomonas aeruginosa attacks cystic firosis patients via airway and once it is in, it uses its flagellum to go to the hypoxic zone, an oxygen-depleted environment. At this location, *P. aeruginosa* undergoes a transition from an aerobic to an anaerobic microbe and starts forming biofilms anaerobically. Once this is formed, the *P. aeruginosa* in this community can sense their population via quorum sensing, where they secret low molecular weight pheromones that enable them to communicate with each other (Holden and

Williams, 2001). One of the enzymes responsible for quorum sensing is tyrosine phosphatase (TpbA). This enzyme relays extracellular quorum sensing signals to polysaccharide production and biofilm formation outside the cells (Uedal and Wood, 2009). This gives them the ability to resist many defenses, including anti-Pseudomonas antibiotics such as ticarcillin, ceftazidime, tobramycin, and ciprofloxacin, because once the bacteria sense that their outer layer of biofilm is being destroyed, the inner layers will grow stronger to reestablish the community (Smiley and Hassett, 2006).

2.4.6 *Staphylococcus aureus*

Scientific Classification:

Domain: Bacteria

Phylum: Firmicutes

Class: Bacilli

Order: Bacillales

Family: Staphylococcaceae

Genus: *Staphylococcus*

Species: *S. aureus*

Binomial name: *Staphylococcus aureus*

The name *Staphylococcus* comes from the Greek *Staphyle*, meaning a bunch of grapes, and *kokkos*, meaning berry. *Staphylococci* are Gram-positive, facultative anaerobic, usually unencapsulated cocci. *Staphylococcus aureus* is a bacterium that is a member of the Firmicutes, and is frequently found in the human respiratory tract and on the skin. *Staphylococcus aureus* appears as grape-like clusters when viewed through a microscope, and has large, round, golden-yellow colonies, often with hemolysis, when grown on blood agar plates (Ryan and Ray, 2004).

Staphylococcus aureus is catalase-positive (meaning it can produce the enzyme catalase), so is able to convert hydrogen peroxide (H_2O_2) to water and oxygen. This test is sometimes used to distinguish *Staphylococci* from *Enterococci* and *Streptococci*. Previously *S. aureus* was differentiated from other *Staphylococci* by the coagulase test. However it is now known that not all *S. aureus* are coagulase positive (Ryan and Ray, 2004), and that incorrect species identification can impact effective treatment and control measures (Matthews *et al*., 1997). Some strains of *S. aureus* are capable of producing staphyloxanthin - a golden coloured carotenoid pigment. This pigment acts as a virulence factor, primarily by being a bacterial antioxidant which helps the microbe evade the reactive oxygen species which the host immune system uses to kill pathogens (Clauditz *et al*., 2006; Liu *et al*., 2005).

2.4.6.1 Significance and Health Implications of Staphylococcus aureus

It is estimated that 20% of the human population are long-term carriers of *S. aureus* (Kluytmans *et al*., 1997) which can be found as part of the normal skin flora and in anterior nares of the nasal passages (Kluytmans *et al*., 1997; Cole *et al*., 2001). *Staphylococcus aureus* can cause a range of illnesses, from minor skin infections, such as pimples, impetigo, boils (furuncles), cellulitis folliculitis, carbuncles, scalded skin syndrome, and abscesses, to life-threatening diseases such as pneumonia, meningitis, osteomyelitis, endocarditis, Toxic Shock Syndrome (TSS), bacteremia, and sepsis. Its incidence ranges from skin, soft tissue, respiratory, bone, joint, endovascular to wound infections. It is still one of the five most common causes of nosocomial infections and is often the cause of postsurgical wound infections. Each year, about 500,000 patients in American hospitals contract a Staphylococcal infection (Bowersox, 1999).

Although *S. aureus* is not always pathogenic, it is a common cause of skin infections (e.g. boils), respiratory disease (e.g. sinusitis), and food poisoning. *S. aureus* can survive from

hours to weeks, or even months, on dry environmental surfaces, depending on strain (Cimolai, 2008).

Staphylococcus aureus infections can spread through contact with pus from an infected wound, skin-to-skin contact with an infected person by producing hyaluronidase that destroys tissues, and contact with objects such as towels, sheets, clothing, or athletic equipment used by an infected person. Deeply penetrating *S. aureus* infections can be severe. Prosthetic joints put a person at particular risk of septic arthritis, and staphylococcal endocarditis (infection of the heart valves) and pneumonia. *Staphylococcus aureus* can host phages, such as Panton-Valentine Leukocidin, that increase its virulence (Cenci-Goga *et al*., 2003).

Staphylococcus aureus is extremely prevalent in atopic dermatitis patients. It is mostly found in fertile, active places, including the armpits, hair, and scalp. Large pimples that appear in those areas may exascerbate the infection if lacerated. This can lead to Staphylococcal Scalded Skin Syndrome (SSSS). A severe form of this, Ritter's disease, can be observed in neonates (Cenci-Goga *et al*., 2003).

Although *S. aureus* can be present on the skin of the host, a large proportion of its carriage is through the anterior nares of the nasal passages (Kluytmans *et al*., 1997). The ability of the nasal passages to harbour *S. aureus* results from a combination of a weakened or defective host immunity and the bacteria's ability to evade host innate immunity (Quinn and Cole, 2007).

Staphylococcus aureus is an incredibly hardy bacterium, as was shown in a study where it survived on polyester for just under three months (Neely and Maley, 2000); polyester is the main material used in hospital privacy curtains.

The bacteria are transported on the hands of healthcare workers, who may pick them up from a seemingly healthy patient carrying a benign or commensal strain of *S. aureus*, and then pass it on to the next patient being treated. Introduction of the bacteria into the bloodstream can lead to various complications, including, but not limited to, endocarditis, meningitis, and, if it is widespread, sepsis. An important and previously unrecognized means of community-associated MRSA colonization and transmission is during sexual contact (Cook *et al*., 2007).

The emergence of antibiotic-resistant forms of pathogenic *S. aureus* (e.g. MRSA, VRSA) is a worldwide problem in clinical medicine. Spread of *S. aureus* (including MRSA, VRSA) generally is through human-to-human contact, although recently some veterinarians have discovered the infection can be spread through pets (Sing *et al*., 2008), with environmental contamination thought to play a relatively unimportant part. Emphasis on basic hand washing techniques are, therefore, effective in preventing its transmission. The use of disposable aprons and gloves by staff reduces skin-to-skin contact and, therefore, further reduces the risk of transmission.

The treatment of choice for *S. aureus* infection is penicillin; in most countries, though, penicillin resistance is extremely common, and first-line therapy is most commonly a penicillinase-resistant β-lactam antibiotic (for example, oxacillin or flucloxacillin). Combination therapy with gentamicin may be used to treat serious infections, such as endocarditis (Bayer *et al*., 1998), but its use is controversial because of the high risk of damage to the kidneys (Bayer *et al*., 1998). The duration of treatment depends on the site of infection and on severity.

Ethanol has proven to be an effective topical sanitizer against MRSA. Quaternary ammonium can be used in conjunction with ethanol to increase the duration of the sanitizing action.

2.5 Antibiotic Resistance

Bacteria and other microorganisms that cause infections are remarkably resilient and can develop ways to survive drugs meant to kill or weaken them. This antibiotic resistance, also known as antimicrobial resistance, is due largely to the increasing use of antibiotics. So, antibiotic resistance is the ability of a microorganism to withstand the effects of an antibiotic.

Recent findings show that there is no necessity of large populations of bacteria for the appearance of antibiotic resistance (Science Daily, 2012). Any heterogeneous environment with respect to nutrient and antibiotic gradients may facilitate the development of antibiotic resistance in small bacterial populations and this is also true for the human body. Several studies have demonstrated that patterns of antibiotic usage greatly affect the number of resistant organisms which develop. For the past 70 years, antimicrobial drugs, such as antibiotics, have been successfully used to treat patients with bacterial and infectious diseases. Over time, however, many infectious organisms have adapted to the drugs designed to kill them, making the products less effective (Science Daily, 2012).

Antibiotics are one of the most important therapeutic discoveries in medical history. They have revolutionized the way patients with bacterial infections are being treated and have contributed to reducing the mortality and morbidity from bacteria related diseases. They are also an essential tool for modern medicine and common procedures such as transplantation, chemotherapy for cancer and even orthopaedic surgery could not be

performed without the availability of potent antibiotics. Unfortunately, antibiotics have been liable to misuse. They are often unnecessarily prescribed for viral infections, against which they have no effect. Similarly when diagnoses are not accurately made, more often than not, broad-spectrum antibiotics, i.e. antibiotics that kill a large proportion of various bacteria and not only the bacteria responsible for the disease, are prescribed because the causative micro-organism is not known. And this brings about resistance even to the broad spectrum antibiotics. That is why Colistin, an older antibiotic once removed from use, is treating drug-resistant bacterial infections (Science Daily, 2012).

Although there were low levels of pre-existing antibiotic-resistant bacteria before the widespread use of antibiotics (Caldwell and Lindberg, 2011), evolutionary pressure from their use has played a role in the development of multidrug resistance varieties and the spread of resistance between bacterial species (Hawkey and Jones, 2009). In medicine, the major problem of the emergence of resistant bacteria is due to misuse and overuse of antibiotics (WHO, 2002). In some countries, antibiotics are sold over the counter without a prescription, which also leads to the development of resistant strains. Other practices contributing towards resistance include the addition of antibiotics to livestock feed (Ferber, 2002; Matthew, 2007). Household use of antibacterials in soaps and other products, although not clearly contributing to resistance, is also discouraged (as not being effective at infection control) (CDC, 2009). Also, unsound practices in the pharmaceutical manufacturing industry can contribute towards the likelihood of creating antibiotic-resistant strains (Larsson and Fick, 2009).

Misuse of antibiotics leads to the emergence and selection of resistant bacteria. Doctors in Europe and worldwide now are sometimes facing situations where infected patients cannot be treated adequately because the responsible bacterium is totally resistant to available antibiotics (ECDC, 2013a). Overuse of broad-spectrum antibiotics, such as

second and third-generation Cephalosporins greatly hastens the development of methicillin resistance.

Antibiotic resistance is a serious and growing phenomenon in contemporary medicine and has emerged as one of the eminent public health concerns of the 21st century. The sheer volume of antibiotics prescribed is the major factor in increasing rates of bacterial resistance rather than compliance with antibiotics (Pechère, 2001). A single dose of antibiotics leads to a greater risk of resistant organisms to that antibiotic in the person for up to a year (Costelloe, 2010).

Inappropriate prescribing of antibiotics has been attributed to a number of cases, including people who insist on antibiotics, physicians who simply prescribe them as they feel they do not have time to explain why they are not necessary, and physicians who do not know when to prescribe antibiotics or else are overly cautious for medical legal reasons (Arnold and Straus, 2005). Common cold is the most common reasons antibiotics are prescribed (Ronald and Olaf, 2009) even though antibiotics are completely useless against viruses.

A large number of people do not finish a course of antibiotics primarily because they feel better (varying from 10 % to 44 %, depending on the country) (Pechère *et al*., 2007). Compliance with once-daily antibiotics is better than with twice-daily antibiotics (Kardas, 2007). Patients taking less than the required dosage or failing to take their doses within the prescribed timing results in decreased concentration of antibiotics in the blood-stream and tissues, and, in turn, exposure of bacteria to sub-optimal antibiotic concentrations increases the frequency of antibiotic resistant organisms (Thomas *et al*., 1998). Advice to always complete a course of antibiotics is not based on strong evidence, and some researchers discourage the use of the prescription label “Finish all this medication unless otherwise

directed by prescriber". Often, antibiotics can be safely stopped 72 hours after symptoms resolve (McCormack and Allan, 2012). However, some infections require treatment long after symptoms are gone, and in all cases, an insufficiency course of antibiotics may lead to relapse (with an infection that is now more antibiotic resistant).

There is evidence that naturally occurring antibiotic resistance is common (Wright, 2010). The genes that confer this resistance are known as the environmental resistome (Wright, 2010). These genes may be transferred from non-disease-causing bacteria to those that do cause disease, leading to clinically significant antibiotic resistance (Wright, 2010). It may take the form of spontaneous or induced genetic mutation, or the acquisition of resistance genes from other bacterial species by horizontal gene transfer via conjugation, transduction, or transformation. Many antibiotic resistance genes reside on transmissible plasmids, facilitating their transfer. Exposure to an antibiotic naturally selects for the survival of the organisms with the genes for resistance. In this way, a gene for antibiotic resistance may readily spread through an ecosystem of bacteria. Antibiotic-resistance plasmids frequently contain genes conferring resistance to several different antibiotics. Genes for resistance to antibiotics, like the antibiotics themselves, are ancient (D'Costa *et al*., 2011).

However, the increasing prevalence of antibiotic-resistant bacterial infections seen in clinical practice stems from antibiotic use both within human medicine and Veterinary medicine. The resistant bacteria in animals due to antibiotic exposure can be transmitted to humans via three pathways, those being through the consumption of meat, from close or direct contact with animals, or through the environment (Schneider and Garrett, 2009). Any use of antibiotics can increase selective pressure in a population of bacteria to allow the resistant bacteria to thrive and the susceptible bacteria to die off. As resistance towards antibiotics becomes more common, a greater need for alternative treatments arises. However, despite a push for new antibiotic therapies, there has been a continued decline in

the number of newly approved drugs (Donadio *et al*., 2010). Antibiotic resistance therefore poses a significant problem.

In 2001, the Union of Concerned Scientists estimated that greater than 70 % of the antibiotics used in the US are given to food animals (for example, chickens, pigs and cattle), in the absence of disease (Union of Concerned Scientists, 2001). Hence, the amounts given are termed "sub-therapeutic", i.e., insufficient to combat disease-because no demonstrable disease is present. Sub-therapeutic dosages kills some, but not all, of the bacterial organisms in the animal-leaving those that are naturally antibiotic-resistant.

Nosocomial infections overwhelmingly dominate cases where MDR pathogens are implicated, but multidrug-resistant infections are also becoming increasingly prevalent in the community. However, Phage therapy may prove as an important alternative to antibiotics for treating multidrug resistant pathogens (Mathur *et al*., 2003).

Examples of common multidrug-resistant bacteria:

Methicillin-resistant *Staphylococcus aureus* (MRSA)

Vancomycin-resistant *Staphylococcus aureus* (VRSA)

Vancomycin-resistant enterococci (VRE)

Extended-spectrum beta-lactamase (ESBL)-producing *Enterobacteriaceae* (examples of common *Enterobacteriaceae* are *Escherichia coli* and *Klebsiella pneumoniae*)

Carbapenemase-producing *Enterobacteriaceae* (*e.g. Klebsiella pneumoniae*)

Multidrug-resistant *Pseudomonas aeruginosa*

Clostridium difficile

Antibiotic-Resistant *Mycobacterium tuberculosis (TB)*

Multidrug-Resistant *Neisseria gonorrhoeae* (Gonorrhea)

2.5.1 Mechanisms of antibiotic resistance

Bacteria may demonstrate any of the Five General Mechanisms of Antibiotic Resistance:

Lack of entry; Decreased cell permeability

Greater exit; Active efflux

Enzymatic inactivation of the antibiotic

Altered target; Modification of drug receptor site

Synthesis of resistant metabolic pathway

These mechanisms can be grouped into three broad categories:

Permeability mechanisms:

Lack of entry: Decreased cell permeability to the drug/antibiotic (Li and Nikadio, 2009).

Greater exit; Active efflux: by increasing active efflux (pumping out) of the drugs across the cell surface (Li and Nikadio, 2009)

Enzymatic inactivation of the antibiotic:

Altered target or pathway;

Altered target: for example, alteration of PBP—the binding target site of penicillins—in MRSA and other penicillin-resistant bacteria.

Modification of drug receptor site: for example, enzymatic deactivation of penicillin G in some penicillin-resistant bacteria through the production of β-lactamases.
Synthesis of resistant metabolic pathway: for example, some sulfonamide-resistant bacteria do not require para-aminobenzoic acid (PABA), an important precursor for the synthesis of folic acid and nucleic acids in bacteria inhibited by sulfonamides, instead, like mammalian cells, they turn to using preformed folic acid.

Aminoglycoside antibiotics, such as kanamycin, gentamicin, streptomycin, etc., were once effective against staphylococcal infections until strains evolved mechanisms to inhibit the aminoglycosides' action, which occurs via protonated amine and/or hydroxyl interactions with the ribosomal RNA of the bacterial 30S ribosomal subunit (Carter *et al.*, 2000). In *S. aureus*, the best-characterized aminoglycoside-modifying enzyme is aminoglycoside adenylyltransferase 4' IA (*ANT (4') IA*). This enzyme has been solved by X-ray crystallography (Sakon *et al*., 1993).

Some types of efflux pumps can act to decrease intracellular quinolone concentration (Morita *et al*., 1998). In Gram-negative bacteria, plasmid-mediated resistance genes produce proteins that can bind to DNA gyrase, protecting it from the action of quinolones. Finally, mutations at key sites in DNA gyrase or topoisomerase IV can decrease their binding affinity to quinolones, decreasing the drug's effectiveness (Robicsek *et al*., 2006). Research has shown that, bacterial protein LexA may play a key role in the acquisition of bacterial mutations giving resistance to quinolones and rifampicin (Cirz *et al*., 2005). However, the major mechanism of resistance involves the introduction of mutations in genes encoding penicillin-binding proteins.

CHAPTER THREE

3.0 Materials and Methods

3.1 Materials

3.1.1 Equipment

Microscope (Wild M11, Switzerland), Colony counters (NAPCO Model 630 Portland, Oregon, U.S.A.), Refrigerator (NAPCO Model 630 Portland, Oregon, U.S.A.), Incubator (Natural appliance:Aheinicke Company Portland, Oregon- made in U.S.A.Model-630), Autoclave (Adelphi MFG Co Ltd,Portland autoclave), Electronic weighing balance(Top balance digital, U.S.A, Ohaus, PA313-model), Digital Water

bath (Mc Donald Scientific International, England), Hot-Air-Oven (Baird and Tatlock, England) and Wire (or inoculating) loop (KD Surgicals, India).

3.1.2 Glasswares

Petri-dishes (Pyrex, England), Test-tubes (Pyrex, England), Beakers (Pyrex, England), Conical flask (Pyrex, England) and Measuring cylinder (Pyrex, England).

3.1.3 Reagents

Crystal violet (May and Baker, England), Lugol's iodine (May and Baker, England), neutral red (May and Baker Ltd. Dagenham, England), oil immersion (BDH Chemicals, England), Potassium Hydroxide (May and Baker, England), Methyl red (May and Baker, England), Alpha Naphthol (Fisher Scientific, United Kingdom), Sodium deoxycholate (BDH, England), Hydrogen peroxide (SKG Pharma., Nigeria), Tween 80: (BDH, England) and Oxidase reagent/strip (Liofilchem s.r.l. Bacteriology products, Italy).

3.1.4 Culture Media

MacConkey Agar (Oxoid, England), Nutrient Agar (Biotec Laboratories, United Kingdom), Urea Agar Base (Oxoid, England), Cetrimide Agar (Merck, Germany), Simmon's Citrate (Oxoid, England), Mueller-Hinton Agar (Oxoid, England), Nutrient Broth (Fluka, U.S.A.), Peptone water (Fluka, U.S.A.) and MRVP Broth (Oxoid, England).

3.1.5 Antibiotic Discs

Ceftriaxone 30µg (Oxoid, UK), Amoxicillin-Clavulanic Acid 20/10µg (Oxoid, UK), Gentamicin 10µg (Oxoid, UK), Levofloxacin 5µg (Oxoid, UK) and Azithromycin 15µg (Oxoid, UK). Other antibiotics used are Bacitracin (Liofilchem s.r.l. Bacteriology products, Italy) and Optochin (Liofilchem s.r.l. Bacteriology products, Italy).

3.1.6 Standard Antibiotic Formulations

Ceftriaxone 500mg (Evans Medical PLC, Nigeria), Augmentin® 650mg (Beecham Pharmaceuticals, Brentford, England), Gentamicin 80mg (Gentalek), Levofloxacin 500mg (Evans Medical PLC RC1161, Nigeria) and Azithromycin 250mg (Greenlife Pharmaceuticals Ltd., Nigeria).

3.1.7 Test Organisms (Clinical isolates)

Streptococcus pneumoniae, *Streptococcus pyogene, Pseudomonas aeruginosa*, *Staphylococcus aureus, Klebsiella pneumoniae* and *Haemophilus influenzae*.

3.2 Methodology

3.2.1 Study Area

The study area was Zaria, Kaduna State, Nigeria. However, the research was conducted in the Faculty of Pharmaceutical Sciences, Department of Pharmaceutics and Pharmaceutical Microbiology, Ahmadu Bello University (A.B.U.), Zaria, Nigeria.

3.2.2 Collection of Materials

Pure honey was collected from Taraba State. However, the lemon was obtained from the Staff quarters in Area-A, A.B.U., Zaria.

3.2.2.1 Preliminary Test for the Confirmation of the Purity of the Honey

Basically the following tests were used to ascertain the purity of the honey used:

The Flame Test: This involves lighting up a cotton bud dipped into the honey with a match-stick flame; or lighting up a match-stick dipped into a honey.

Dissolution Test: This involves adding a tablespoon of honey into the water. If the honey is impure, it will dissolve in the water at top layer. If it is pure, the honey will

stick together and sink as a solid lump to the bottom of the glass. This is because pure honey does not immediately dissolve in water; you will notice that it takes a bit of effort to stir it in the water to dissolve the lumpy bits, whereas sugar tends to dissolve easily in a jiggery as you drop them into the water.

Crystallization Test: This involves subjecting the honey sample to a very low temperature (refrigerating temperature). Even below 5 °C, pure honey will not crystallize and, thus, the original texture and flavor can be preserved indefinitely (Tomasik, 2004).

3.2.2.2 The Retrospective Study

Ethical approval for the collection of samples was obtained from University Health Services (see appendix V).

Patients' records were obtained from the laboratory record books of Medical Microbiology Department, Ahmadu Bello University Zaria and Microbiology Department, Ahmadu Bello University Health Services Samaru Campus, Zaria. Information obtained include sex, age, types of samples collected, commonly isolated bacteria and common antibiotics they were susceptible or resistant to. The period observed was January, 2011 to February, 2012.

3.2.3 Isolation and Identification of Bacteria from Respiratory Tract Infections

Clinical isolates were collected from throat swab, ear swab, nasal secretions and sputum samples from patients presented with RTIs in Ahmadu Bello University Teaching Hospital (ABUTH), Zaria and Ahmadu Bello University Health Services (ABUHS) Samaru Campus, Zaria. The samples were then inoculated on Blood Agar, Chocolate agar, MacConkey agar and cetrimide agar, and the plates incubated at 37 ^{0}C for 24-48 hours.

Initial identification of the growing microorganisms was done by colony morphology and Gram-Staining method. Pure colonies were sub-cultured on Blood agar, Nutrient agar and Chocolate agar media. Further identification or confirmation was carried out using Biochemical tests as recommended by Cheesbrough (2010).

Culture Media Preparation:

The media were prepared according to the manufacturer's direction, and sterilized at 121 ^{0}C for 15 minutes. The media were allowed to cool to about 47 ^{0}C and poured into sterile plates.

Isolation and Purification of Isolates:

Pure colonies were isolated and inoculated into nutrient broth (5 ml), and incubated at 37 ^{0}C for 24 hours.

All organisms used were purified by sequentially streaking a loopful of the resulting overnight broth culture on dried surface of sterile Mannitol salt agar, Blood agar, Chocolate agar, Mac Conkey agar, and Cetrimide agar to give well distinct isolate colonies after incubation at 37 ^{0}C for 24-48 hrs. The discrete colonies were then picked and inoculated on nutrient agar slants for subsequent use (Stephen *et al*., 2004).

Gram Staining:

The Gram staining was carried out in accordance with the standard method (Cheesbrough, 2010).

3.2.4 Biochemical Tests

3.2.4.1 Methyl Red-Voges Proskauer Test

Procedure:

A colony of the test organism was inoculated into 5 ml of MR-VP broth and incubated for 48-72 hours at 37 ^{0}C.

After this period of incubation, about 2 ml of the inoculated broth was transferred to a sterile test tube.

To this small quantity, 2-3 drops of methyl red was added.

Development of a red colour on the addition of the indicator signified positive methyl red test.

A yellow colour signified a negative test.

To the rest of the inoculated broth in the original test tube, 3 drops of 5 % α-Naphthol reagent was added, followed by 40 % KOH and shaken vigorously losing the cap of the test tube and placed in a sloping position.

The development of red colour starting from the liquid air interphase within 1hour indicated a VP positive test.

No colour change occurs signified a VP negative test.

3.2.4.2 Indole Test

Procedure:

The organism was grown in 5 ml peptone water for 24 hours.

After the 24 hours of incubation, 3-4 drops of Kovac's indole reagent was added.

The development of a red colour in the reagent layer above the broth within 1minute indicated a positive reaction.

In a negative reaction, the indole reagent retained its yellow colour.

3.2.4.3 Bacitracin Sensitivity Test

Procedure:

Plates of blood agar were streaked with the culture under test for confluent growth.

A bacitracin disc was placed in the centre of the streaked area and the plates incubated in candle jar at 37 ^{0}C.

The plates were observed after 24 hours of incubation.

A wide zone of inhibition indicated a positive test (i.e. sensitivity to bacitracin).

Absence of a zone of inhibition indicated a negative result.

3.2.4.4 Urease Test

Procedure:

The test organism was inoculated into Christerifens Urea Agar slant (originally yellow in color).

This was then incubated at 37 ^{0}C for 24-48 hours.

After the period of incubation, color changes were noted (yellow to pink indicated positive test).

In negative test, the agar retained its color.

3.2.4.5 Optochin Sensitivity Test

Procedure:

Plates of blood agar were streaked with the culture under test for confluent growth.

An optochin disc was placed in the centre of the streaked area and the plates incubated in candle jar at 37 ^{0}C.

The plates were observed after 24 hours of incubation.

A wide zone of inhibition indicated a positive test (i.e. sensitivity to optochin).

Absence of a zone of inhibition indicated a negative result.

3.2.4.6 Bile solubility test

This helps to differentiate *Streptococcus pneumoniae*, which is soluble in bile and bile salts, from other *alpha-haemolytic Streptococci* (*Viridans Streptococci*) which are insoluble.

Tube method:

Although the bile solubility test can be performed by testing colonies directly on a culture plate or on a slide, a tube technique is recommended because the results are easier to read:

Several colonies of the test organism were emulsified in a tube containing 2 ml sterile physiological saline, to give a turbid suspension.

The suspension was divided into two test tubes.

To one tube, 2 drops of sodium deoxycholate reagent were added and mixed.

To the other tube (negative control); 2 drops of sterile distilled water were added and mixed.

Both tubes were incubated at 37 ^{0}C for 10–15 minutes.

Clearing of turbidity was observed in the tube containing the sodium deoxycholate.

The test tube with cleared turbidity indicated a positive result.

3.2.4.7 Catalase test

This test is used to differentiate those bacteria that produce the enzyme catalase, such as *Staphylococci*, from non-catalase producing bacteria such as *Streptococci*.

Method:

About 2–3 ml of 6 % hydrogen peroxide solution was poured into a test tube.

Sterile glass rod was used to remove several colonies of the test organism and immersed in the hydrogen peroxide solution.

Immediate bubbling was observed for catalase positive organism.

3.2.4.8 Citrate utilization test

This test is one of several techniques used occasionally to assist in the identification of *Enterobacteria*. The test is based on the ability of an organism to use citrate as its only source of carbon.

Method using Simmon's citrate agar:

Slopes of the medium were prepared in bijou bottles as recommended by the manufacturer.

A sterile straight wire-loop was used to first streak the slope with a saline suspension of the test organism and then the butt was stabbed.

This was then incubated at 37 ^{0}C for 48 hours.

A bright blue colour in the medium (i.e. change in the color of the medium from green to bright blue) indicated the presence of citrate positive organism.

3.2.4.9 Coagulase test

This test was used to identify *S. aureus* which produces the enzyme coagulase.

Slide test method (detects bound coagulase):

1. A drop of sterile distilled water was placed on two separate slides.

2. A colony of the test organism (previously checked by Gram staining) was emulsified in each of the drops to make two thick suspensions.

3. A loopful of plasma was added to one of the suspensions, and mixed gently.

4. Clumping of the organisms within 10 seconds, indicates the presence of coagulase positive organism.

5. No plasma was added to the second suspension. This was used to differentiate any granular appearance of the organism from true coagulase clumping.

Tube test method (detects free coagulase):

1. Three small test tubes were labeled as follows:

T=Test organism (18–24 h broth culture).

Pos=Positive control (18–24 h *S. aureus* broth culture).

Neg=Negative control (sterile broth).

2. 0.2 ml of plasma was pipetted into each tube.

3. 0.8 ml of the test organism 'broth culture' was added to tube T.

(i) 0.8 ml of the *S. aureus* culture (control) was added to the tube labeled 'Pos'.

(ii) 0.8 ml of sterile broth was added to the tube labeled 'Neg'.

4. After mixing gently, the three tubes were incubated at 37 ^{0}C. This was examined for clotting after 1 hour. If no clotting has occurred, it was examine after 3 hours. If the test is still negative, the tubes were kept at room temperature overnight and examined again.

3.2.4.10 Oxidase test

Cytochrome oxidase test:

The oxidase test was used to assist in the identification of *Pseudomonas*, *Neisseria*, *Vibrio*, *Brucella*, and *Pasteurella* species, all of which produce the enzyme cytochrome oxidase.

Method using an oxidase reagent strips:

1. The strip was moistened with a drop of sterile water.

2. A piece of sterile glass rod was used to remove a colony of the test organism and rubbed on the strip.

3. Red-purple colour within 20 seconds indicates the presence of oxidase positive organism.

3.2.5 Antibacterial Activity Testing

3.2.5.1 Standardization of inocula

Overnight cultures of the test organisms were diluted in sterile normal saline to match 0.5 Mc Farland turbidity as used by (Samie *et al.,* 2005). At this point, the organisms should be at a concentrations of approximately 10^5 to 10^6 cfu/ml.

3.2.5.2 Susceptibility testing of the bacteria isolates to Honey, Lemon juice and the Standard antibiotic formulations

The honey was diluted with sterile distilled water to concentrations of between 25 % (v/v) to 50 % (v/v). The lemon was washed with water (to remove sand and other particles) and then washed with sterile distilled water and then cut using sterile knife before the juice was squeezed out and sieved. The sieving was done to remove the seeds and other particles. The juice was then diluted with sterile distilled water to concentrations of between 25 % (v/v) to 50 % (v/v).

For combination studies, rato of mixtures (Lemon juice:Honey:water) was as follows 10:50:40; 20:50:30; 30:50:20; 40:50:10; 50:50:0 (v/v) concentrations and Honey:Lemon juice:water at 10:50:40; 20:50:30; 30:50:20; 40:50:10; 50:50:0 (v/v) concentrations.

Agar well diffusion technique as used by Adeniyi *et al.,* (1996) and Adeshina *et al*., (2010) was used to determine the antibacterial activities of the Honey, Lemon and the combinations of the two agents.

Mueller-Hinton agar were prepared and poured into sterile petri-dishes, and then allowed to set. The prepared inocula were then spread thinly with sterile swab stick on the surface of the agar. Thereafter, holes were bored using sterile cork-borer (number 4) to make uniform wells on the inoculated agar. The bottom of the hole was then sealed with 2 drops of molten sterile Mueller Hinton agar and then filled with the test antibacterial agent (honey, lemon, honey/lemon). Care was taken to ensure that the correct measure of concentration of the agent was added into each of the holes and to avoid spillage.

The standard antibiotic discs were also placed at some points in the same petri dishes for them to undergo the same conditions. Pre-incubation diffusion time (45 minutes to 1 hour) was allowed, after which the petri-dishes were incubated at 37 ^{o}C for 18-24 hrs.

After the incubation period, the diameters of the zones of inhibition were measured in millimeters (with the aid of a metre rule) and recorded accordingly. Interpretation of zones sizes in terms of sensitivity or susceptibility, and resistance was based on the values provided by Clinical and Laboratory Standards Institute (CLSI), (2008).

3.2.5.3 Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the agents

The MIC was carried out using the broth dilution method as used by (Kabir *et al*., 2005) and as described by (CLSI, 2008). Stock solutions of 125 µg/ml (i.e. 50 mg of the standard antibiotic formulation powder was weighed using digital weighing balance and mixed with 200 ml of sterile distilled water) was prepared for CRO, AMC, and LEV; 100 µg/ml and 50 µg/ml was also prepared for CN and AZM respectively based on their different MIC break point values. Two-fold serial dilution of the stock solutions were made in eight (8) test tubes (plus three control test tubes; one containing Mueller Hinton broth and the test bacteria, another containing Mueller-Hinton broth and the reference antibiotics and the

other containing Mueller-Hinton broth and sterile distilled water) of Mueller-Hinton broth, with the first test tube being a double strength and the others single strength to obtain concentrations between 125-0.98 µg/ml, 100-0.78 µg/ml and 50.0-0.39 µg/ml.

For the Honey and Lemon, 100 µg/ml, 90 µg/ml, 80 µg/ml, 70 µg/ml and 60 µg/ml of stock solutions were prepared after sieving under sterile condition. Two-fold serial dilution of the stock solutions were also made in eight (8) test tubes (plus three control test tubes; one containing Mueller Hinton broth and the test bacteria, another containing Mueller-Hinton broth and the test agent and the other containing Mueller-Hinton broth and sterile distilled water) of Mueller-Hinton broth with the first test tube being a double strength and the others single strength to obtain concentrations between 100-0.78 µg/ml, 90.0-0.71 µg/ml, 80.0-0.63 µg/ml, 70.0-0.55 µg/ml and 60.0-0.47 µg/ml.

One hundred microliter (100 µl) of the standardized inoculums (10^5 to 10^6 cfu/ml) was then inoculated to the different dilutions of the agents and the antibiotics in the 8 test tubes plus the organism-control test tube. The organisms were then incubated at 37 ^{0} C for 24 hours. The lowest concentration (highest dilution) of the honey and/or lemon or the antibiotics which showed clear solution or no visible bacterial growth (i.e. no turbidity) when compared with the control tubes was regarded as the Minimum Inhibitory Concentration (MIC).

A Minimum Bactericidal Concentration (MBC) was determined from the broth dilution tests by sub-culturing to antibiotic-free Mueller Hinton agar (i.e. Mueller Hinton Agar+5 % v/v tween 80) from tubes showing no visible growth after overnight (24 hours) incubation at 37 ^{0}C. The lowest concentration of an antibacterial agent that kills more than 99.9 % of the initial inoculation after the 24 hours incubation represents the MBC as used by (Aboaba *et al*., 2006).

3.2.5.4 Rate of Kill

A 0.1 ml aliquot of standardized overnight culture of the test organisms that were susceptible and those that were resistant to the standard antibiotics, honey, lemon and mixture of both (approximately 10^6 cfu/ml) was added to 9.9 ml each of test antibacterial agents (honey and/or lemon) formulated with sterile distilled water (using the concentrations of Sub-MIC, Around-MIC, and Above-MIC). This was mixed thoroughly and kept inside Digital shaker bath at 37 ^oC. At different time interval (0, 30, 60, 120, 240, 360 and 1440 minutes) 1 ml test organism/extract admixture was taken and ten-fold dilution protocol performed with sterile inactivated normal saline (i.e. normal saline with 5 % Tween 80). These dilutions were then plated out in duplicates on sterile molten Mueller – Hinton agar supplemented with 5 % Tween 80. The agar plates were then incubated at 37 ^oC for 24 hours. After the period of incubation, colonies observed were counted with the aid of a Colony Counter (Batch *et al*., 1998; Adeshina *et al*., 2010).

These procedures were repeated for Sub-MIC, Around-MIC, and Above-MIC values of levofloxacin and ceftriaxone; as the standard antibacterial agents.

3.2.6 Interaction studies

The MIC values obtained in the MIC Tests were used to determine interaction effects in the combination of honey and lemon juice. The MIC of honey, lemon juice and that of the combination of honey and lemon juice were evaluated. To evaluate the effect of each test agent in the combination (i.e. honey and lemon), the fractional inhibitory concentration (FIC) was calculated for each of the agents in the combination. The ∑FICs were calculated as follows:

∑FIC=FIC A + FIC B.

Where FIC A is the MIC of drug A in the combination/MIC of drug A alone, and FIC B is the MIC of drug B in the combination/MIC of drug B alone (Gani *et al*., 2005).

A Fractional Inhibitory Concentration Index (FICI) was used to interpret the results (Eugene *et al*., 2003). A combination is considered synergistic when the Fractional Inhibitory Concentration (∑FIC) index is ≤0.5; Additive is indicated by a FIC index >0.5 to ≤1.0; Indifference is indicated by a FIC index >1.0 to ≤4, while antagonism is when the ∑FIC is >4 (Prinsloo *et al*., 2008 and Sopirala *et al*., 2010).

3.2.7 Statistical Analysis

Using the null hypothesis, data obtained in this study were expressed as Mean ± Standard Error of Mean (SEM). The honey and lemon juice mixtures were compared with their use concentrations separately by Analysis of Variance (ANOVA) using 'Tukey post-hoc test'. This is to see if the combination of honey and lemon juice has significant antibacterial activity against bacteria isolated from the RTIs than when used separately. Differences were considered significant if $P<0.05$.

CHAPTER FOUR

4.0 Results

4.1 Retrospective Studies

The retrospective studies were carried out in order to know the commonly isolated bacteria related to the Respiratory Tract Infections (RTIs) and the common antibiotics used in the selected hospitals from January, 2011 to February, 2012.

From the Laboratory record book at Medical Microbiology Ahmadu Bello University Teaching Hospital (ABUTH), most of the isolates were obtained from Sputum samples (61.0 %), followed by Throat Swab samples (24.4 %) and the least was Nasal samples (0.41 %). The most common isolates were *Streptococcus pneumoniae* (59.3 %), followed by *Staphylococcus aureus* (20.2 %) and the least were *Klebsiella pneumoniae* (5.68 %) (Table 4.1). From the records obtained at the Microbiology Laboratory Ahmadu Bello University Health Services (ABUHS), Zaria, most of the organisms isolated were also from sputum samples (69 %), followed by throat swab samples (20 %) and non from nasal secretions (0

%). The most commonly isolated bacteria were *Streptococcus pneumoniae* (54.5 %), followed by *Staphylococcus aureus* (32.4 %) and the least were *Pseudomonas aeruginosa* (2.76 %) (Table 4.2).

Common antibiotics used for susceptibility testing at the ABUTH and ABUHS, Zaria include: Ciprofloxacin: CIP, Tetracycline: TE, Ofloxacin: OFX, Erythromycin: E, Ceftriaxone: CRO, Gentamicin: CN, Amoxicillin: AMX, Cefuroxime: CXM, Ampicillin: AMP, Amoxicillin-Clavulanic acid: AMC and Cotrimoxazole: COT. From their records at ABUTH, most of the organisms were sensitive to CN, CIP, and AMX; but resistant to COT, AMC and E (Table 4.3). However, at ABUHS, most of the organisms were sensitive to OFX, CIP and CN; but showed resistance to AMC, COT and E (Table 4.4).

Table 4.1: Retrospective Study of Bacteria Isolates from Respiratory Tract at Medical Microbiology Department, ABUTH from January, 2011 to February, 2012

Bacterial isolates	**Number of isolates from Sputum samples**	**Number of isolates from Nasal samples**	**Number of isolates from Throat Swab samples**	**Number of isolates from Ear Swab samples**	**Total**
Streptococcus pneumoniae	477	0	91	17	585 (59.3 %)
Staphylococcus aureus	63	3	74	59	199 (20.2 %)
Pseudomonas aeruginosa	4	0	3	54	61 (6.19 %)
Klebsiella pneumoniae	43	1	3	9	56 (5.68 %)
Streptococcus pyogene	14	0	70	1	85 (8.62 %)
Total	601 (61%)	4 (0.41%)	241 (24.4%)	140 (14.2%)	**Grand Total=986 (100%)**

Table 4.2: Retrospective Study of Bacteria Isolates from Respiratory Tract at Microbiology Department, ABUHS, Zaria from January, 2011 to February, 2012

Bacterial isolates	Number of isolates from Sputum samples	Number of isolates from Nasal samples	Number of isolates from Throat Swab samples	Number of isolates from Ear Swab samples	Total
Streptococcus pneumoniae	66	0	13	0	79 (54.5 %)
Staphylococcus aureus	21	0	15	11	47 (32.4 %)
Pseudomonas aeruginosa	1	0	0	3	4 (2.76 %)
Klebsiella pneumoniae	8	0	0	2	10 (6.90 %)
Streptococcus pyogene	4	0	1	0	5 (3.45 %)
Total	**100 (69%)**	**0 (0%)**	**29 (20%)**	**16 (11%)**	**Grand Total=145 (100%)**

Table 4.3: Retrospective Study of the sensitivity and resistance profile of the bacteria isolates to the commonly used antibiotics at Medical Microbiology Department, ABUTH, Zaria from January, 2011 to February, 2012.

Bacterial isolates	Common antibiotics the bacterial isolates are sensitive to.	Common antibiotics the bacterial isolates are resistant to.
Streptococcus pneumoniae	CXM, CN, AMX.	COT, CRO.
Staphylococcus aureus	CN, CIP, AMX.	AMC, AMP.
Pseudomonas aeruginosa	CIP, CN.	E, AMC, TE.
Klebsiella pneumoniae	CN, CIP.	COT, CXM.
Streptococcus pyogene	TE, CIP, CN.	COT, E.

Table 4.4: Retrospective Study of the sensitivity and resistance profile of the bacteria isolates to the commonly used antibiotics at Microbiology Department ABUHS, Zaria from January, 2011 to February, 2012.

Bacterial isolates	Common antibiotics the bacterial isolates are sensitive to.	Common antibiotics the bacterial isolates are resistant to.
Streptococcus pneumoniae	CIP, CN, OFX.	COT, AMC, E.
Staphylococcus aureus	OFX, CIP, CN.	COT, E, AMC.
Pseudomonas aeruginosa	CIP, CN, OFX.	AMC, COT.
Klebsiella pneumoniae	CIP, CN, OFX.	AMC, COT.
Streptococcus pyogene	CIP, CN.	AMC, COT.

4.2 Prospective Studies

4.2.1 Sample Collection

Data on samples collected including the age, sex of patients presented with respiratory tract infections, sample source and sample number can be seen in Appendix V.

A total of 126 clinical isolates were collected. Twenty six (26) from throat swab, three (3) from nasal secretion, fourteen (14) from Ear swab and eighty three (83) from Sputum samples. The isolates identified and confirmed include 15 *Klebsiella pneumoniae* (26.8 %), 14 *Staphylococcus aureus* (25.0 %), 2 *Haemophilus influenzae* (3.57 %), 12 *Pseudomonas aeruginosa* (21.4 %), 7 *Streptococcus pneumoniae* (12.5 %), 6 *Streptococcus pyogenes* (10.7 %).

4.2.2 Susceptibility Pattern of the Bacterial Isolates to Test Standard Antibiotic Formulations

Figure 4.1 shows the percentage susceptibility pattern of the test organisms; *Haemophilus influenzae* were found to be 100 % sensitive to all the antibiotics; *Klebsiella pneumoniae* were 100 % sensitive to CRO; *Pseudomonas aeruginosa* were 100 % sensitive to CRO, CN, AZM and LEV, while 75 % resistance to AMC; *Streptococcus pneumoniae* were 100 % sensitive to CRO, CN, and LEV; *Streptococcus pyogene* were 100 % sensitive to CN and LEV.

Figure 4.2 shows the percentage susceptibility profile of all the test bacterial isolates to the various antibiotics used. All the bacterial isolates were 98.2 %, 96.4 %, 94.6 %, 85.7 %, and 67.9 % sensitive to LEV, CRO, CN, AZM and AMC respectively (Fig. 4.2).

Figure 4.3 shows the percentage susceptibility profile of the test bacterial isolates as per different sample sources. All the isolates from ear swab were sensitive to CRO, CN, LEV, and AZM; Similarly, all isolates from Nasal secretion were sensitive to CN, LEV, and AZM, while being resistance to AMC; All isolates from throat swab were sensitive to CRO and LEV.

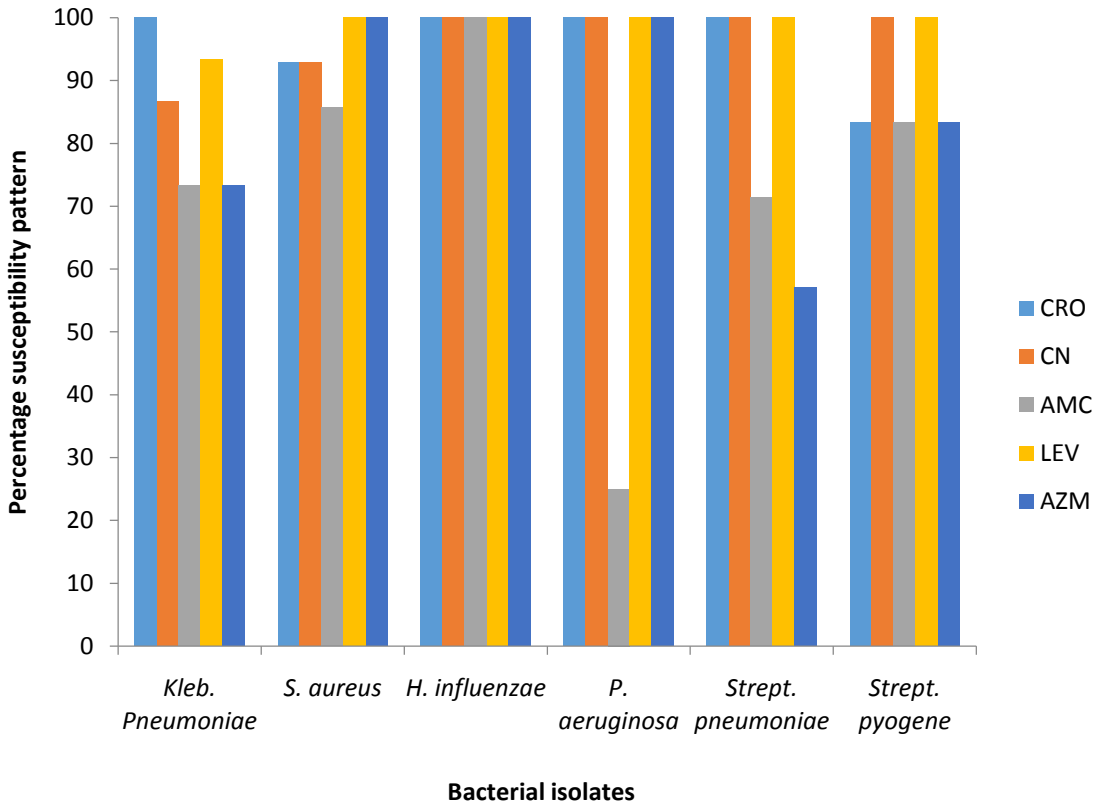


Figure 4.1: The antibiotic susceptibility pattern of bacteria isolates from RTI patients in ABUTH & ABUHS, Zaria.

Key: CN=Gentamicin, CRO=Ceftriaxone, AMC=Amoxicillin-Clavulanic acid, LEV=Levofloxacin, AZM=Azithromycin.

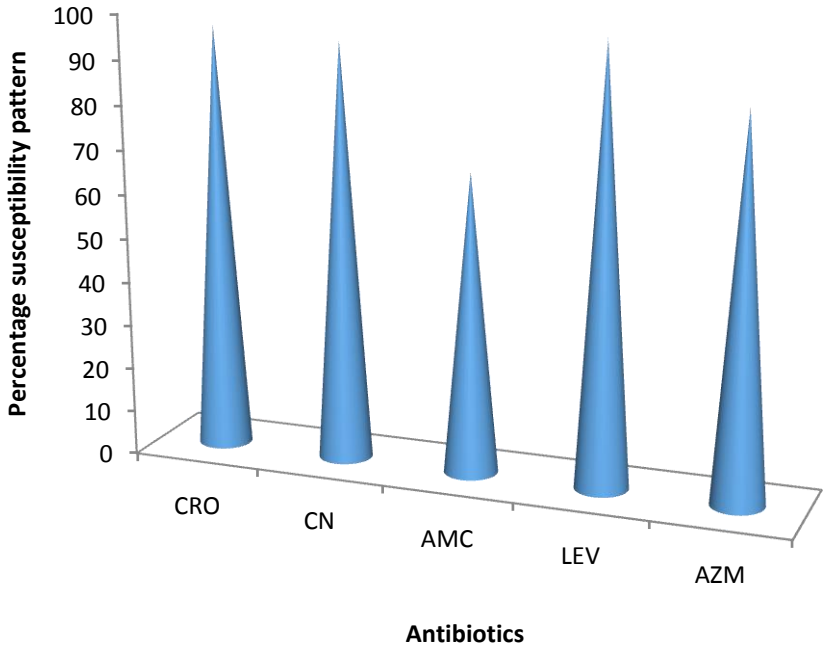


Figure 4.2: The antibiotic susceptibility pattern of bacteria isolates from RTI patients in ABUTH & ABUHS, Zaria.

Key: CN=Gentamicin, CRO=Ceftriaxone, AMC=Amoxicillin-Clavulanic acid, LEV=Levofloxacin, AZM=Azithromycin.

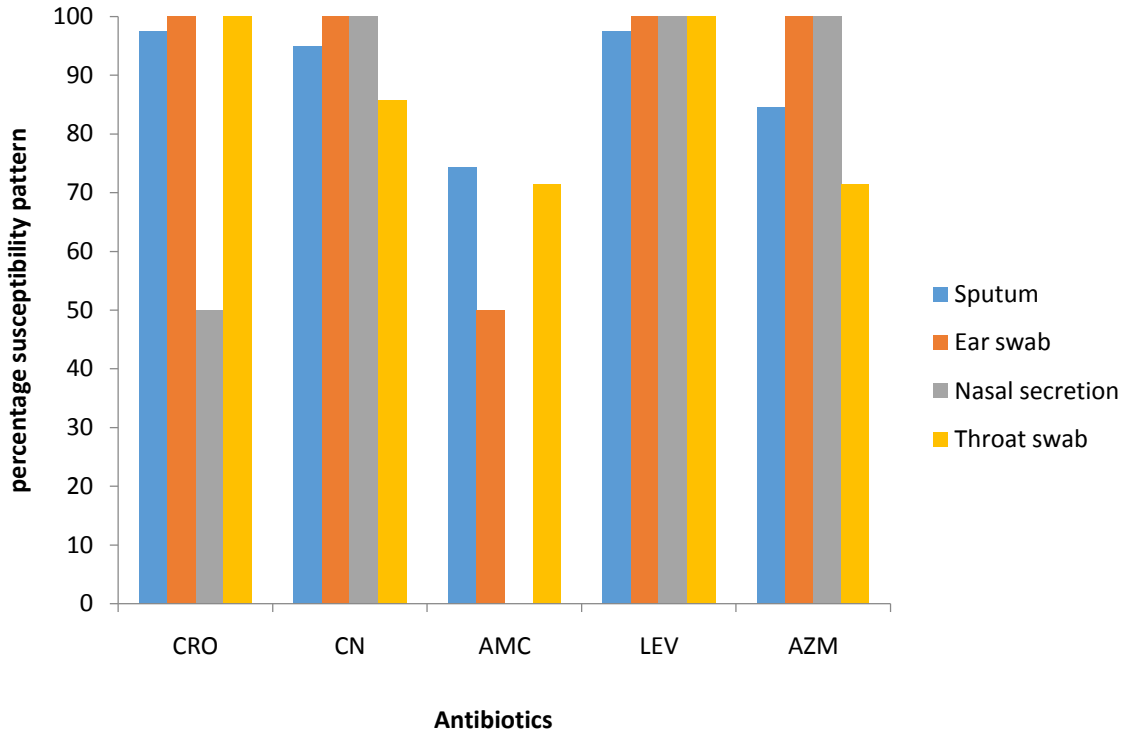


Figure 4.3: Susceptibility pattern of the test bacteria isolates to standard antibiotics as per source.

Key: CN=Gentamicin, CRO=Ceftriaxone, AMC=Amoxicillin-Clavulanic acid, LEV=Levofloxacin, AZM=Azithromycin.

4.2.3 Susceptibility Pattern of the Bacterial Isolates to Honey and Lemon Juice.

Figure 4.4 shows the percentage susceptibility pattern of all the test bacterial isolates to Honey, Lemon and the mixture of Honey and Lemon. All the test bacterial isolates were susceptible to pure Honey, undiluted Lemon juice, Honey/Lemon juice at crude concentrations; 98.2 % and 94.6 % susceptible to Lemon juice and Honey respectively, at 50 % v/v dilution in water; and 23.2 % and 67.9 % susceptible to Honey and Lemon respectively, at 25 % v/v dilution in water.

Figure 4.5 shows the susceptibility pattern of isolates species to Honey, Lemon and Honey/Lemon juice mixture. All isolates of *Haemophilus influenzae, Streptococcus Pneumoniae,* and *Streptococcus pyogenes* were sensitive to the undiluted Honey and Lemon juice as well as 50 % v/v dilution in water, and Lemon at 25 % v/v dilution in water. However, *H. influenzae, K. Pneumoniae*, *S. pneumoniae* and *S. Pyogene* were 100 %, 86.7 %, 85.7 % and 83.3 % resistance to Honey at 25 % v/v concentrations respectively. *Staphylococcus aureus* and *Pseudomonas aeruginosa* are 50.0 %, 83.3 % resistance to Lemon juice at 25 % v/v dilution in water respectively and 64.3 %, 66.7 % resistant to Honey at 25 % v/v dilution in water.

Figure 4.6 shows the susceptibility pattern of the test organisms to Honey, Lemon and the mixture of Honey and Lemon juice. All isolates from ear swab were sensitive to undiluted Honey and Lemon and 50% dilutions, while 87.5% and 62.5% resistance to Honey and Lemon at 25% dilution; All isolates from nasal secretion were sensitive to undiluted Honey and Lemon juice, while all were resistant to Honey at 25% dilution; All isolates from throat swab were sensitive to undiluted Honey and Lemon juice and 50% diluted, while 57.1% resistance to Honey at 25% dilution.

Plate IV_a shows the susceptibility plate of *Streptococus pneumoniae* (Sample number S232), to Lemon (100-25 % v/v concentrations), CN, AMC and CRO showing various zones of inhibition. The largest zone of inhibition was shown with undiluted Lemon; while the lowest zone of inhibition was shown by AMC.

Plate IV_b shows the susceptibility plate of *Streptococus pneumoniae* (Sample number S232), to Honey (100-25 % v/v concentrations), Honey and Lemon mixture (30:50 % v/v concentration), LEV and AZM showing various zones of inhibition. The largest zone was observed with Honey and Lemon juice mixture, followed by LEV and undiluted Honey.

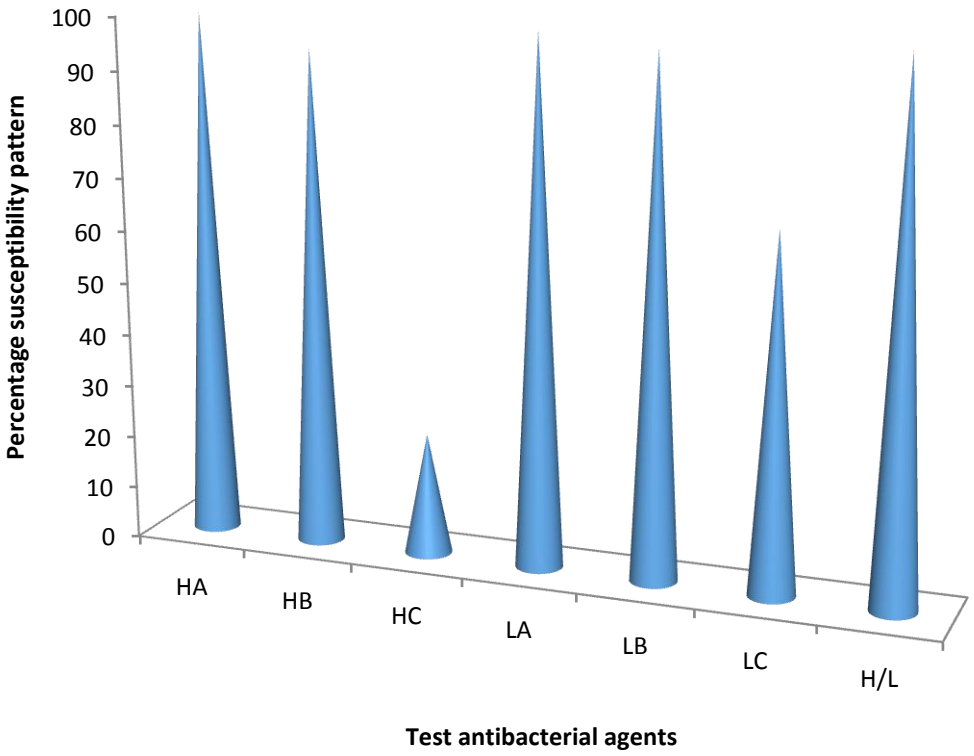


Figure 4.4: Susceptibility pattern of the test bacterial isolates to honey, lemon juice and mixture of honey/lemon juice.

Key: H: Honey, L: Lemon juice, H/L: Honey and Lemon juice**,** HA: Pure Honey, LA: Crude Lemon juice**,** HB: 50% Honey, LB: 50% Lemon**,** HC: 25% Honey, LC: 25% Lemon.

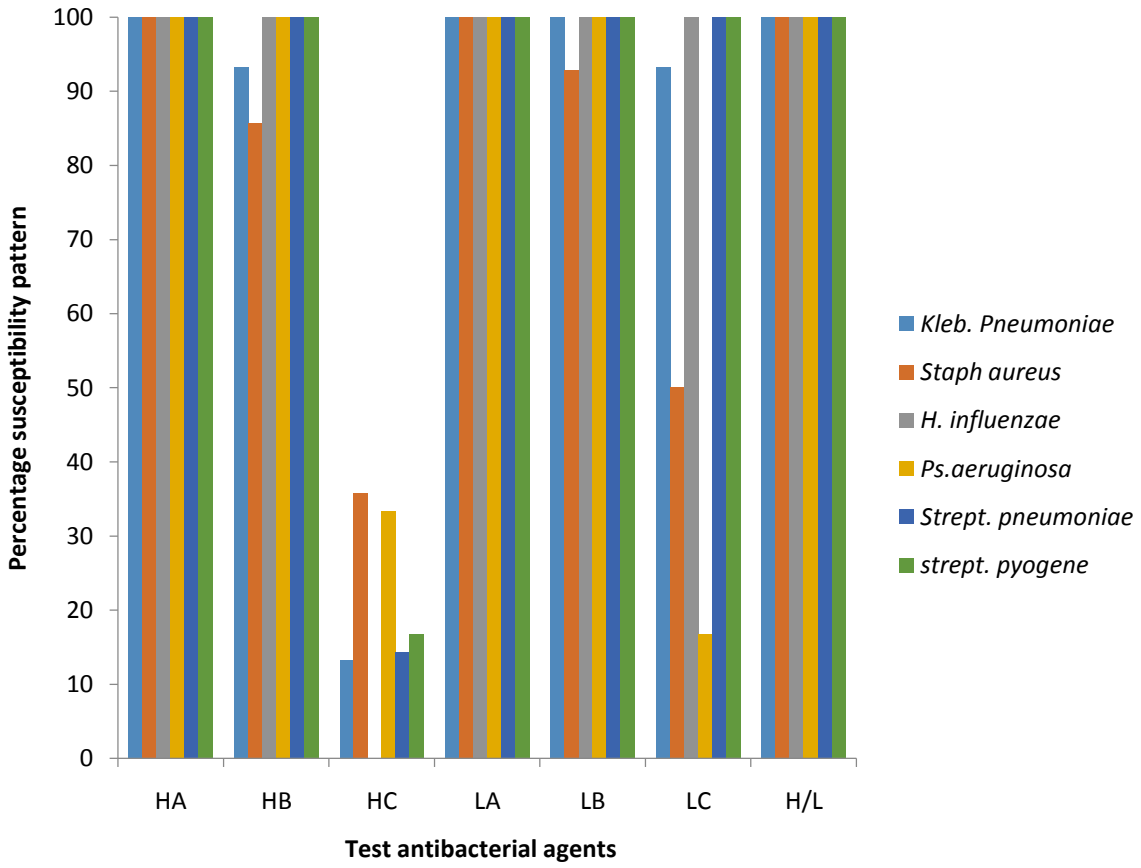


Figure 4.5: Susceptibility pattern of each of the bacterial species to Honey, Lemon juice & Honey/Lemon mixture.

Key: H: Honey, L: Lemon juice, H/L: Honey and Lemon juice, HA: 100% Honey, LA: 100% Lemon, HB: 50% Honey, LB: 50% Lemon, HC: 25% Honey, LC: 25% Lemon.

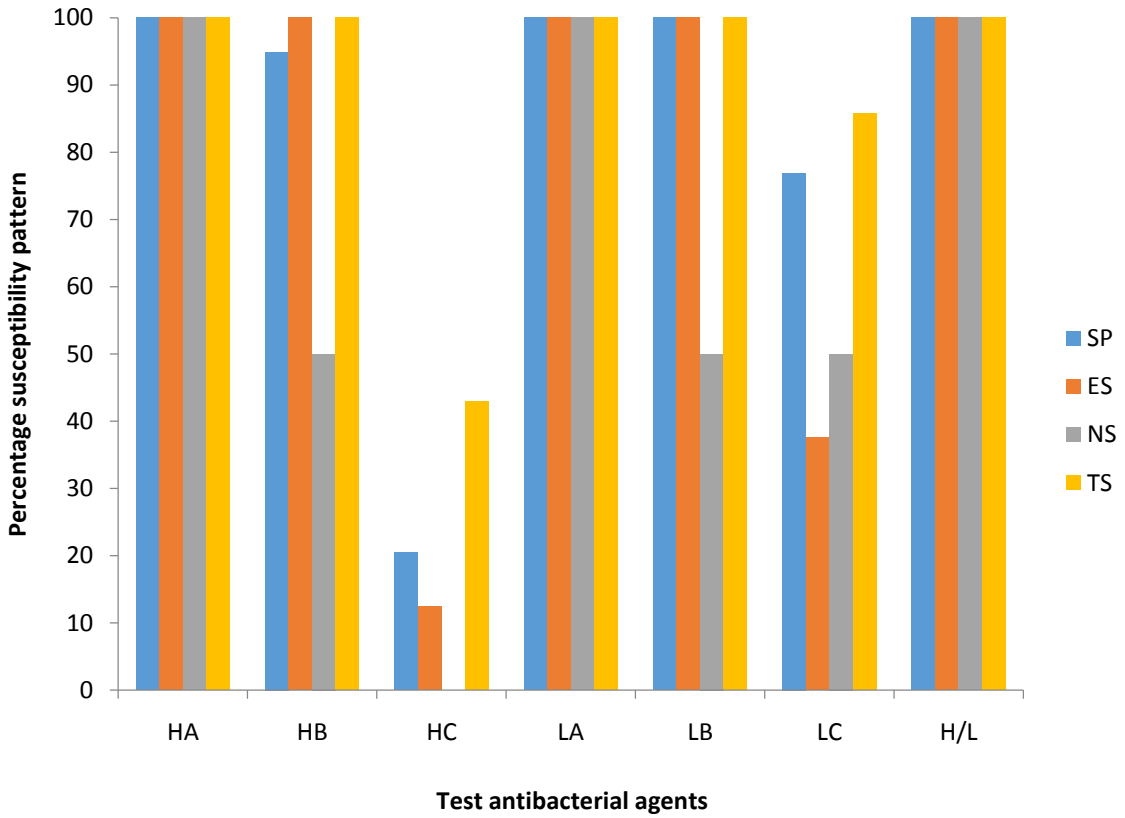


Figure 4.6: Susceptibility pattern of the test bacterial isolates to honey, lemon and mixture of honey/lemon as per source.

Key: H: Honey, L: Lemon, H/L: Honey and Lemon, HA: 100% Honey, LA: 100% Lemon, HB: 50% Honey, LB: 50% Lemon, HC: 25% Honey, LC: 25% Lemon.

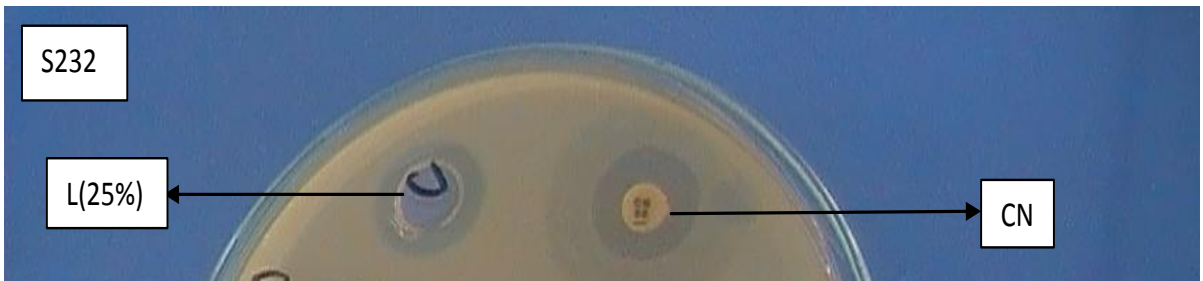


Plate IV_a: Susceptibility Test plate of S*treptococcus pneumoniae*_1.

Key: L=Lemon juice, CN=Gentamicin, AMC=Amoxicillin-Clavulanic Acid and CRO=Ceftriaxone.

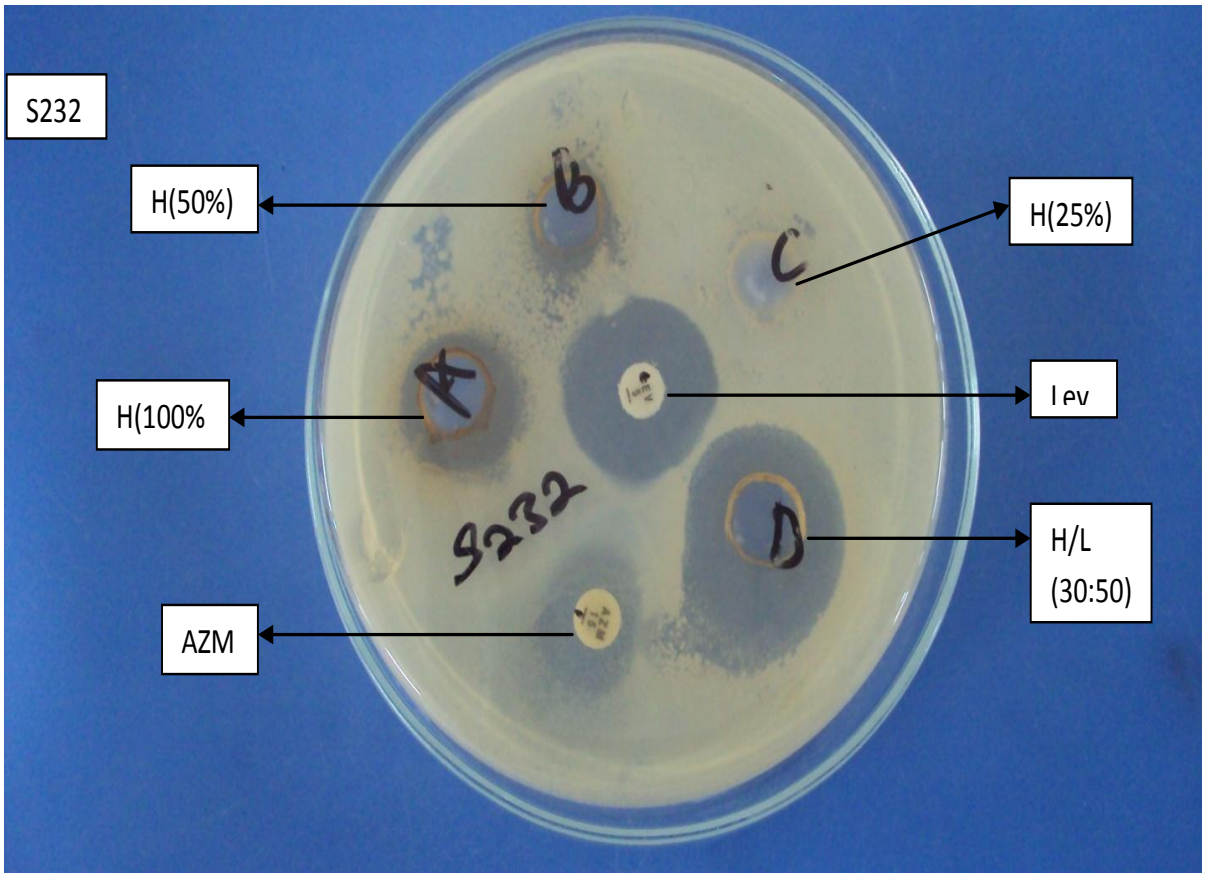

S232
H(50%)
H(25%)
H(100%
Lev
S232
H/L
(30:50)
AZM

Plate IV_b: Susceptibility Test plate of *Streptococcus pneumonia*e_2.

Key: H=Honey, Lev=Levofloxacin, AZM=Azithromycin, H/L=Honey and Lemon juice.

4.3 Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentrations (MBC)

4.3.1 The Percentage Resistance Profile For the MIC Tests using the Standard Antibiotic Formulations

The percentage resistance profile to the standard antibiotics for all the bacterial isolates are as shown in Figure 4.7. See appendix II for details.

4.3.2 Comparing the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) Results between Honey, Lemon juice and Honey/Lemon juice mixture

Generally, there is a reduction in MIC and MBC in the mixture of Honey and Lemon juice compared to the Honey alone (see appendix II).

There is increase in MIC and MBC in the mixture of Honey and Lemon juice compared to the Lemon juice alone (see appendix II).

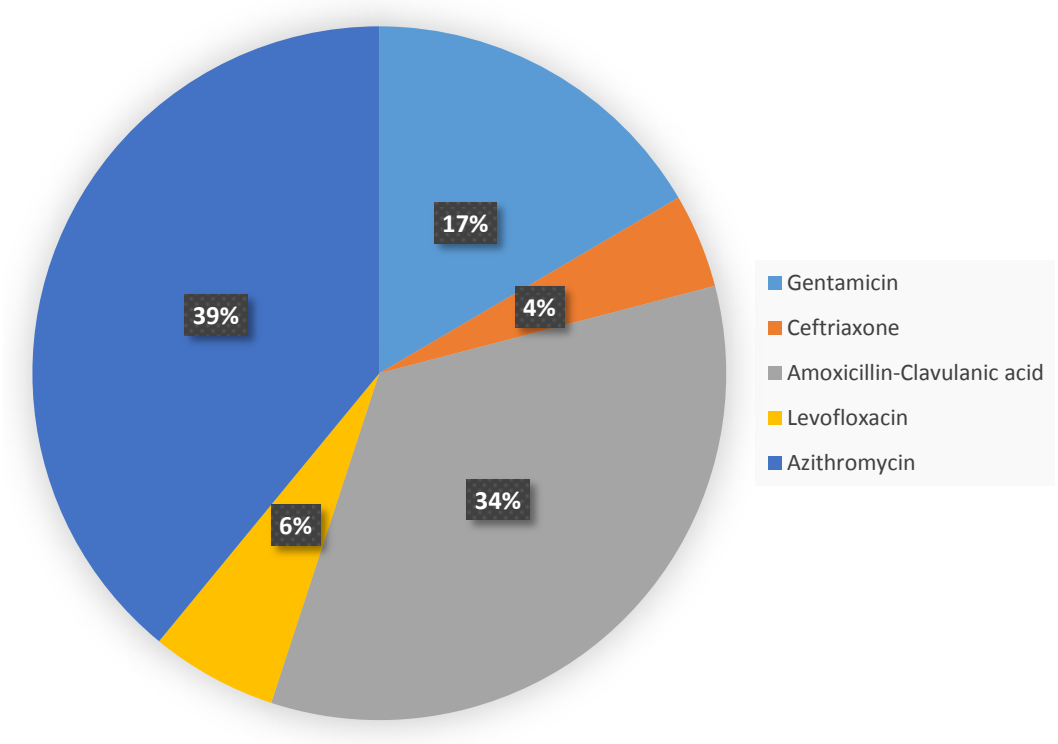


Figure 4.7: Resistance profile of the test bacterial isolates to the standard antibiotics.

4.4 Rate of Kill

Lemon effected complete killing at 240 minutes which is depicted by gradual decrease in cell population from 30 minutes to 120 minutes, and a sharp decrease at 240 minutes; Honey and Lemon however effected complete killing at 360 minutes, as also depicted by gradual decrease in cell population from 30 minutes to 240 minutes and a sharp decrease at 360 minutes, while CEF, LEV, HONEY could not produce complete killing even at 1440 minutes for the resistant *Klebsiella pneumoniae* (Fig. 4.8).

Lemon effected complete killing at 120 minutes, as depicted by gradual decrease in cell population from 30 minutes to 90 minutes and a sharp decrease down at 120 minutes; Honey and Lemon mixture effected complete killing at 240 minutes, as shown by a steady decrease in cell population from 30 minutes to 120 minutes, and gradual decrease down at 240 minutes; while CEF, LEV and Honey produced complete killing at 1440 minutes, as observed by a gradual decrease from 30 minutes to 360 minutes and a steady decrease down to 1440 minutes for the susceptible *Klebsiella pneumoniae* (Fig. 4.9).

Honey and Lemon mixture effected complete killing at 1440 minutes, as observed by the gradual decrease in cell population from 30 minutes to 360 minutes and a steady decrease to 1440 minutes; while CEF, LEV, Honey, Lemon could not produce complete killing even at 1440 minutes, (LEV and CRO produced similar effect with a gradual decrease in cell population from 30-120 minutes, and no decrease from 360-1440 minutes) for the resistant *Streptococcus pyogenes* (Fig. 4.10).

Lemon, Honey and Lemon mixture effected complete killing at 120 minutes, as depicted by gradual decrease in the cell population from 30 minutes to 90 minutes and a steady decrease at 120 minutes; while CEF, LEV and Honey produced complete killing at

1440 minutes, as depicted by gradual decrease in cell populations from 30 minutes to 360 minutes and a steady decrease to 1440 minutes for the susceptible *Streptococcus pneumoniae* (Fig. 4.11).

However, for the detail of the rate of kill results for the sub-MIC and above-MIC concentrations see appendix I.

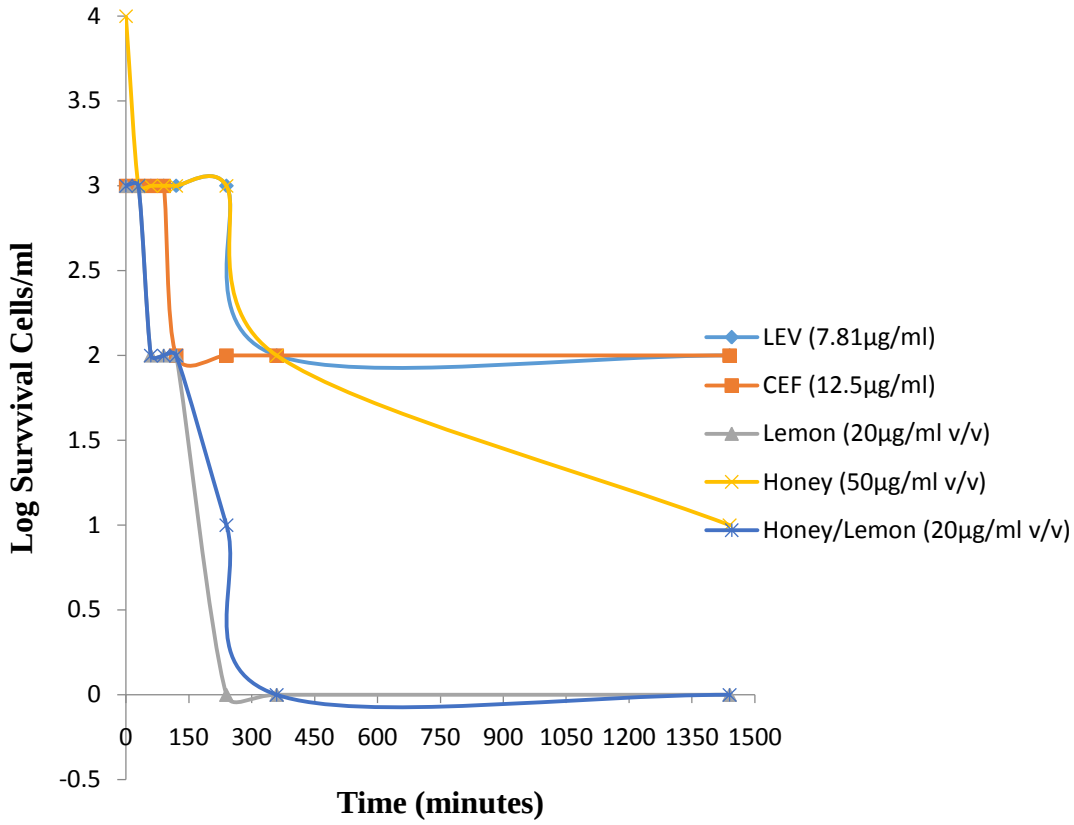


Figure 4.8: The Log of Survival Cells/ml of *Klebsiella pneumoniae* (Resistant) on exposure to standard antibiotics and honey, lemon and honey/lemon juice mixture.

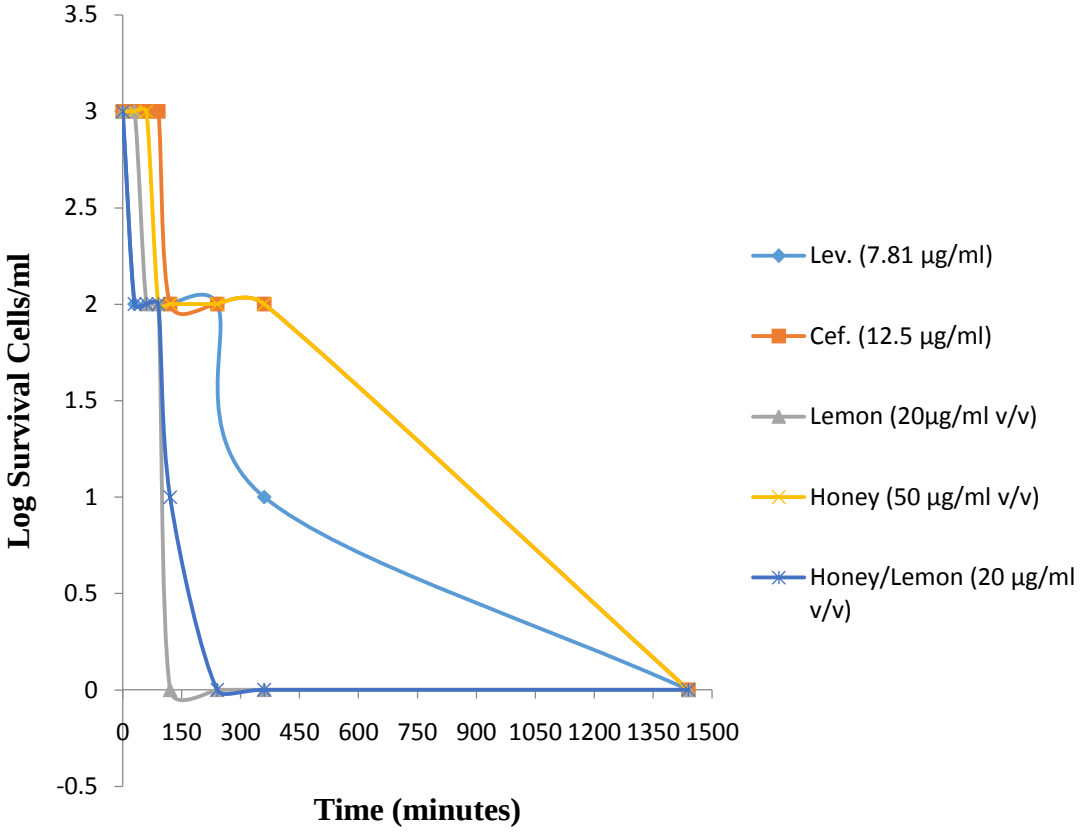


Figure 4.9: The Log of Survival Cells/ml of *Klebsiella pneumoniae* (Sensitive) on exposure to standard antibiotics and honey, lemon and honey/lemon juice mixture.

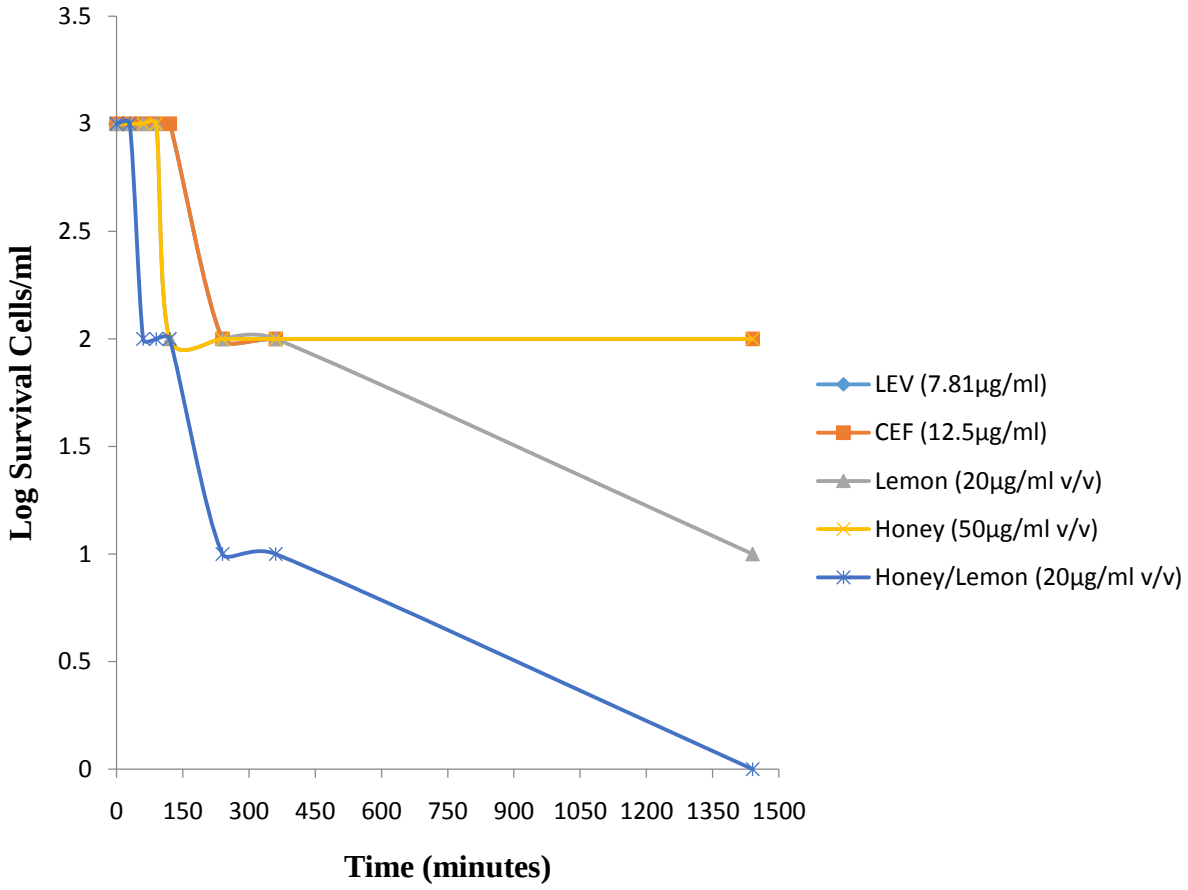


Fig. 4.10: The Log of Survival cells/ml of *Streptococcus pyogene* (Resistant) on exposure to standard antibiotics and honey, lemon and honey/lemon juice mixture.

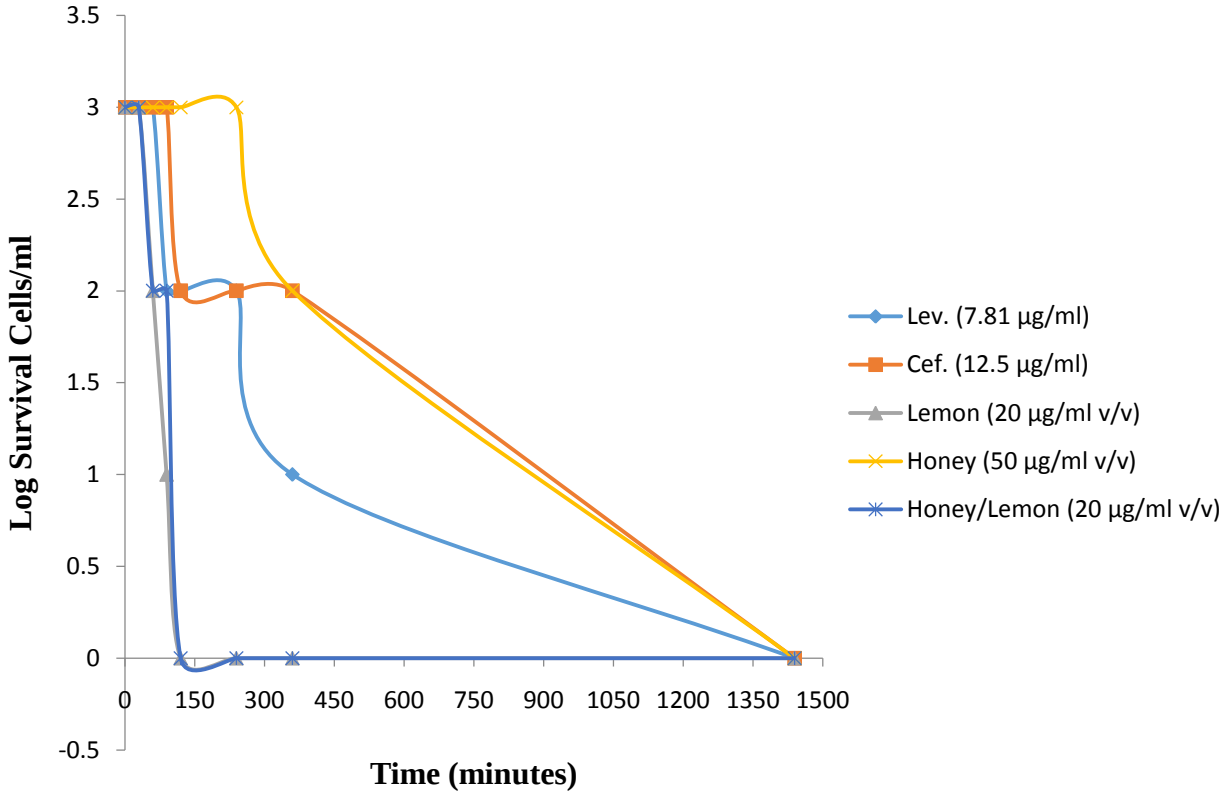


Fig. 4.11: The Log of Survival cells/ml of *Streptococcus pneumoniae* (Sensitive) on exposure to standard antibiotics and honey, lemon and honey/lemon juice mixture.

4.5 Results for the Interaction Studies

The activities of the combination of Honey and Lemon were found to be additive and not antagonistic. It falls within the additive range of the FIC Index interpretation (>0.5 to ≤1.0) and indifference range of (>1.0 to ≤4) (see Table 4.8 and 4.9). Of which the results observed in Table 4.8 and Table 4.9 were obtained from the evaluation of the values in Table 4.5 to Table 4.7 using the synergy formula ∑FIC=FIC A + FIC B as described earlier in the Methodology.

Table 4.5: Average Minimum Inhibitory Concentration (MIC) (µg/ml) values for the test agents (Honey and Lemon juice) against the bacteria isolates

Bacterial isolates	Honey	Lemon juice
Klebsiella pneumoniae	43.0	21.3
Staphylococcus aureus	47.1	17.9
Haemophilus influenzae	26.3	15.0
Pseudomonas aeruginosa	32.5	16.7
Streptococcus pneumoniae	37.1	31.1
Streptococcus pyogenes	43.3	34.6

Table 4.6: Average Minimum Inhibitory Concentration (MIC) (µg/ml) values for the test agents (Honey/Lemon juice mixture) against the bacterial isolates

Bacterial isolates	10:50	20:50	30:50	40:50	50:50
Klebsiella pneumoniae	19.3	21.3	25.3	27.6	28.3
Staphylococcus aureus	18.0	21.4	20.2	21.6	20.0
Haemophilus influenzae	32.5	17.5	15.0	17.5	17.5
Pseudomonas aeruginosa	21.9	22.3	16.6	17.5	19.6
Streptococcus pneumoniae	19.6	20.0	20.4	19.6	19.6

Streptococcus pyogenes	20.4	19.6	17.9	17.9	18.8

Table 4.7: Average Minimum Inhibitory Concentration (MIC) (µg/ml) values for the test agents (Lemon juice/Honey mixture) against the bacterial isolates

Bacterial isolates	10:50	20:50	30:50	40:50	50:50

Klebsiella pneumoniae	33.7	32.0	29.3	28.8	28.3
Staphylococcus aureus	32.1	28.2	22.1	21.3	20.0
Haemophilus influenzae	30.0	27.5	27.5	23.8	17.5
Pseudomonas aeruginosa	25.8	23.1	20.2	18.1	19.6
Streptococcus pneumoniae	43.6	38.6	27.1	23.9	19.6
Streptococcus pyogenes	32.9	27.9	25.8	22.5	18.8

Table 4.8: ∑FIC values for the test agents (Honey/Lemon juice mixture) against the bacterial isolates

Bacterial isolates	10:50	20:50	30:50	40:50	50:50
Klebsiella pneumoniae	1.35	1.50	1.78	1.94	1.99
Staphylococcus aureus	1.39	1.65	1.56	1.67	1.54
Haemophilus influenzae	3.41	1.84	1.57	1.84	1.84
Pseudomonas aeruginosa	1.98	2.03	1.50	1.59	1.77
Streptococcus pneumoniae	1.16	1.18	1.21	1.16	1.16
Streptococcus pyogenes	1.06	1.02	0.93	0.93	0.97

Table 4.9: ∑FIC values for the test agents (Lemon juice/Honey mixture) against the bacterial isolates

Bacterial isolates	10:50	20:50	30:50	40:50	50:50
Klebsiella pneumoniae	2.36	2.24	2.05	2.02	1.99
Staphylococcus aureus	2.47	2.18	1.70	1.64	1.54
Haemophilus influenzae	3.14	2.88	2.88	2.49	1.84
Pseudomonas aeruginosa	2.33	2.09	1.83	1.64	1.77
Streptococcus pneumoniae	2.58	2.28	1.60	1.41	1.16
Streptococcus pyogenes	1.71	1.45	1.35	1.17	0.97

4.6 Comparative Analysis of the Mean Zones of Inhibition

There is significant difference at P≤0.05 between the mean zone of inhibition of undiluted Honey and 30:50, 40:50, 50:50 % v/v concentrations of Honey/Lemon juice mixtures against *Klebsiella pneumoniae* (Fig. 4.12).

There is also significant difference at P≤0.05 between the mean zone of inhibition of undiluted Honey and 10:50, 20:50, 30:50, 40:50, 50:50 % v/v concentrations of Honey and Lemon mixtures against *Staphylococcus aureus* (Fig. 4.13).

There is also significant difference at P≤0.05 between the mean zone of inhibition of undiluted Honey and 10:50, 20:50, 40:50, 50:50 % v/v concentrations of Honey and Lemon

juice mixtures against *Streptococcus pneumoniae* and *Streptococcus pyogenes* (Fig. 4.14; Fig. 4.15).

In addition, there is significant difference at P≤0.05 between the mean zones of inhibition of undiluted Honey, undiluted Lemon and 50:50 % v/v concentrations of Honey and Lemon juice mixtures against *Staphylococcus aureus* and *Streptococcus pyogenes* (Fig. 4.16; 4.18).

There is significant difference at P≤0.05 between the mean zone of inhibition of undiluted Lemon juice and 10:50, 20:50, 30:50, 40:50 and 50:50 % v/v concentrations of Honey and Lemon juice mixture against *Pseudomonas aeruginosa* (Fig. 4.17).

However, for the other organisms and the other concentrations, there is no significant difference at P≤0.05 between 100 % v/v concentration of Honey, 100 % v/v concentration of Lemon juice and the Honey/Lemon juice mixture (see appendix III & IV).

Furthermore, the mean zone of inhibition between Honey and lemon juice and the standard antibiotic formulations has been compared. There is no significant difference at P≤0.05 between the standard antibiotic formulations and undiluted honey, undiluted lemon juice, honey/lemon juice mixture; except for AMC, and AZM in some, 25 % v/v concentration of Honey (Table 4.10 to 4.26).

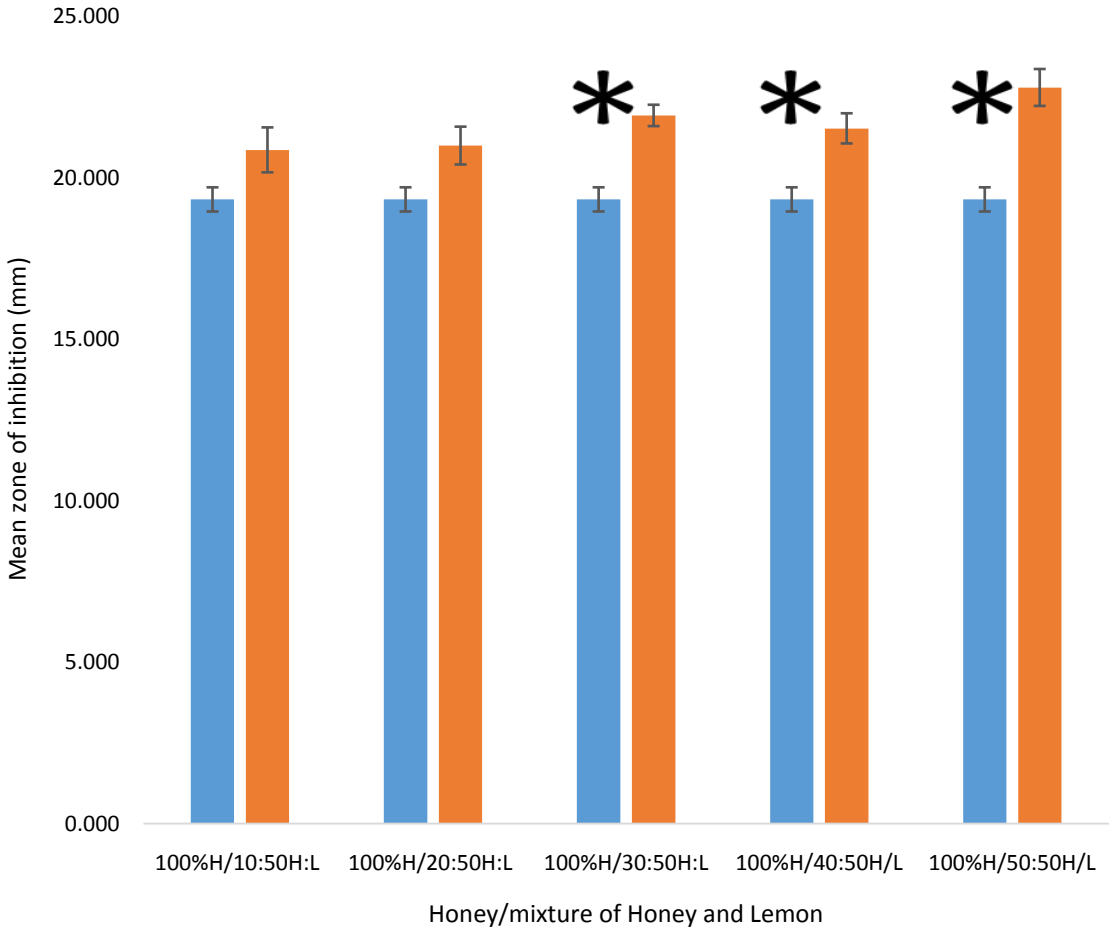


Figure 4.12: Comparing the mean zones of inhibition of Honey and the mixture of Honey and Lemon juice against *Klebsiella pneumoniae*.

Key: *=Significant difference, H=Honey, L=Lemon juice.

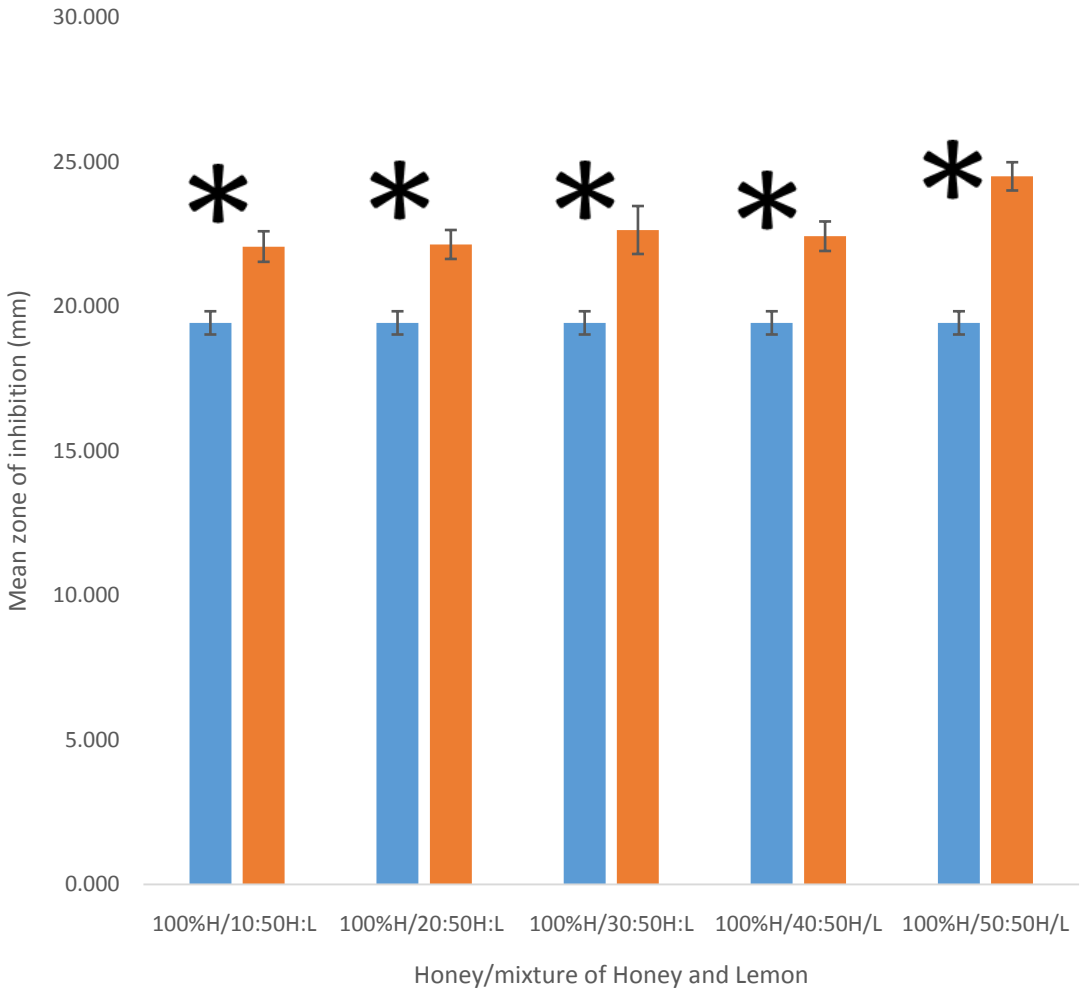


Figure 4.13: Comparing the mean zones of inhibition of Honey and the mixture of Honey and Lemon juice against *Staphylococcus aureus*.

Key: *=Significant difference, H=Honey, L=Lemon juice.

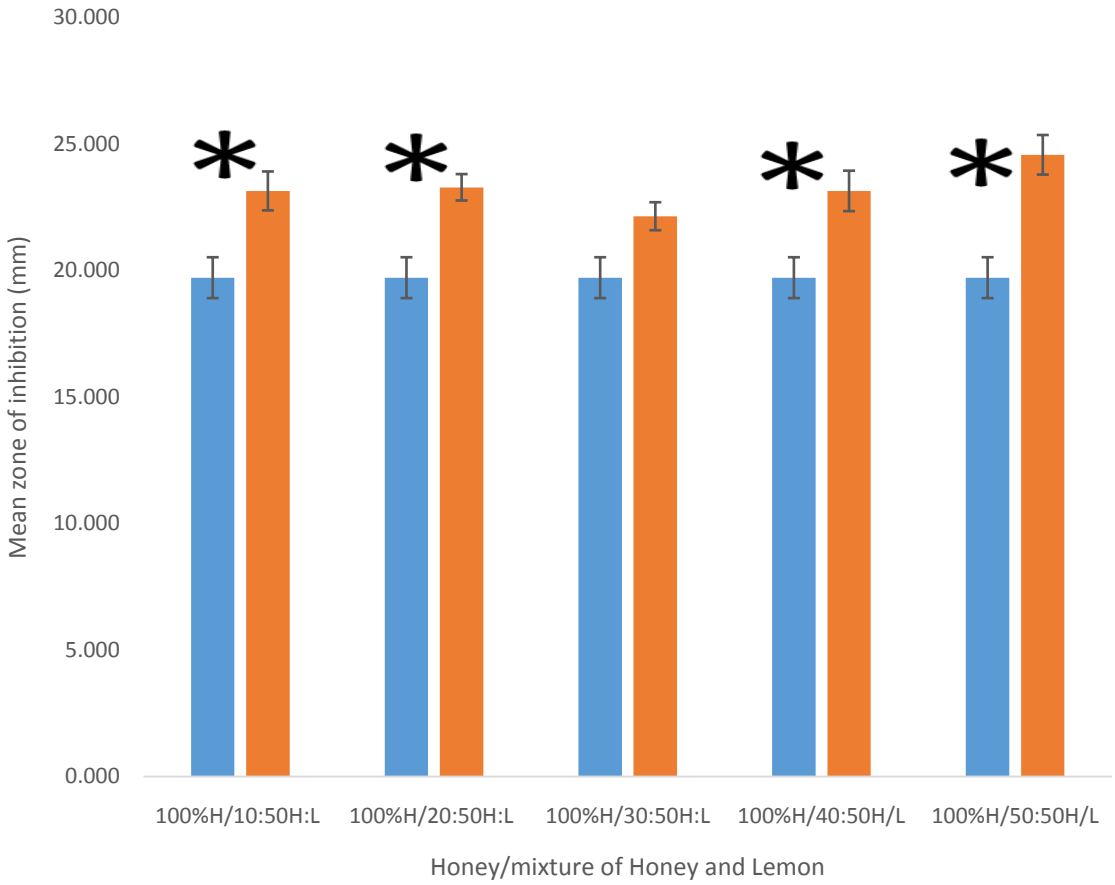


Figure 4.14: Comparing the mean zones of inhibition of Honey and the mixture of Honey and Lemon juice against *Streptococcus Pneumoniae*.

Key: *=Significant difference, H=Honey, L=Lemon juice.

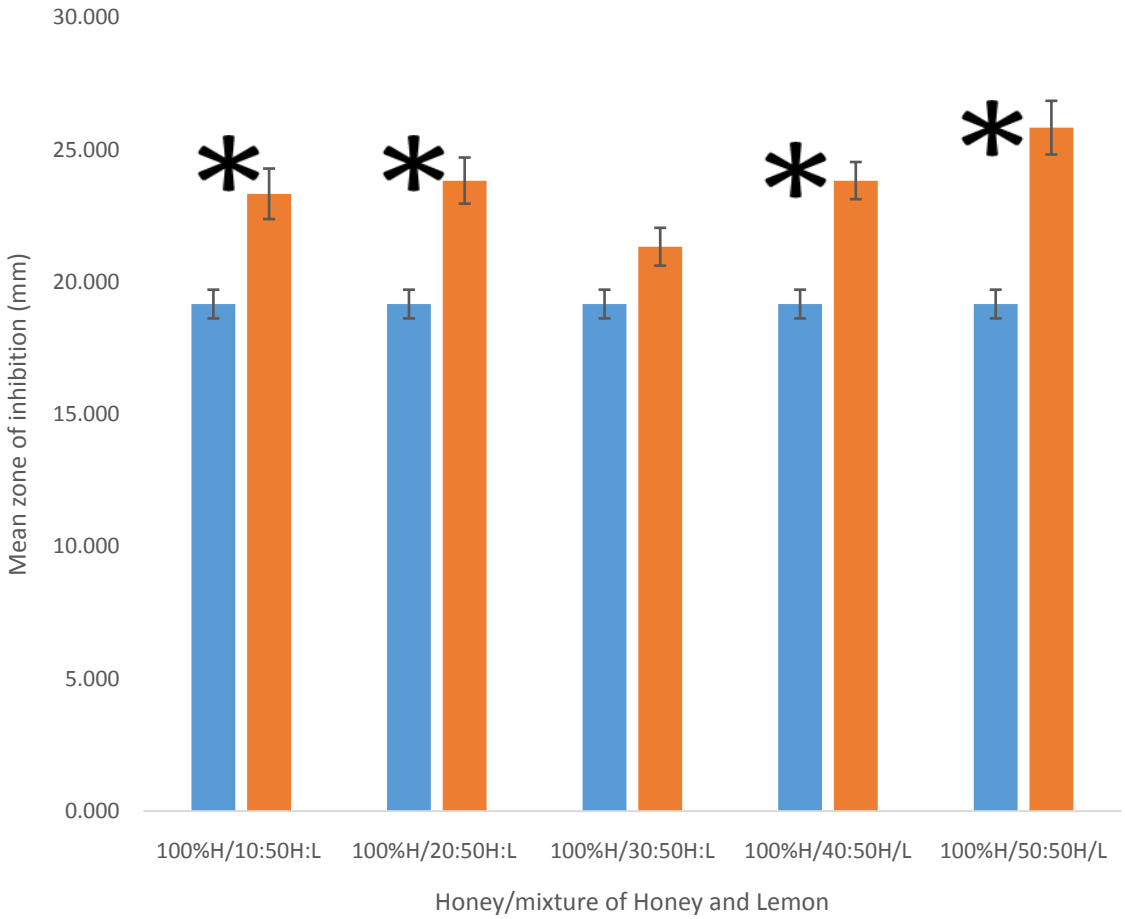


Figure 4.15: Comparing the mean zones of inhibition of Honey and the mixture of Honey and Lemon juice against *Streptococcus pyogenes*.

Key: *=Significant difference, H=Honey, L=Lemon juice.

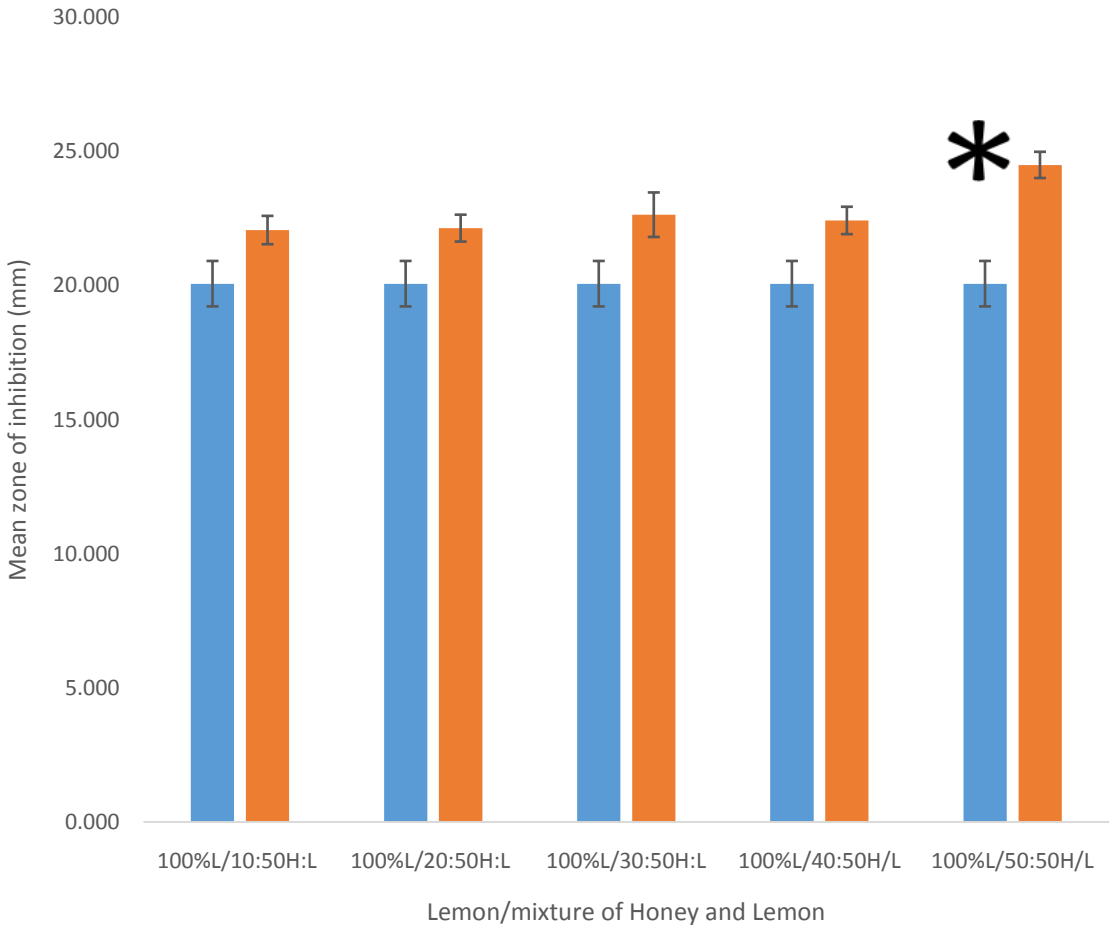


Figure 4.16: Comparing the mean zones of inhibition of Lemon juice and the mixture of Honey and Lemon juice against *Staphylococcus aureus*.

Key: *=Significant difference, H=Honey, L=Lemon juice.

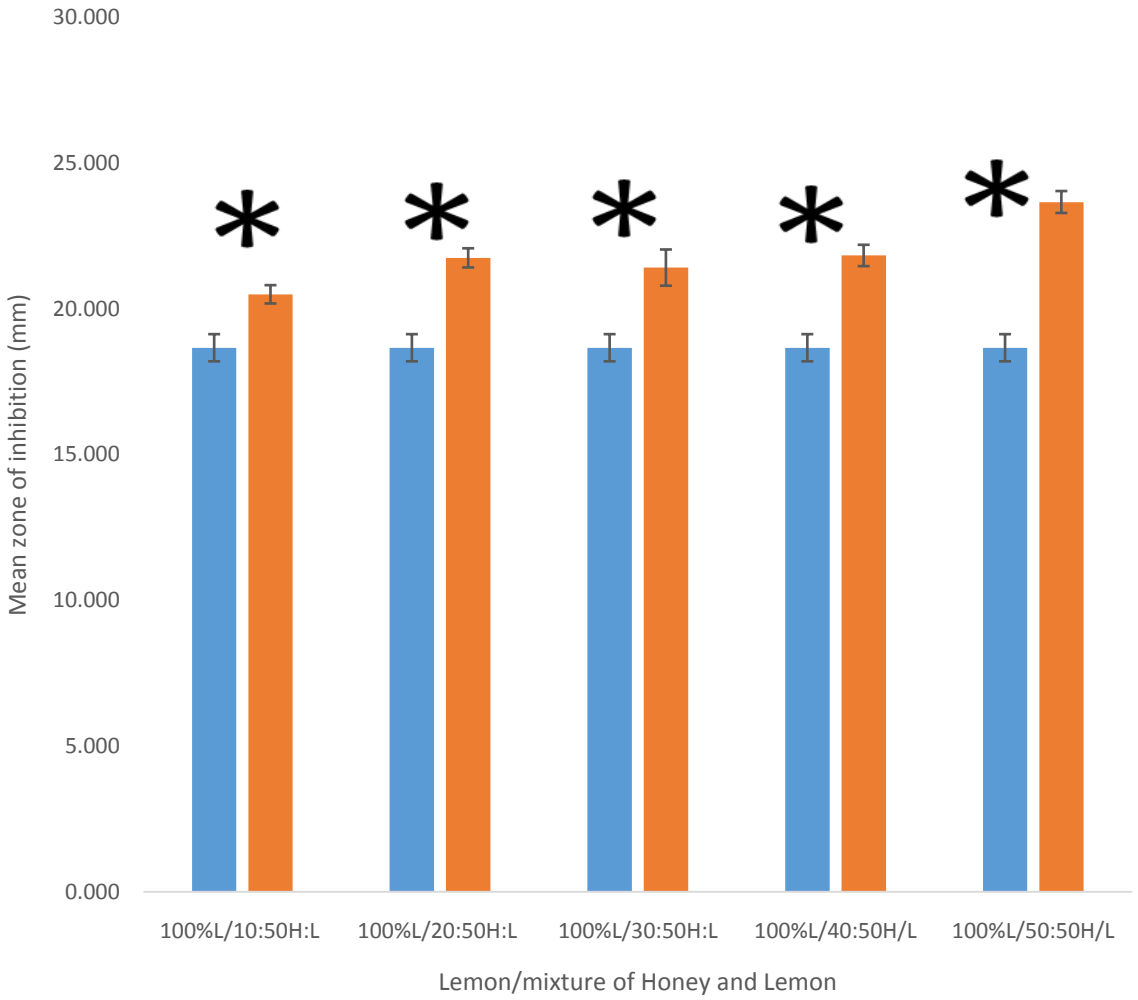


Figure 4.17: Comparing the mean zones of inhibition of Lemon juice and the mixture of Honey and Lemon juice against *Pseudomonas aeruginosa*.

Key: *=Significant difference, H=Honey, L=Lemon juice.

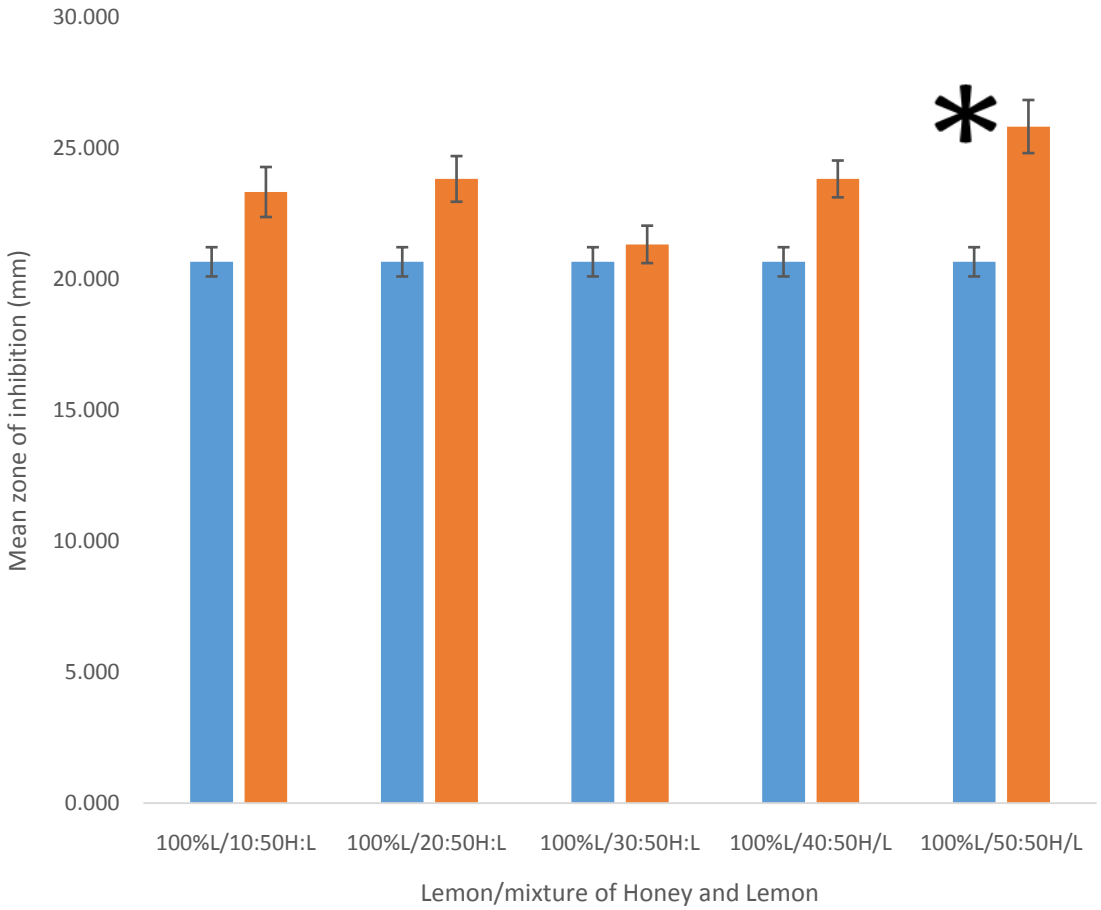


Figure 4.18: Comparing the mean zones of inhibition of Lemon juice and the mixture of Honey and Lemon juice against *Streptococcus pyogenes*.

Key: *=Significant difference, H=Honey, L=Lemon juice.

(I) Group	(J) Group	Mean±SEM	P-value	LOS

Table 4.10:

Comparing the Mean zone of inhibition of Honey and the Standard antibiotic formulations against *Klebsiella pneumoniae*

Honey (100%)	CRO	20.667±1.511	0.991	NS
	CN	19.267±1.523	1.000	NS
	AMC	17.667±1.670	0.968	NS
	LEV	19.867±1.171	1.000	NS
	AZM	15.467±1.050	0.249	NS
	Honey (50%)	16.000±0.439	0.439	NS
	Honey (25%)	11.733±0.442	0.000	SF
Honey (50%)	CRO	20.667±1.511	0.082	NS
	CN	19.267±1.523	0.466	NS
	AMC	17.667±1.670	0.968	NS
	LEV	19.867±1.171	0.249	NS
	AZM	15.467±1.050	1.000	NS
	Honey (100%)	19.333±0.374	0.439	NS
	Honey (25%)	11.733±0.442	0.148	NS
Honey (25%)	CRO	20.667±1.511	0.000	SF
	CN	19.267±1.523	0.000	SF
	AMC	17.667±1.670	0.008	SF
	LEV	19.867±1.171	0.000	SF
	AZM	15.467±1.050	0.291	NS
	Honey (100%)	19.333±0.374	0.000	SF
	Honey (50%)	16.000±0.439	0.148	NS

Key**:** LOS=Level of Significance; NS=Not significantly different;

SF=Significantly different.

Table 4.11: Comparing the Mean zone of inhibition of Honey and the Standard antibiotic formulations against *Staphylococcus aureus*

(I) Group	(J) Group	Mean±SEM	P-value	LOS
Honey (100%)	CRO	20.000±1.129	1.000	NS
	CN	21.571±1.073	0.831	NS
	AMC	20.286±1.669	0.999	NS
	LEV	25.857±1.148	0.001	SF
	AZM	19.643±1.243	1.000	NS
	Honey (50%)	15.714±0.438	0.201	NS
	Honey (25%)	12.500±0.542	0.000	SF
Honey (50%)	CRO	20.000±1.129	0.082	NS
	CN	21.571±1.073	0.003	SF
	AMC	20.286±1.669	0.050	SF
	LEV	25.857±1.148	0.000	SF

	AZM	19.643±1.243	0.147	NS
	Honey (100%)	19.429±0.402	0.201	NS
	Honey (25%)	12.500±0.542	0.374	NS
	CRO	20.000±1.129	0.000	SF
	CN	21.571±1.073	0.000	SF
	AMC	20.286±1.669	0.000	SF
Honey (25%)	LEV	25.857±1.148	0.000	SF
	AZM	19.643±1.243	0.000	SF
	Honey (100%)	19.429±0.402	0.000	SF
	Honey (50%)	15.714±0.542	0.374	NS

Key**:** LOS=Level of Significance; NS=Not significantly different;

SF=Significantly different.

Table 4.12: Comparing the Mean zone of inhibition of Honey and the Standard antibiotic formulations against *Haemophilus influenzae*

(I) Group	(J) Group	Mean±SEM	P-value	LOS

	CRO	25.500±2.500	0.055	SF
	CN	20.500±0.500	1.000	NS
	AMC	18.000±0.000	0.829	NS
Honey (100%)	LEV	22.500±0.500	0.654	NS
	AZM	14.500±0.500	0.055	SF
	Honey (50%)	16.000±1.000	0.210	NS
	Honey (25%)	10.000±0.000	0.002	SF
	CRO	25.500±2.500	0.002	SF
	CN	20.500±0.500	0.135	NS
	AMC	18.000±0.000	0.829	NS
Honey (50%)	LEV	22.500±0.500	0.023	SF
	AZM	14.500±0.500	0.949	NS
	Honey (100%)	20.000±0.000	0.210	NS
	Honey (25%)	10.000±0.000	0.035	SF
	CRO	25.500±2.500	0.000	SF
	CN	20.500±0.500	0.001	SF
	AMC	18.000±0.000	0.007	SF
Honey (25%)	LEV	22.500±0.500	0.000	SF
	AZM	14.500±0.500	0.135	NS
	Honey (100%)	20.000±0.000	0.002	SF
	Honey (50%)	16.000±1.000	0.035	SF

Key**:** LOS=Level of Significance; NS=Not significantly different;

SF=Significantly different.

Table 4.13: Comparing the Mean zone of inhiition of Honey and the Standard antibiotic formulations against *Pseudomonas aeruginosa*

(I) Group	(J) Group	Mean±SEM	P-value	LOS
Honey (100%)	CRO	20.083±0.701	0.795	NS
	CN	20.333±0.541	0.882	NS
	AMC	10.083±1.510	0.000	SF
	LEV	26.333±0.948	0.060	NS
	AZM	18.083±0.802	0.070	NS
	Honey (50%)	18.167±0.991	0.081	NS
	Honey (25%)	12.583±0.417	0.000	SF
Honey (50%)	CRO	20.083±0.701	0.856	NS
	CN	20.333±0.541	0.761	NS
	AMC	10.083±1.510	0.000	SF
	LEV	26.333±0.948	0.000	SF
	AZM	18.083±0.802	1.000	NS
	Honey (100%)	22.167±1.325	0.081	NS
	Honey (25%)	12.583±0.417	0.002	SF
Honey (25%)	CRO	20.083±0.701	0.000	SF
	CN	20.333±0.541	0.000	SF
	AMC	10.083±1.510	0.606	NS
	LEV	26.333±0.948	0.000	SF

AZM	18.083±0.802	0.003	SF
Honey (100%)	22.167±1.325	0.000	SF
Honey (50%)	18.167±0.991	0.002	SF

Key**:** LOS=Level of Significance; NS=Not significantly different;

SF=Significantly different.

Table 4.14: Comparing the Mean zone of inhibition of Honey and the Standard antibiotic formulations against *Streptococcus pneumoniae*

(I) Group	(J) Group	Mean±SEM	P-value	LOS
	CRO	21.714±1.924	0.978	NS
	CN	20.429±1.556	1.000	NS
Honey (100%)	AMC	19.286±2.067	1.000	NS
	LEV	21.286±1.322	0.995	NS
	AZM	16.143±1.945	0.677	NS

	Honey (50%)	15.429±0.528	0.455	NS
	Honey (25%)	11.286±0.680	0.004	SF
Honey (50%)	CRO	21.714±1.924	0.072	NS
	CN	20.429±1.556	0.263	NS
	AMC	19.286±2.067	0.588	NS
	LEV	21.286±1.322	0.115	NS
	AZM	16.143±1.945	1.000	NS
	Honey (100%)	19.714±0.808	0.455	NS
	Honey (25%)	11.286±0.680	0.498	NS
Honey (25%)	CRO	21.714±1.924	0.000	SF
	CN	20.429±1.556	0.001	SF
	AMC	19.286±2.067	0.008	SF
	LEV	21.286±1.322	0.000	SF
	AZM	16.143±1.945	0.297	NS
	Honey (100%)	19.714±0.808	0.004	SF
	Honey (50%)	15.429±0.528	0.498	NS

Key**:** LOS=Level of Significance; NS=Not significantly different;

SF=Significantly different.

Table 4.15: Comparing the Mean zone of inhibition of Honey and the Standard antibiotic formulations against *Strepto*coccus *pyogenes*

(I) Group	(J) Group	Mean±SEM	P-value	LOS
Honey (100%)	CRO	20.667±2.275	0.996	NS
	CN	21.833±0.946	0.911	NS
	AMC	16.333±2.431	0.882	NS
	LEV	19.500±1.803	1.000	NS
	AZM	15.667±1.430	0.723	NS
	Honey (50%)	15.833±0.477	0.768	NS
	Honey (25%)	11.500±0.619	0.018	SF
Honey (50%)	CRO	20.667±2.275	0.336	NS
	CN	21.833±0.946	0.120	NS
	AMC	16.333±2.431	1.000	NS
	LEV	19.500±1.803	0.675	NS
	AZM	15.667±1.430	1.000	NS
	Honey (100%)	19.167±0.543	0.768	NS
	Honey (25%)	11.500±0.619	0.474	NS
Honey (25%)	CRO	20.667±2.275	0.002	SF
	CN	21.833±0.946	0.000	SF
	AMC	16.333±2.431	0.336	NS
	LEV	19.500±1.803	0.012	SF
	AZM	15.667±1.430	0.524	NS
	Honey (100%)	19.167±0.543	0.018	SF
	Honey (50%)	15.833±0.477	0.474	NS

Key**:** LOS=Level of Significance; NS=Not significantly different;

SF=Significantly different.

Table 4.16: Comparing the Mean zone of inhibition of Lemon juice and the Standard antibiotic formulations against *Klebsiella pneumoniae*

(I) Group	(J) Group	Mean±SEM	P-value	LOS
Lemon (100%)	CRO	20.667±1.511	1.000	NS
	CN	19.267±1.523	0.902	NS
	AMC	17.667±1.670	0.313	NS
	LEV	19.867±1.171	0.984	NS
	AZM	15.467±1.050	0.009	SF
	Lemon (50%)	18.333±0.374	0.576	NS
	Lemon (25%)	15.133±0.435	0.005	SF
Lemon (50%)	CRO	20.667±1.511	0.831	NS
	CN	19.267±1.523	0.999	NS
	AMC	17.667±1.670	1.000	NS

	LEV	19.867±1.171	0.980	NS
	AZM	15.467±1.050	0.632	NS
	Lemon (100%)	21.333±0.374	0.576	NS
	Lemon (25%)	15.133±0.435	0.492	NS
	CRO	20.667±1.511	0.018	SF
	CN	19.267±1.523	0.177	NS
	AMC	17.667±1.670	0.763	NS
Lemon (25%)	LEV	19.867±1.171	0.073	NS
	AZM	15.467±1.050	1.000	NS
	Lemon (100%)	21.333±0.374	0.005	SF
	Lemon (50%)	18.333±0.374	0.492	NS

Key**:** LOS=Level of Significance; NS=Not significantly different;

SF=Significantly different.

Table 4.17: Comparing the Mean zone of inhibition of Lemon juice and the Standard antibiotic formulations against *Staphylococcus aureus*

(I) Group	(J) Group	Mean±SEM	P-value	LOS
	CRO	20.000±1.129	1.000	NS
	CN	21.571±1.073	0.980	NS
	AMC	20.286±1.669	1.000	NS
Lemon (100%)	LEV	25.857±1.148	0.009	SF
	AZM	19.643±1.243	1.000	NS
	Lemon (50%)	17.500±0.918	0.734	NS
	Lemon (25%)	13.714±0.615	0.003	SF
	CRO	20.000±1.129	0.761	NS
	CN	21.571±1.073	0.178	NS
	AMC	20.286±1.669	0.648	NS
Lemon (50%)	LEV	25.857±1.148	0.000	SF
	AZM	19.643±1.243	0.875	NS
	Lemon (100%)	20.071±0.843	0.734	NS
	Lemon (25%)	13.714±0.615	0.256	NS
	CRO	20.000±1.129	0.003	SF
	CN	21.571±1.073	0.000	SF
	AMC	20.286±1.669	0.002	SF
Lemon (25%)	LEV	25.857±1.148	0.000	SF
	AZM	19.643±1.243	0.007	SF
	Lemon (100%)	20.071±0.843	0.003	SF
	Lemon (50%)	17.500±0.918	0.256	NS

Key: LOS=Level of Significance; NS=Not significantly different;

SF=Significantly different.

Table 4.18: Comparing the Mean zone of inhibition of Lemon juice and the Standard antibiotic formulations against *Haemophilus influenzae*

(I) Group	(J) Group	Mean±SEM	P-value	LOS
Lemon (100%)	CRO	25.500±2.500	0.152	NS
	CN	20.500±0.500	1.000	NS
	AMC	18.000±0.000	0.769	NS
	LEV	22.500±0.500	0.900	NS
	AZM	14.500±0.500	0.069	NS
	Lemon (50%)	17.500±0.500	0.609	NS
	Lemon (25%)	16.000±1.000	0.224	NS
Lemon (50%)	CRO	25.500±2.500	0.015	SF
	CN	20.500±0.500	0.609	NS
	AMC	18.000±0.000	1.000	NS
	LEV	22.500±0.500	0.152	NS
	AZM	14.500±0.500	0.609	NS

	Lemon (100%)	20.500±1.500	0.609	NS
	Lemon (25%)	16.000±1.000	0.974	NS
	CRO	25.500±2.500	0.005	SF
	CN	20.500±0.500	0.224	NS
	AMC	18.000±0.000	0.900	NS
Lemon (25%)	LEV	22.500±0.500	0.047	SF
	AZM	14.500±0.500	0.974	NS
	Lemon (100%)	20.500±1.500	0.224	NS
	Lemon (50%)	17.500±0.500	0.974	NS

Key: LOS=Level of Significance; NS=Not significantly different;

SF=Significantly different.

Table 4.19: Comparing the Mean zone of inhibition of Lemon juice and the Standard antibiotic formulations against *Pseudomonas aeruginosa*

(I) Group	(J) Group	Mean±SEM	P-value	

				LOS
	CRO	20.083±0.701	0.913	NS
	CN	20.333±0.541	0.818	NS
	AMC	10.083±1.510	0.000	SF
Lemon (100%)	LEV	26.333±0.948	0.000	SF
	AZM	18.083±0.802	1.000	NS
	Lemon (50%)	16.250±0.429	0.398	NS
	Lemon (25%)	12.667±0.310	0.000	SF
	CRO	20.083±0.701	0.022	SF
	CN	20.333±0.541	0.011	SF
	AMC	10.083±1.510	0.000	SF
Lemon (50%)	LEV	26.333±0.948	0.000	SF
	AZM	18.083±0.802	0.735	NS
	Lemon (100%)	18.667±0.466	0.398	NS
	Lemon (25%)	12.667±0.310	0.042	SF
	CRO	20.083±0.701	0.000	SF
	CN	20.333±0.541	0.000	SF
	AMC	10.083±1.510	0.312	NS
Lemon (25%)	LEV	26.333±0.948	0.000	SF
	AZM	18.083±0.802	0.000	SF
	Lemon (100%)	18.667±0.466	0.000	SF
	Lemon (50%)	16.250±0.429	0.042	SF

Key: LOS=Level of Significance; NS=Not significantly different;

SF=Significantly different.

Table 4.20: Comparing the Mean zone of inhibition of Lemon juice and the Standard antibiotic formulations against *Streptococcus pneumoniae*

(I) Group	(J) Group	Mean±SEM	P-value	LOS
Lemon (100%)	CRO	21.714±1.924	0.999	NS
	CN	20.429±1.556	0.919	NS
	AMC	19.286±2.067	0.638	NS
	LEV	21.286±1.322	0.991	NS
	AZM	16.143±1.945	0.038	SF
	Lemon (50%)	18.286±0.184	0.339	NS
	Lemon (25%)	15.429±0.297	0.015	SF
Lemon (50%)	CRO	21.714±1.924	0.724	NS
	CN	20.429±1.556	0.968	NS
	AMC	19.286±2.067	1.000	NS
	LEV	21.286±1.322	0.836	NS
	AZM	16.143±1.945	0.968	NS
	Lemon (100%)	23.000±0.195	0.339	NS
	Lemon (25%)	15.429±0.297	0.867	NS

	CRO	21.714±1.924	0.074	NS
	CN	20.429±1.556	0.269	NS
	AMC	19.286±2.067	0.593	NS
Lemon (25%)	LEV	21.286±1.322	0.118	NS
	AZM	16.143±1.945	1.000	NS
	Lemon (100%)	23.000±0.195	0.015	SF
	Lemon (50%)	18.286±0.184	0.867	NS

Key: LOS=Level of Significance; NS=Not significantly different;

SF=Significantly different.

Table 4.21: Comparing the Mean zone of inhibition of Lemon juice and the Standard antibiotic formulations against *Strept*ococcus *pyogenes*

(I) Group	(J) Group	Mean±SEM	P-value	LOS

	CRO	18.500±2.473	0.982	NS
	CN	20.167±1.887	1.000	NS
	AMC	14.667±2.216	0.205	NS
Lemon (100%)	LEV	18.833±2.151	0.993	NS
	AZM	15.667±1.430	0.418	NS
	Lemon (50%)	17.833±0.543	0.926	NS
	Lemon (25%)	15.333±0.558	0.337	NS
	CRO	18.500±2.473	1.000	NS
	CN	20.167±1.887	0.973	NS
	AMC	14.667±2.216	0.876	NS
Lemon (50%)	LEV	18.833±2.151	1.000	NS
	AZM	15.667±1.430	0.982	NS
	Lemon (100%)	20.667±0.558	0.926	NS
	Lemon (25%)	15.333±0.558	0.961	NS
	CRO	18.500±2.473	0.876	NS
	CN	20.167±1.887	0.461	NS
	AMC	14.667±2.216	1.000	NS
Lemon (25%)	LEV	18.833±2.151	0.810	NS
	AZM	15.667±1.430	1.000	NS
	Lemon (100%)	20.667±0.558	0.337	NS
	Lemon (50%)	17.833±0.543	0.961	NS

Key: LOS=Level of Significance; NS=Not significantly different;

SF=Significantly different.

Table 4.22: Comparing the Mean zone of inhibition of Honey/Lemon juice mixture and the Standard antibiotic formulations against *Klebsiella pneumoniae*

(I) Group	(J) Group	Mean±SEM	P-value	LOS
H/L; 10:50 (21.600±0.321)	CRO	20.667±1.511	1.000	NS
	CN	19.267±1.523	0.833	NS
	AMC	17.667±1.670	0.168	NS
	LEV	19.867±1.171	0.970	NS
	AZM	15.467±1.050	0.001	SF
H/L; 20: 50 (21.933±0.248)	CRO	20.667±1.511	0.997	NS
	CN	19.267±1.523	0.697	NS
	AMC	17.667±1.670	0.096	NS
	LEV	19.867±1.171	0.912	NS
	AZM	15.467±1.050	0.001	SF
H/L; 30:50 (22.000±0.218)	CRO	20.667±1.511	0.995	NS
	CN	19.267±1.523	0.666	NS
	AMC	17.667±1.670	0.085	NS
	LEV	19.867±1.171	0.895	NS
	AZM	15.467±1.050	0.000	SF
	CRO	20.667±1.511	0.993	NS
	CN	19.267±1.523	0.634	NS

H/L; 40:50 (22.067±0.300)	AMC	17.667±1.670	0.075	NS
	LEV	19.867±1.171	0.876	NS
	AZM	15.467±1.050	0.000	SF
H/L; 50:50 (24.067±0.358)	CRO	20.667±1.511	0.352	NS
	CN	19.267±1.523	0.034	SF
	AMC	17.667±1.670	0.001	SF
	LEV	19.867±1.171	0.108	NS
	AZM	15.467±1.050	0.000	SF

Key: LOS=Level of Significance; NS=Not significantly different;

SF=Significantly different; L=Lemon juice, H=Honey.

Table 4.23: Comparing the Mean zone of inhibition of Honey/Lemon juice mixture and the Standard antibiotic formulations against *Staphylococcus aureus*

(I) Group	(J) Group	Mean±SEM	P-value	LOS
H/L; 10:50 (22.071±0.529)	CRO	20.000±1.129	0.898	NS
	CN	21.571±1.073	1.000	NS
	AMC	20.286±1.669	0.957	NS
	LEV	25.857±1.148	0.183	NS
	AZM	19.643±1.243	0.773	NS
H/L; 20: 50 (22.143±0.501)	CRO	20.000±1.129	0.877	NS
	CN	21.571±1.073	1.000	NS
	AMC	20.286±1.669	0.946	NS
	LEV	25.857±1.148	0.204	NS

	AZM	19.643±1.243	0.742	NS
H/L; 30:50 (22.643±0.829)	CRO	20.000±1.129	0.677	NS
	CN	21.571±1.073	0.999	NS
	AMC	20.286±1.669	0.802	NS
	LEV	25.857±1.148	0.398	NS
	AZM	19.643±1.243	0.501	NS
H/L; 40:50 (22.429±0.510)	CRO	20.000±1.129	0.773	NS
	CN	21.571±1.073	1.000	NS
	AMC	20.286±1.669	0.877	NS
	LEV	25.857±1.148	0.306	NS
	AZM	19.643±1.243	0.607	NS
H/L; 50:50 (24.500±0.489)	CRO	20.000±1.129	0.051	SF
	CN	21.571±1.073	0.536	NS
	AMC	20.286±1.669	0.088	NS
	LEV	25.857±1.148	0.993	NS
	AZM	19.643±1.243	0.024	SF

Key: LOS=Level of Significance; NS=Not significantly different;

SF=Significantly different; L=Lemon juice, H=Honey.

Table 4.24: Comparing the Mean zone of inhibition of Honey/Lemon juice mixture and the Standard antibiotic formulations against *Haemophilus influenzae*

(I) Group	(J) Group	Mean±SEM	P-value	LOS

H/L; 10:50 (23.500±0.500)	CRO	25.500±2.500	0.981	NS
	CN	20.500±0.500	0.841	NS
	AMC	18.000±0.000	0.233	NS
	LEV	22.500±0.500	1.000	NS
	AZM	14.500±0.500	0.018	SF
H/L; 20: 50 (23.000±2.000)	CRO	25.500±2.500	0.933	NS
	CN	20.500±0.500	0.933	NS
	AMC	18.000±0.000	0.324	NS
	LEV	22.500±0.500	1.000	NS
	AZM	14.500±0.500	0.026	SF
H/L; 30:50 (21.000±1.000)	CRO	25.500±2.500	0.439	NS
	CN	20.500±0.500	1.000	NS
	AMC	18.000±0.000	0.841	NS
	LEV	22.500±0.500	0.997	NS
	AZM	14.500±0.500	0.114	NS
H/L; 40:50 (24.000±2.000)	CRO	25.500±2.500	0.997	NS
	CN	20.500±0.500	0.714	NS
	AMC	18.000±0.000	0.164	NS
	LEV	22.500±0.500	0.997	NS
	AZM	14.500±0.500	0.012	SF
H/L; 50:50 (25.500±1.500)	CRO	25.500±2.500	1.000	NS
	CN	20.500±0.500	0.324	NS
	AMC	18.000±0.000	0.054	SF
	LEV	22.500±0.500	0.841	NS
	AZM	14.500±0.500	0.004	SF

Key: LOS=Level of Significance; NS=Not significantly different; SF=Significantly different; L=Lemon juice, H=Honey.

Table 4.25: Comparing the Mean zone of inhibition of Honey/Lemon juice mixture and the Standard antibiotic formulations against *Pseudomonas aeruginosa*

(I) Group	(J) Group	Mean±SEM	P-value	LOS
H/L; 10:50 (20.500±0.314)	CRO	20.083±0.701	1.000	NS
	CN	20.333±0.541	1.000	NS
	AMC	10.083±1.510	0.000	SF
	LEV	26.333±0.948	0.000	SF
	AZM	18.083±0.802	0.391	NS
H/L; 20: 50 (21.750±0.329)	CRO	20.083±0.701	0.849	NS
	CN	20.333±0.541	0.938	NS
	AMC	10.083±1.510	0.000	SF
	LEV	26.333±0.948	0.001	SF
	AZM	18.083±0.802	0.022	SF
H/L; 30:50 (21.417±0.621)	CRO	20.083±0.701	0.957	NS
	CN	20.333±0.541	0.989	NS
	AMC	10.083±1.510	0.000	SF
	LEV	26.333±0.948	0.000	SF
	AZM	18.083±0.802	0.056	SF
H/L; 40:50 (21.833±0.366)	CRO	20.083±0.701	0.808	NS
	CN	20.333±0.541	0.914	NS
	AMC	10.083±1.510	0.000	SF
	LEV	26.333±0.948	0.001	SF
	AZM	18.083±0.802	0.017	SF

	CRO	20.083±0.701	0.028	SF
	CN	20.333±0.541	0.056	SF
H/L; 50:50 (23.667±0.376)	AMC	10.083±1.510	0.000	SF
	LEV	26.333±0.948	0.255	NS
	AZM	18.083±0.802	0.000	SF

Key: LOS=Level of Significance; NS=Not significantly different; SF=Significantly different; L=Lemon juice, H=Honey.

Table 4.26: Comparing the Mean zone of inhibition of Honey/Lemon juice mixture and the Standard antibiotic formulations against *Streptococcus pneumoniae*

(I) Group	(J) Group	Mean±SEM	P-value	LOS
	CRO	21.714±1.924	0.999	NS
	CN	20.429±1.556	0.917	NS
H/L; 10:50 (23.143±0.769)	AMC	19.286±2.067	0.593	NS
	LEV	21.286±2.067	0.993	NS
	AZM	16.143±1.945	0.018	SF
	CRO	21.714±1.924	0.998	NS
	CN	20.429±1.556	0.890	NS
H/L; 20: 50 (23.286±0.522)	AMC	19.286±2.067	0.543	NS
	LEV	21.286±2.067	0.988	NS
	AZM	16.143±1.945	0.014	SF
	CRO	21.714±1.924	1.000	NS
	CN	20.429±1.556	0.996	NS

H/L; 30:50 (22.143±0.553)	AMC	19.286±2.067	0.890	NS
	LEV	21.286±2.067	1.000	NS
	AZM	16.143±1.945	0.074	SF
H/L; 40:50 (23.143±0.800)	CRO	21.714±1.924	0.999	NS
	CN	20.429±1.556	0.917	NS
	AMC	19.286±2.067	0.593	NS
	LEV	21.286±2.067	0.993	NS
	AZM	16.143±1.945	0.018	SF
H/L; 50:50 (24.571±0.782)	CRO	21.714±1.924	0.890	NS
	CN	20.429±1.556	0.493	NS
	AMC	19.286±2.067	0.174	NS
	LEV	21.286±2.067	0.782	NS
	AZM	16.143±1.945	0.002	SF

Key: LOS=Level of Significance; NS=Not significantly different; SF=Significantly different; L=Lemon juice, H=Honey.

Table 4.27: Comparing the Mean zone of inhibition of Honey/Lemon juice mixture and the Standard antibiotic formulations against *Streptococcus pyogenes*

(I) Group	(J) Group	Mean±SEM	P-value	LOS
H/L; 10:50 (23.333±0.955)	CRO	20.667±2.275	0.949	NS
	CN	21.833±0.946	0.999	NS
	AMC	16.333±2.431	0.038	SF

	LEV	19.500±1.803	0.687	NS
	AZM	15.667±1.430	0.016	SF
H/L; 20: 50 (23.833±0.872)	CRO	20.667±2.275	0.867	NS
	CN	21.833±0.946	0.992	NS
	AMC	16.333±2.431	0.020	SF
	LEV	19.500±1.803	0.526	NS
	AZM	15.667±1.430	0.008	SF
H/L; 30:50 (21.333±0.715)	CRO	20.667±2.275	1.000	NS
	CN	21.833±0.946	1.000	NS
	AMC	16.333±2.431	0.325	NS
	LEV	19.500±1.803	0.996	NS
	AZM	15.667±1.430	0.176	NS
H/L; 40:50 (23.833±0.703)	CRO	20.667±2.275	0.867	NS
	CN	21.833±0.946	0.992	NS
	AMC	16.333±2.431	0.020	SF
	LEV	19.500±1.803	0.526	NS
	AZM	15.667±1.430	0.008	SF
H/L; 50:50 (25.833±1.014)	CRO	20.667±2.275	0.282	NS
	CN	21.833±0.946	0.635	NS
	AMC	16.333±2.431	0.001	SF
	LEV	19.500±1.803	0.086	NS
	AZM	15.667±1.430	0.000	SF

Key: LOS=Level of Significant; NS=Not significantly different; SF=Significantly different; L=Lemon juice, H=Honey.

CHAPTER FIVE

5.0 Discussion

The bacterial isolates in the retrospective study were *Streptococcus pneumoniae, Staphylococcus aureus, Klebsiella pneumoniae, Streptococcus pyogenes, Pseudomonas aeruginosa and Haemophilus infleunzae.* In a study by Dilnawaz *et al*., (1995), various Gram negative and Gram positive organisms similar to those reported in this work, were isolated from patients presented with respiratory infections.

The common antibiotics used for susceptibility testing at the ABUTH and UHS A.B.U., Zaria include: Ciprofloxacin (CIP), Tetracycline (TE), Ofloxacin (OFX), Erythromycin (E), Ceftriaxone (CEF), Gentamicin (CN), Amoxicillin (AMX), Cefuroxime (CXM), Ampicillin (AMP), Amoxicillin-Clavulanic acid (AMC) and Cotrimoxazole (COT). From their records at ABUTH, *Streptococcus pneumoniae* were found to be mostly susceptible to CN, CXM, and AMX but mostly resistant to COT, CRO; *Staphylococcus aureus* were found to be sensitive to CN, CIP, and AMX but showed resistance to AMC and AMP; *Pseudomonas aeruginosa* isolates were sensitive to CIP, CN but showed resistance to E, AMC, and TE; *Klebsiella pneumoniae* isolates were sensitive to CN and CIP but showed resistance to COT and CXM; and *Streptococcus pyogenes* were mostly sensitive to CIP, CN, TE and E but showed resistance to COT. However, at UHS *Streptococcus pneumoniae* were found to be mostly susceptible to CN, CIP, and OFX but mostly resistant to COT, AMC and E; *Staphylococcus aureus* were found to be sensitive to OFX, CN, and CIP but showed resistance to AMC and COT; *Pseudomonas aeruginosa* isolates were sensitive to CIP, CN and OFX but showed resistance to AMC, and COT; *Klebsiella pneumoniae* isolates were sensitive to OFX, CN and CIP but showed resistance to COT and AMC; and *Streptococcus pyogenes* were mostly sensitive to CIP and CN but showed resistance to COT and AMC. This is because most of the antibiotics the isolates were sensitive to are out of reach of the poor due to the cost and even for some of

the antibiotics that are cheaper, they are mostly administered parentally, hence less abused (for example, Gentamicin).

However, in the prospective study, the highest percentage of *Klebsiella pneumoniae*, *Staphylococcus aureus* as predominant organisms isolated is in close proximity with several other findings such as a study conducted in Benin City by Ophori and Wemabu (2010). They identified bacteria isolates such as *H. influenzae, Klebsiella pneumoniae, Streptococcus pneumoniae, Moraxella catarrhalis* and *Streptococcus pyogenes*. However, *H. influenzae* had the highest percentage prevalence of 20.8 %, followed by *Klebsiella pneumoniae* (19.2 %), *Streptococcus pneumoniae* (12.0 %), *Moraxella catarrhalis* (10.0 %) and *Streptococcus pyogenes* (2.00 %).

Haemophilus influenzae were found to be completely susceptible to all the tested antibiotics; which is in contrast to work done by Lysenko *et* al., (2005) and Chang *et al*., (2010), who observed fluoroquinolone-resistance among *Haemophilus influenzae* species used in their research. *Pseudomonas aeruginosa* however, were susceptible to CRO, CN, LEV, and AZM, but showed remarkable resistance to AMC. *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Streptococcus pyogenes* were more sensitive to LEV. Generally, all the bacterial isolates were more susceptible to LEV, CRO, and CN; but less susceptible to AMC and AZM.

All the tested bacterial isolates were completely susceptible to the crude concentrations of Honey, Lemon juice and the mixture of Honey and Lemon juice. They were also moderately susceptible to Honey at 50 % v/v concentration, Lemon juice at 25 % v/v concentration; but showed resistance to Honey at 25 % v/v concentration. This is in agreement to works done by Kawaii *et al*., (2000) and Ifra and Sheikh, (2009) who reported that the stock solution of the honey samples inhibited the growth of all the bacterial isolates, but when the dilutions were made the efficacy reduced.

This activity of honey is attributed to its antibacterial property with regards to its high osmolarity, acidity (low pH) and content of hydrogen peroxide (H_2O_2) and non-peroxide components, i.e., the presence of phytochemical components like methylglyoxal (MGO) (Weston, 2000; Mavric *et al*., 2008). The antimicrobial agents in honey are predominantly hydrogen peroxide, of which the concentration is determined by relative levels of glucose oxidase, synthesized by the bee and catalase originating from flower pollen (Mavric *et al*., 2008). Most types of honey generate Hydrogen peroxide (H_2O_2) when diluted, because of the activation of the enzyme glucose oxidase that oxidizes glucose to gluconic acid and H_2O_2, which thus attributes the antimicrobial activity (Bang *et al*., 2003).

Apart from honey and lemon being active against the tested bacteria in this study, it has also been found to be active against other bacteria by other researchers; for example, research conducted on manuka (*L. scoparium*) honey, demonstrated effectiveness against several human pathogens, including *Escherichia coli*, *Enterobacter aerogenes*, *Salmonella typhimurium*, *Staphylococcus aureus* (Visavadia *et al.,* 2006). Laboratory studies have revealed that honey is effective against methicillin-resistant *Staphylococcus aureus* (MRSA), β-haemolytic *Streptococci* and vancomycin resistant *Enterococci* (VRE) (Allen *et al*., 2000; Kingsley, 2001). The zone diameter of inhibition of different honey samples (5-20 % concentration) has been determined against *E. coli* O157:H7 and *Salmonella typhimurium,* where (12 mm - 24 mm) and (0 mm – 20 mm) zones of inhibition were obtained respectively (Badawy *et al*., 2004). This is contrary to results obtained in this study especially for the 25% dilution. Agbagwa and Frank-Peterside (2010) examined different honey samples: Western Nigerian honey, Southern Nigerian honey, Eastern Nigerian honey and Northern Nigerian honey, and compared their abilities to inhibit the growth of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Proteus mirabilis* with an average of zone diameter of inhibition (5.3-11.6) mm, (1.4-15.4) mm, (4.4-13.5) mm and (9.1-17) mm, respectively, and with honey concentrations of 80%-100%.

Using the MIC for the resistance profile, it was found that the bacterial isolates were more resistant to Amoxicillin-Clavulanic Acid and Azithromycin due to increase in the MIC and MBC values. The bacterial isolates were more sensitive to Ceftriaxone and Levofloxacin; and moderately sensitive to Gentamicin as observed with the decrease in MIC and MBC values. Generally, there was reduction in MIC and MBC in the mixture of Honey and Lemon compared to the Honey alone. However, there was increase in the MIC and the MBC in the mixture of Honey and Lemon compared to the Lemon alone. This is probably because lemon has low acidity pH than honey. The least MIC values observed for Honey and Lemon juice was 15 (µg/ml) v/v. However, Sherlock *et al*., (2010) reported a lower MIC value in ulmo (*Eucryphia cordifolia*) honey (3.1 - 6.3 % v/v) than in manuka honey (12.5 % v/v) for MRSA isolates; while for the *Escherichia coli* and *Pseudomonas* strains equivalent MIC values were observed (12.5 % v/v). The MIC values for Tualang honey ranged 8.75 – 25 %, while those for manuka honey ranged 8.75 - 20 % against many pathogenic Gram-positive and Gram negative bacteria as reported by (Tan *et al*., 2009).

However, for the standard antibiotics, from the MIC and MBC results, resistance to Ceftriaxone and Levofloxacin is low. This is probably because Ceftriaxone and Levofloxacin are relatively costly, not within the reach of the poor sector of the populace, hence less abused. Thus, high sensitivity profile shown by the organisms to the standard antibiotics used in this study (especially, Ceftriaxone, Gentamicin, and Levofloxacin) is an indication that these drugs are still effective in this region. The aminoglycosides (Gentamicin), though relatively cheap, are only available as injectables and hence not easily abused.

The bacterial isolates less susceptible to Amoxicillin-Clavulanic acid and Azithromycin is in agreement to a work done by Abd-el Aal (2007). Abd-El Aal reported in his work that the mean zone of inhibition produced by honey against the isolated Gram negative organisms were significantly higher than that of Amoxicillin-Clavulanic acid.

However, Ceftriaxone, Levofloxacin and Gentamicin were observed to be the most active among the reference antibiotics.

Generally, inadequate antimicrobial treatment defined as ineffective treatment of infection, due to non-completion of dosage regimen upon feeling of relief and use of left-over drugs when disturbed with common cold or catarrh is an important factor in the emergence of antibiotic resistant bacteria. Factors that contribute to inadequate antimicrobial treatment of hospitalized patients includes: the prior use of antibiotics not being prescribed to the patients, use of broad spectrum antibiotics, prolonged hospital stay, antibiotics used in poultry farms. Other factors include the spread of resistant organisms by health-care workers to patients, overcrowding and inadequate hospital infection control practices (Marin, 2000). Due to these factors, the populace especially those from remote areas resort to using the nature's gift around them. Of which honey and lemon has been used by many especially in the treatment of common cold and sore throat and has been found effective.

The pressure of prolonged usage and regular abuse in our society has led to general resistance to many antibiotics. The resistance to Azithromycin and Amoxicillin-Clavulanic could be due to the abuse of these drugs by the populace; since in Nigeria, different antibiotics are readily available from patent medicine shops, street drug vendors, and in open markets; sources where adequate storage conditions are not adhered to. This has also motivated others to resort in the use of honey and lemon which can be obtained in their environment, than spending their money on drugs and at the end will not be found effective due to problem of resistance.

In addition, a significant proportion of the populace are involved in self-medication. As a result, many antibiotics are bought from illegal conduits and such patients only turn to physicians when their self-medication approaches have failed. However, before this stage is

reached, many antibiotics would have been used and in most cases, inadequate dosage regimens, and extremely short durations are the norm, rather than the exception. The contributory effect of this practice (self-medication) on the emergence of resistant organisms is enormous. Many therefore, tends to traditionally use the honey and lemon when the case is not that complicated than buying drugs from the illegal conduits for self-medication and later suffer for their actions. While some people in some part of Nigeria still rely on traditional medicine as they do not believe in orthodox medicine.

The rate of kill provides more accurate description of antimicrobial activity of antimicrobial agents than does the MIC (Mandal *et al*., 2009). The rate of kill of the resistant and even the susceptible *Klebsiella pneumoniae* on exposure to the test agents showed that Lemon juice effected a better killing (evidenced by the sharp decrease in the bacterial cell populations) than the Honey, Honey and Lemon juice mixture, and the standard antibiotics. However, Honey and Lemon juice mixture effected a better decrease in the bacterial cell populations than the Lemon, Honey and the standard antibiotics against the resistant *Streptococcus pyogenes*. This is due to the highly acidic pH of the honey and lemon juice mixture (though their types of acids differs). It was however, observed that Honey/Lemon mixture and Lemon effected similar decrease in the bacterial cell populations against the susceptible *Streptococcus pneumoniae.* Kwakman *et al*., (2008) also reported in their work that antibiotic susceptible and resistant isolates of *S. aureus*, *S. epidermidis*, *Enterococcus faecium*, *E. coli*, *P. aeruginosa*, *E. cloacae*, and *Klebsiella oxytoca* were killed within 24 h by 10 – 40 % (v/v) honey.

The activities of Honey and Lemon mixtures were additive and not antagonistic. Which means the agents are acting additively. This is also because the ratio of the concentration of each of the agent to their various minimum inhibitory concentrations in the mixtures were not the same. This also indicates that the minimum inhibitory concentrations

of the mixture of honey and lemon is lower than the sum of their independent MIC values as used by (Spoorthi *et al*., 2011).

The null hypothesis that, 'honey does not have antibacterial activity against isolates from respiratory tract infections' was therefore rejected; the null hypothesis that, 'lemon juice does not have antibacterial activity against isolates from respiratory tract infections' was also rejected. However, based on the Calculation carried out using the Checkerboard formula for the synergy test, the null hypothesis that, 'honey and lemon when used in combination do not produce synergistic effect against isolates associated with respiratory tract infections' was accepted. This is because the ∑FIC index levels of honey/lemon juice mixture falls basically within the additive and indifference level. That is to say honey and lemon juice has additive effect. There is significant difference in the mean zones of inhibition between the standard antibiotics and lower concentrations of honey and lemon juice used. This is because crude concentrations of the honey and lemon juice produces a better activity than the lower concentrations. Thus, the significant difference between the standard antibiotics and the lower concentrations of the agents.

The higher the concentration of honey and lemon juice, the better the activity. However, as the dilution increases, the efficacy is reduced. This is in agreement to works done by Kawaii *et al*., (2000); Ifra and Sheikh (2009) who reported that the stock solution of the honey samples inhibited the growth of all the bacterial isolates, but when the dilutions were made the efficacy reduced.

Therefore, as vividly demonstrated in this research, Honey, Lemon and Honey/Lemon mixture were found to possess antibacterial activity; but to varying degrees. The mixture in various proportions gave a better activity compared to Honey alone, Lemon alone (in some of the organisms) and relative activity to Lemon in other organisms.

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATION

6.1 Summary

This research evaluated the efficacy of Honey and Lemon juice against bacterial isolates from respiratory tract infections; and evaluated and compared their activities separately and the mixture of the two agents. Their activities in comparison with the standard antibiotic formulations were also compared.

The following were the major findings:

Retrospective study of patients records from 2011-2012 revealed that:

Streptococcus pneumoniae was the major pathogen of the respiratory tract infections followed by *Staphylococcus aureus, Klebsiella pneumoniae, Streptococcus pyogenes,* and *pseudomonas aeruginosa*.

The most commonly prescribed antibiotics were Ceftriaxone, Gentamicin, Amoxicillin-Clavulanic acid (Augmentin®), Ciprofloxacin, Tetracycline, Ofloxacin, Erythromycin, Amoxicillin, Cefuroxime, Ampicillin, and Cotrimoxazole.

The prospective study which was conducted from March, 2012 to March, 2013; showed that:

The major pathogen of the respiratory tract infections was *Klebsiella pneumoniae*, followed by *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Streptococcus pneumoniae*, *Streptococcus pyogene*, and *Haemophilus influenzae*.

Most of the isolates were highly susceptible to Levofloxacin, Ceftriaxone and Gentamicin; less susceptible to Azithromycin and Amoxicillin-Clavulanic acid. Honey and Lemon exerted significant antibacterial activity (*in vitro*) against the various organisms, with Honey and Lemon mixture exerting a better activity than most of the standard antibiotics and a striking similarity in activity to some of them.

Honey and lemon mixture gave a better antibacterial activity against the test bacterial isolates, followed by the Lemon and then the Honey.

6.2 Conclusion

Bacteria isolates associated with respiratory tract infections were found to be *Streptococcus pneumoniae*, *Streptococcus pyogene*, *Haemophilus influenzae*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*.

The bacterial isolates were found to be more susceptible to Levofloxacin, Ceftriaxone, and Gentamicin; and less susceptible to Azithromicin and Amoxicillin-Clavulanic acid.

The activity of Honey and Lemon juice were found to be additive and not antagonistic.

Honey and Lemon juice had more inhibitory effect against all the tested bacterial isolates than the commonly used antibiotics especially Azithromycin and Amoxicillin-Clavulanic acid.

Honey and lemon juice generally possess antibacterial activity against the bacterial isolates. This research therefore has provided a scientific basis for the use of honey and lemon juice mixture as an alternative medicine by the populace in the treatment of respiratory tract infections.

6.3 Recommendation

It is therefore necessary that further investigations be undertaken to discover the possible ways of the clinical effectiveness of honey and lemon in RTIs.

It is also important that when honey is to be used as an antimicrobial agent, it should be selected from honey samples that have been assayed in the laboratory for antimicrobial activity.

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APPENDICES

APPENDIX I: RATE OF KILL RESULTS

Rate of kill (at average MIC values) for *Klebsiella pneumoniae*_Sp894 (Resistant)

Time (minutes)	No of colonies counted in LEV (7.81µg/ml) plate	No of colonies counted in CEF (12.5 µg/ml) plate	No of colonies counted in Lemon (20 µg/ml) plate	No of colonies counted in Honey (50 µg/ml) plate	No of colonies counted in H/L (20 µg/ml) plate
0	6.9×10^3	6.5×10^3	2.7×10^3	1.1×10^4	2.2×10^3
30	3.1×10^3	2.5×10^3	1.3×10^3	8.9×10^3	1.7×10^3
60	2.6×10^3	2.3×10^3	9.1×10^2	7.2×10^3	8.0×10^2
90	1.9×10^3	1.3×10^3	2.0×10^2	6.3×10^3	4.8×10^2
120	1.6×10^3	8.3×10^2	1.3×10^2	5.9×10^3	1.7×10^2
240	1.3×10^3	7.2×10^2	0	3.7×10^3	4×10
360	9.6×10^2	5.6×10^2	0	5.6×10^2	0
1440	4.9×10^2	3.6×10^2	0	9×10	0

Key: Sp=Sputum, MIC=Minimum Inhibitory Concentration, LEV=Levofloxacin, CEF=Ceftriaxone, H=Honey, L=Lemon juice.

Rate of Kill (at average MIC values) for *Klebsiella pneumoniae*_Sp270 (Susceptible)

Time (minutes)	No of colonies counted in LEV (7.81 µg/ml) plate	No of colonies counted in CEF (12.5 µg/ml) plate	No of colonies counted in Lemon (20 µg/ml) plate	No of colonies counted in Honey (50 µg/ml) plate	No of colonies counted in H/L (20 µg/ml) plate
0	1.3×10^3	4.0×10^3	2.0×10^3	4.3×10^3	1.0×10^3
30	3.5×10^2	1.5×10^3	1.2×10^3	1.8×10^3	8.0×10^2
60	2.7×10^2	1.3×10^3	5.0×10^2	1.6×10^3	5.9×10^2
90	2.3×10^2	1.1×10^3	1.0×10^2	8.4×10^2	1.0×10^2
120	2.0×10^2	9.0×10^2	0	6.9×10^2	1×10
240	1.2×10^2	4.7×10^2	0	3.1×10^2	0
360	8×10	2.1×10^2	0	1.8×10^2	0
1440	0	0	0	0	0

Key: Sp=Sputum, MIC=Minimum Inhibitory Concentration, LEV=Levofloxacin, CEF=Ceftriaxone, H=Honey, L=Lemon juice.

Rate of Kill (at average MIC values) for *Streptococcus pyogene*_Sp265 (Resistant)

Time (mins)	No of colonies counted in LEV (7.81 µg/ml) plate	No of colonies counted in CEF (12.5 µg/ml) plate	No of colonies counted in Lemon (20 µg/ml) plate	No of colonies counted in Honey (50 µg/ml) plate	No of colonies counted in H/L (20 µg/ml) plate
0	6.0×10^3	6.1×10^3	7.5×10^3	9.4×10^3	2.7×10^3
30	2.6×10^3	3.2×10^3	2.5×10^3	3.8×10^3	2.0×10^3
60	2.0×10^3	2.2×10^3	1.9×10^3	2.3×10^3	8.0×10^2
90	1.6×10^3	1.8×10^3	1.4×10^3	1.6×10^3	3.7×10^2
120	1.1×10^3	1.0×10^3	9.1×10^2	8.1×10^2	1.8×10^2
240	8.0×10^2	8.7×10^2	4.5×10^2	6.5×10^2	6×10
360	4.3×10^2	3.1×10^2	2.9×10^2	4.5×10^2	1×10
1440	1.1×10^2	1.9×10^2	7×10	2.8×10^2	0

Key: Sp=Sputum, MIC=Minimum Inhibitory Concentration, LEV=Levofloxacin, CEF=Ceftriaxone, H=Honey, L=Lemon juice.

Rate of Kill (at average MIC values) for *Streptococcus pneumoniae*_Sp232 (Susceptible)

Time (mins)	No of colonies counted in LEV (7.81 µg/ml) plate	No of colonies counted in CEF (12.5 µg/ml) plate	No of colonies counted in Lemon (20 µg/ml) plate	No of colonies counted in Honey (50 µg/ml) plate	No of colonies counted in H/L (20 µg/ml) plate
0	2.5×10^3	9.6×10^3	5.8×10^3	6.6×10^3	1.9×10^3
30	1.8×10^3	8.1×10^3	1.4×10^3	3.4×10^3	1.4×10^3
60	1.1×10^3	7.6×10^3	8.0×10^2	2.1×10^3	7.5×10^2
90	6.9×10^3	5.5×10^3	2×10	1.6×10^3	1.6×10^2
120	3.2×10^2	4.3×10^3	0	7.8×10^2	0
240	2.6×10^2	3.8×10^3	0	4.8×10^2	0
360	5×10	5.8×10^2	0	3.4×10^2	0
1440	0	0	0	0	0

Key: Sp=Sputum, MIC=Minimum Inhibitory Concentration, LEV=Levofloxacin, CEF=Ceftriaxone, H=Honey, L=Lemon juice.

Rate of kill (below average MIC values) for *Streptococcus pyogenes*_Sp265 (Resistant)

Time (mins)	No of colonies counted in LEV (3.91µg/ml) plate	No of colonies counted in CEF (6.25 µg/ml) plate	No of colonies counted in Lemon (10 µg/ml) plate	No of colonies counted in Honey (25 µg/ml) plate	No of colonies counted in H/L (10 µg/ml) plate
0	1.6×10^4	2.1×10^5	6.7×10^4	8.1×10^5	5.6×10^4
30	$1.3.\times10^4$	1.6×10^5	5.3×10^4	6.4×10^5	4.1×10^4
60	3.8×10^3	6.3×10^4	2.9×10^4	3.2×10^5	1.3×10^4
90	2.0×10^3	5.9×10^3	7.0×10^3	8.3×10^4	9.1×10^3

120	1.7×10^{3}	3.7×10^{3}	6.3×10^{3}	3.7×10^{4}	8.0×10^{3}
240	1.4×10^{4}	5.2×10^{4}	7.2×10^{4}	8.0×10^{4}	5.1×10^{3}
360	3.4×10^{4}	3.0×10^{5}	1.4×10^{4}	4.1×10^{5}	3.3×10^{4}
1440	1.7×10^{5}	3.4×10^{5}	1.0×10^{5}	3.1×10^{6}	5.8×10^{4}

Key: Sp=Sputum, MIC=Minimum Inhibitory Concentration, LEV=Levofloxacin, CEF=Ceftriaxone, H=Honey, L=Lemon juice.

Rate of kill (below average MIC values) for *Streptococcus pneumoniae*_Sp232 (Susceptible)

Time (mins)	No of colonies counted in LEV (3.91µg/ml) plate	No of colonies counted in CEF (6.25 µg/ml) plate	No of colonies counted in Lemon (10 µg/ml) plate	No of colonies counted in Honey (25 µg/ml) plate	No of colonies counted in H/L (10 µg/ml) plate
0	4.8×10^{3}	6.5×10^{3}	2.7×10^{3}	1.1×10^{5}	1.2×10^{4}
30	3.4×10^{3}	2.5×10^{3}	1.3×10^{3}	8.9×10^{4}	8.7×10^{3}
60	3.3×10^{3}	2.3×10^{3}	9.1×10^{2}	7.2×10^{3}	4.9×10^{3}
90	2.3×10^{3}	1.3×10^{3}	2.0×10^{2}	6.3×10^{3}	4.1×10^{3}
120	1.5×10^{3}	8.3×10^{2}	1.3×10^{2}	5.9×10^{3}	2.4×10^{3}
240	7.0×10^{2}	7.2×10^{2}	2.6×10^{2}	3.7×10^{3}	1.7×10^{3}
360	3.4×10^{2}	5.6×10^{2}	2.4×10^{2}	5.6×10^{4}	1.1×10^{3}
1440	1.7×10^{2}	3.6×10^{2}	1.9×10^{2}	9.0×10^{4}	1.1×10^{3}

Key: Sp=Sputum, MIC=Minimum Inhibitory Concentration, LEV=Levofloxacin, CEF=Ceftriaxone, H=Honey, L=Lemon juice.

Rate of kill (below average MIC values) for *Klebsiella pneumoniae*_Sp894 (Resistant)

Time (mins)	No of colonies counted in LEV (3.91µg/ml) plate	No of colonies counted in CEF (6.25 µg/ml) plate	No of colonies counted in Lemon (10 µg/ml) plate	No of colonies counted in Honey (25 µg/ml) plate	No of colonies counted in H/L (10 µg/ml) plate
0	9.2×10^5	2.6×10^6	7.1×10^4	3.4×10^6	9.5×10^4
30	$6.3.\times10^4$	1.6×10^5	6.0×10^4	9.2×10^5	9.2×10^4
60	2.8×10^4	8.3×10^4	4.2×10^4	5.3×10^5	8.9×10^3
90	1.5×10^3	4.5×10^3	8.3×10^3	1.3×10^5	6.8×10^3
120	3.9×10^3	7.3×10^3	5.9×10^2	7.4×10^4	7.5×10^2
240	4.3×10^4	7.0×10^4	5.1×10^3	7.9×10^4	6.2×10^2
360	6.1×10^5	1.3×10^4	2.7×10^4	3.3×10^5	4.8×10^3
1440	5.3×10^5	1.7×10^5	1.8×10^5	2.9×10^6	3.0×10^4

Key: Sp=Sputum, MIC=Minimum Inhibitory Concentration, LEV=Levofloxacin, CEF=Ceftriaxone, H=Honey, L=Lemon juice.

Rate of kill (below average MIC values) for *Klebsiella pneumoniae*_Sp270 (Susceptible)

Time (mins)	No of colonies counted in LEV (3.91µg/ml) plate	No of colonies counted in CEF (6.25 µg/ml) plate	No of colonies counted in Lemon (10 µg/ml) plate	No of colonies counted in Honey (25 µg/ml) plate	No of colonies counted in H/L (10 µg/ml) plate
0	7.3×10^{3}	9.5×10^{3}	8.4×10^{3}	4.1×10^{5}	4.2×10^{4}
30	7.0×10^{3}	8.2×10^{3}	7.5×10^{3}	8.0×10^{4}	3.8×10^{4}
60	5.8×10^{3}	7.3×10^{3}	9.6×10^{2}	6.4×10^{4}	6.9×10^{3}
90	9.3×10^{2}	7.1×10^{3}	7.0×10^{2}	7.3×10^{3}	7.1×10^{2}
120	7.1×10^{2}	8.2×10^{2}	5.2×10^{2}	6.2×10^{3}	3.4×10^{2}
240	4.6×10^{2}	6.6×10^{3}	6.3×10^{2}	7.7×10^{3}	6.7×10^{2}
360	6.6×10^{3}	4.3×10^{3}	4.5×10^{3}	3.5×10^{4}	3.3×10^{3}
1440	8.7×10^{3}	8.6×10^{3}	7.9×10^{3}	2.0×10^{5}	5.1×10^{3}

Key: Sp=Sputum, MIC=Minimum Inhibitory Concentration, LEV=Levofloxacin, CEF=Ceftriaxone, H=Honey, L=Lemon juice.

Rate of kill (above average MIC values) for *Klebsiella pneumoniae*_Sp894 (Resistant)

Time (mins)	No of colonies counted in LEV (15.62 µg/ml) plate	No of colonies counted in CEF (25 µg/ml) plate	No of colonies counted in Lemon (40 µg/ml) plate	No of colonies counted in Honey (100 µg/ml) plate	No of colonies counted in H/L (40 µg/ml) plate
0	6.9×10^{3}	2.8×10^{3}	2.7×10^{3}	2.4×10^{4}	2.2×10^{2}
30	3.1×10^{2}	5.3×10^{2}	1.3×10^{2}	4.2×10^{3}	1.1×10
60	6.0×10	3.0×10	3.1×10	7.2×10^{2}	0
90	0	0	0	1.3×10^{1}	0
120	0	0	0	5.0×10^{0}	0
240	0	0	0	0	0
360	0	0	0	0	0
1440	0	0	0	0	0

Key: Sp=Sputum, MIC=Minimum Inhibitory Concentration, LEV=Levofloxacin, CEF=Ceftriaxone, H=Honey, L=Lemon juice.

Rate of Kill (above average MIC values) for *Klebsiella pneumoniae*_Sp270 (Susceptible)

Time (mins)	No of colonies counted in LEV (15.62 µg/ml) plate	No of colonies counted in CEF (25 µg/ml) plate	No of colonies counted in Lemon (40 µg/ml) plate	No of colonies counted in Honey (100 µg/ml) plate	No of colonies counted in H/L (40 µg/ml) plate
0	1.9×10^3	1.6×10^3	3.9×10^2	3.7×10^3	6.5×10^2
30	7.1×10^2	3.4×10^2	1.0×10	2.3×10^2	3.0×10
60	5.3×10	2.1×10	0	1.5×10^2	0
90	0	0	0	3.2×10	0

120	0	0	0	0	0
240	0	0	0	0	0
360	0	0	0	0	0
1440	0	0	0	0	0

Key: Sp=Sputum, MIC=Minimum Inhibitory Concentration, LEV=Levofloxacin, CEF=Ceftriaxone, H=Honey, L=Lemon juice.

Rate of Kill (above average MIC values) for *Streptococcus pyogene*_Sp265 (Resistance)

Time (mins)	**No of colonies counted in LEV (15.62 µg/ml) plate**	**No of colonies counted in CEF (25 µg/ml) plate**	**No of colonies counted in Lemon (40 µg/ml) plate**	**No of colonies counted in Honey (100 µg/ml) plate**	**No of colonies counted in H/L (40 µg/ml) plate**
0	4.3×10^3	7.4×10^3	8.1×10^3	6.9×10^3	8.3×10^2

30	1.8×10^{3}	5.0×10^{3}	2.3×10^{2}	2.4×10^{3}	2.0×10
60	1.7×10^{2}	3.1×10^{2}	1.7×10	5.8×10^{2}	0
90	2.0×10	1.8×10	0	2.6×10^{2}	0
120	0	1.0×10	0	8.1×10	0
240	0	0	0	0	0
360	0	0	0	0	0
1440	0	0	0	0	0

Key: Sp=Sputum, MIC=Minimum Inhibitory Concentration, LEV=Levofloxacin, CEF=Ceftriaxone, H=Honey, L=Lemon juice.

Rate of Kill (above average MIC values) for *Streptococcus pneumoniae*_Sp232

Time (mins)	No of colonies counted in LEV (15.62 µg/ml) plate	No of colonies counted in CEF (25 µg/ml) plate	No of colonies counted in Lemon (40 µg/ml) plate	No of colonies counted in Honey (100 µg/ml) plate	No of colonies counted in H/L (40 µg/ml) plate
0	2.5×10^3	1.4×10^3	1.2×10^3	7.2×10^3	5.1×10^2
30	6.3×10	6.5×10^2	1.4×10	6.1×10^2	4.2×10
60	0	7.1×10	0	3.2×10	0
90	0	0	0	0	0
120	0	0	0	0	0
240	0	0	0	0	0
360	0	0	0	0	0
1440	0	0	0	0	0

Key: Sp=Sputum, MIC=Minimum Inhibitory Concentration, LEV=Levofloxacin, CEF=Ceftriaxone, H=Honey, L=Lemon juice.

APPENDIX II: MIC AND MBC RESULTS FOR HONEY, LEMON JUICE, HONEY/LEMON JUICE MIXTURE AND THE STANDARD ANTIBIOTICS FORMULATIONS

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) Test of Honey and Lemon juice (µg/ml) against *Klebsiella pneumoniae*

Sample No.	MIC	MBC	MIC	MBC
	Honey		Lemon	
ES 848	45.0	100	20.0	50.0
SP 224	40.0	70.0	20.0	40.0
SP 229	55.0	NB	20.0	50.0
SP 270	30.0	60.0	15.0	25.0
SP 340	50.0	NB	15.0	40.0
SP 867	40.0	100	35.0	50.0
SP 870	50.0	NB	20.0	25.0
SP 871	35.0	100	15.0	25.0
SP 892	40.0	100	20.0	25.0
SP 894	40.0	100	20.0	25.0
SP1348	30.0	90.0	15.0	20.0
SP1386	20.0	100	35.0	50.0
SP1715	60.0	NB	35.0	50.0
TS 320	45.0	100	15.0	50.0
TS1234	65.0	NB	20.0	50.0

Key: MIC=Minimum Inhibitory Concentration, MBC= Minimum Bactericidal Concentration, ES=Ear Swab, SP=Sputum, TS=Throat Swab, NB=Not Bactericidal.

MIC and MBC Test of Honey and Lemon juice (µg/ml) against *Staphylococcus aureus*

Sample No.	MIC	MBC	MIC	MBC
	Honey		Lemon	
NS 256	47.5	95.0	17.5	45.0
NS 893	45.0	90.0	15.0	25.0
SP 260	35.0	90.0	15.0	35.0
SP 277	30.0	100	15.0	45.0
SP 278	42.5	90.0	15.0	50.0
SP 307	55.0	NB	20.0	60.0
SP 334	50.0	95.0	35.0	45.0
SP1096	75.0	NB	17.5	70.0
SP1308	37.5	70.0	15.0	25.0
SP1347	60.0	NB	15.0	90.0
SP1382	25.0	100	15.0	22.5

TS 227	47.5	80.0	15.0	45.0
TS 312	50.0	NB	17.5	60.0
TS1097	60.0	NB	22.5	32.5

Key: MIC=Minimum Inhibitory Concentration, MBC= Minimum Bactericidal Concentration, NS=Nasal Secretion, SP=Sputum, TS=Throat Swab, NB=Not Bactericidal.

MIC and MBC Test of Honey and Lemon juice (µg/ml) against *Haemophilus influenzae*

Sample No.	MIC	MBC	MIC	MBC
	Honey		Lemon	
SP 267	22.5	90.0	15.0	35.0
SP1270	30.0	90.0	15.0	30.0

Key: MIC=Minimum Inhibitory Concentration, MBC= Minimum Bactericidal Concentration, SP=Sputum.

MIC and MBC Test of Honey and Lemon juice (µg/ml) against *Pseudomonas aeruginosa*

Sample No.	MIC	MBC	MIC	MBC
	Honey		Lemon	
ES 225	30.0	70.0	17.5	25.0
ES 235	30.0	70.0	15.0	50.0
ES 350	30.0	70.0	17.5	40.0
ES 876	25.0	60.0	15.0	25.0
ES 877	45.0	100	15.0	30.0
ES 895	50.0	NB	17.5	70.0
ES1243	30.0	60.0	17.5	60.0
SP 259	30.0	60.0	17.5	60.0
SP 897	25.0	100	15.0	25.0
SP1203	30.0	70.0	17.5	25.0
SP1204	30.0	100	17.5	40.0
SP1205	35.0	70.0	17.5	50.0

Key: MIC=Minimum Inhibitory Concentration, MBC= Minimum Bactericidal Concentration, ES=Ear Swab, SP=Sputum, NB=Not Bactericidal.

MIC and MBC Test of Honey and Lemon juice (µg/ml) against *Streptococcus pneumoniae*

Sample No.	MIC	MBC	MIC	MBC
	Honey		Lemon	
SP 228	55.0	90.0	22.5	40.0
SP 232	32.5	50.0	35.0	45.0

SP 285	22.5	60.0	40.0	70.0
SP 803	60.0	NB	22.5	45.0
SP 903	20.0	60.0	30.0	60.0
TS 328	25.0	90.0	45.0	70.0
TS 901	45.0	80.0	22.5	35.0

Key: MIC=Minimum Inhibitory Concentration, MBC= Minimum Bactericidal Concentration, SP=Sputum, TS=Throat Swab, NB=Not Bactericidal.

MIC and MBC Test of Honey and Lemon juice (µg/ml) against *Streptococcus pyogenes*

Sample No.	MIC	MBC	MIC	MBC
	Honey		Lemon	
SP 265	20.0	60.0	30.0	50.0
SP1349	60.0	NB	42.5	70.0
SP 275	22.5	90.0	22.5	35.0
SP 321	75.0	NB	47.5	95.9
SP 266	60.0	NB	40.0	90.0
SP 269	22.5	90.0	25.0	50.0

Key: MIC=Minimum Inhibitory Concentration, MBC= Minimum Bactericidal Concentration, SP=Sputum, NB=Not Bactericidal.

MIC and MBC Test (µg/ml) of Honey and Lemon juice mixture against *Klebsiella pneumoniae*

Sample No.	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
	10:50		20:50		30:50		40:50		50:50	
ES 848	35.0	60.0	35.0	60.0	35.0	60.0	40.0	60.0	50.0	80.0
SP 1348	25.0	45.0	25.0	45.0	25.0	40.0	25.0	45.0	25.0	50.0
SP 1386	15.0	25.0	17.5	25.0	22.5	40.0	25.0	50.0	22.5	40.0
SP 1715	17.5	25.0	20.0	35.0	30.0	50.0	30.0	60.0	35.0	50.0
SP 224	17.5	25.0	20.0	30.0	30.0	45.0	30.0	50.0	17.5	25.0
SP 229	15.0	25.0	17.5	25.0	20.0	40.0	25.0	40.0	25.0	50.0

SP 270	15.0	25.0	17.5	25.0	20.0	40.0	25.0	40.0	15.0	25.0
SP 340	17.5	25.0	20.0	30.0	25.0	40.0	22.5	40.0	25.0	45.0
SP 867	20.0	30.0	25.0	40.0	30.0	45.0	35.0	50.0	25.0	50.0
SP 870	20.0	35.0	20.0	40.0	22.5	35.0	25.0	45.0	17.5	25.0
SP 871	15.0	25.0	15.0	25.0	25.0	40.0	30.0	45.0	35.0	60.0
SP 892	22.5	30.0	25.0	40.0	22.5	35.0	25.0	40.0	25.0	50.0
SP 894	17.5	25.0	20.0	30.0	20.0	35.0	25.0	50.0	40.0	70.0
TS 1234	20.0	30.0	25.0	40.0	22.5	35.0	22.5	35.0	45.0	70.0
TS 320	17.5	25.0	17.5	25.0	30.0	45.0	30.0	50.0	22.5	40.0

Key: MIC=Minimum Inhibitory Concentration, MBC= Minimum Bactericidal Concentration, ES=Ear Swab, SP=Sputum, TS=Throat Swab.

MIC and MBC Test (µg/ml) of Honey and Lemon juice mixture against *Staphylococcus aureus*

Sample No.	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
	10:50		20:50		30:50		40:50		50:50	
NS 256	15.0	25.0	17.5	25.0	25.0	45.0	25.0	50.0	20.0	35.0
NS 893	20.0	30.0	22.5	30.0	17.5	25.0	20.0	35.0	30.0	50.0
SP 1096	15.0	25.0	20.0	30.0	25.0	50.0	30.0	60.0	17.5	25.0
SP 1308	20.0	35.0	30.0	50.0	35.0	50.0	30.0	60.0	17.5	25.0
SP 1347	15.0	25.0	17.5	25.0	20.0	30.0	20.0	35.0	25.0	50.0
SP 1382	22.5	40.0	25.0	50.0	22.5	35.0	25.0	40.0	15.0	25.0
SP 260	17.5	30.0	22.5	35.0	25.0	45.0	30.0	50.0	17.5	25.0
SP 277	17.5	25.0	17.5	25.0	17.5	25.0	17.5	25.0	15.0	25.0
SP 278	20.0	30.0	25.0	45.0	15.0	25.0	17.5	25.0	15.0	25.0
SP 307	17.5	25.0	17.5	25.0	15.0	25.0	17.5	25.0	17.5	25.0
SP 334	15.0	25.0	20.0	30.0	20.0	40.0	20.0	45.0	20.0	30.0
TS 1097	25.0	40.0	30.0	45.0	15.0	25.0	17.5	30.0	17.5	25.0
TS 227	15.0	25.0	17.5	30.0	15.0	25.0	15.0	25.0	35.0	50.0
TS 312	17.5	25.0	17.5	25.0	15.0	25.0	17.5	25.0	17.5	30.0

Key: MIC=Minimum Inhibitory Concentration, MBC= Minimum Bactericidal Concentration, NS=Nasal Secretion, SP=Sputum, TS=Throat Swab.

MIC and MBC Test (µg/ml) of Honey and Lemon juice mixture against *Haemophilus influenzae*

Sample No.	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
	10:50		20:50		30:50		40:50		50:50	
SP 267	30.0	45.0	17.5	25.0	15.0	25.0	17.5	25.0	17.5	25.0
SP 1270	35.0	50.0	17.5	25.0	15.0	22.5	17.5	22.5	17.5	25.0

Key: MIC=Minimum Inhibitory Concentration, MBC= Minimum Bactericidal Concentration, SP=Sputum.

MIC and MBC Test (µg/ml) of Honey and Lemon juice mixture against *Pseudomonas aeruginosa*

Sample No.	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
	10:50		20:50		30:50		40:50		50:50	
ES 1243	15.0	25.0	30.0	50.0	15.0	25.0	15.0	25.0	15.0	22.5
ES 225	17.5	25.0	17.5	25.0	17.5	25.0	15.0	25.0	15.0	22.5
ES 235	35.0	50.0	25.0	40.0	17.5	25.0	17.5	25.0	15.0	22.5
ES 350	17.5	25.0	15.0	25.0	17.5	25.0	15.0	22.5	15.0	22.5
ES 876	30.0	45.0	25.0	40.0	17.5	25.0	17.5	25.0	25.0	40.0

ES 877	30.0	45.0	25.0	45.0	17.5	25.0	30.0	45.0	25.0	50.0
ES 895	15.0	25.0	17.5	25.0	17.5	25.0	17.5	25.0	25.0	45.0
SP 1203	17.5	25.0	15.0	25.0	15.0	22.5	17.5	25.0	15.0	22.5
SP 1204	15.0	25.0	15.0	25.0	17.5	25.0	15.0	25.0	17.5	25.0
SP 1205	17.5	25.0	35.0	50.0	15.0	25.0	15.0	25.0	25.0	40.0
SP 259	17.5	25.0	17.5	25.0	17.5	25.0	17.5	25.0	25.0	40.0
SP 897	35.0	50.0	30.0	45.0	15.0	25.0	17.5	25.0	17.5	25.0

Key: MIC=Minimum Inhibitory Concentration, MBC= Minimum Bactericidal Concentration, ES=Ear Swab, SP=Sputum.

MIC and MBC Test (µg/ml) of Honey and Lemon juice mixture against *Streptococcus pneumoniae*

Sample No.	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
	10:50		20:50		30:50		40:50		50:50	
SP 228	25.0	40.0	25.0	45.0	25.0	40.0	25.0	45.0	25.0	60.0
SP 232	17.5	25.0	20.0	35.0	17.5	25.0	17.5	25.0	17.5	25.0
SP 285	17.5	25.0	17.5	25.0	17.5	25.0	17.5	25.0	17.5	25.0
SP 803	25.0	40.0	25.0	45.0	25.0	40.0	25.0	45.0	25.0	50.0
SP 903	17.5	25.0	17.5	25.0	17.5	25.0	17.5	25.0	17.5	25.0
TS 328	17.5	25.0	17.5	25.0	17.5	25.0	17.5	25.0	17.5	25.0
TS 901	17.5	25.0	17.5	25.0	22.5	35.0	17.5	25.0	17.5	25.0

Key: MIC=Minimum Inhibitory Concentration, MBC= Minimum Bactericidal Concentration, SP=Sputum, TS=Throat Swab.

MIC and MBC Test (µg/ml) of Honey and Lemon juice mixture against *Streptococcus pyogenes*

Sample No.	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
	10:50		20:50		30:50		40:50		50:50	
SP 265	17.5	25.0	20.0	30.0	17.5	25.0	17.5	25.0	17.5	25.0
SP 1349	17.5	25.0	20.0	35.0	17.5	25.0	17.5	25.0	17.5	25.0
SP 275	17.5	25.0	17.5	25.0	17.5	25.0	17.5	25.0	17.5	25.0
SP 321	35.0	50.0	25.0	50.0	20.0	30.0	20.0	35.0	25.0	40.0
SP 266	17.5	25.0	17.5	25.0	17.5	25.0	17.5	25.0	17.5	25.0
SP 269	17.5	25.0	17.5	25.0	17.5	25.0	17.5	25.0	17.5	25.0

Key: MIC=Minimum Inhibitory Concentration, MBC= Minimum Bactericidal Concentration, SP=Sputum.

MIC and MBC Test (µg/ml) of Lemon juice and Honey mixture against *Klebsiella pneumoniae*

Sample No.	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
	10:50		20:50		30:50		40:50		50:50	
ES 848	30.0	45.0	25.0	40.0	35.0	70.0	45.0	80.0	50.0	80.0
SP 1348	25.0	40.0	20.0	40.0	30.0	50.0	30.0	50.0	25.0	50.0

SP 1386	30.0	60.0	25.0	50.0	25.0	40.0	25.0	40.0	22.5	40.0
SP 1715	20.0	40.0	20.0	40.0	30.0	45.0	30.0	50.0	35.0	50.0
SP 224	30.0	50.0	30.0	50.0	30.0	50.0	25.0	50.0	17.5	25.0
SP 229	30.0	60.0	35.0	50.0	25.0	40.0	25.0	45.0	25.0	50.0
SP 270	25.0	50.0	25.0	40.0	25.0	50.0	22.5	40.0	15.0	25.0
SP 340	45.0	60.0	45.0	60.0	22.5	40.0	20.0	40.0	25.0	45.0
SP 867	45.0	60.0	40.0	60.0	30.0	60.0	25.0	60.0	25.0	50.0
SP 870	50.0	70.0	40.0	60.0	22.5	40.0	20.0	40.0	17.5	25.0
SP 871	30.0	50.0	30.0	50.0	40.0	60.0	45.0	70.0	35.0	60.0
SP 892	40.0	60.0	35.0	50.0	25.0	50.0	30.0	60.0	25.0	50.0
SP 894	30.0	60.0	40.0	60.0	35.0	60.0	40.0	70.0	40.0	70.0
TS 1234	50.0	70.0	45.0	60.0	40.0	60.0	30.0	70.0	45.0	70.0
TS 320	25.0	40.0	25.0	40.0	25.0	40.0	20.0	35.0	22.5	40.0

Key: MIC=Minimum Inhibitory Concentration, MBC= Minimum Bactericidal Concentration, ES=Ear Swab, SP=Sputum, TS=Throat Swab.

MIC and MBC Test (µg/ml) of Lemon juice and Honey mixture against *Staphylococcus aureus*

Sample No.	MIC 10:50	MBC	MIC 20:50	MBC	MIC 30:50	MBC	MIC 40:50	MBC	MIC 50:50	MBC
NS 256	25.0	45.0	20.0	40.0	15.0	25.0	20.0	35.0	20.0	35.0
NS 893	35.0	50.0	30.0	50.0	25.0	45.0	30.0	50.0	30.0	50.0
SP 1096	40.0	60.0	30.0	60.0	25.0	50.0	20.0	35.0	17.5	25.0
SP 1308	20.0	35.0	20.0	35.0	20.0	30.0	17.5	25.0	17.5	25.0
SP 1347	40.0	70.0	35.0	70.0	25.0	50.0	25.0	50.0	25.0	50.0
SP 1382	35.0	50.0	30.0	50.0	17.5	25.0	20.0	35.0	15.0	25.0
SP 260	45.0	70.0	40.0	60.0	17.5	25.0	17.5	30.0	17.5	25.0
SP 277	35.0	60.0	30.0	60.0	35.0	60.0	15.0	25.0	15.0	25.0
SP 278	25.0	45.0	25.0	40.0	15.0	25.0	17.5	30.0	15.0	25.0
SP 307	25.0	40.0	20.0	35.0	25.0	40.0	17.5	30.0	17.5	25.0
SP 334	20.0	35.0	20.0	35.0	30.0	50.0	22.5	35.0	20.0	30.0
TS 1097	30.0	50.0	25.0	40.0	25.0	40.0	20.0	35.0	17.5	25.0
TS 227	25.0	45.0	30.0	50.0	17.5	25.0	35.0	45.0	35.0	50.0
TS 312	50.0	70.0	40.0	60.0	17.5	25.0	20.0	40.0	17.5	30.0

Key: MIC=Minimum Inhibitory Concentration, MBC= Minimum Bactericidal Concentration, NS=Nasal Secretion, SP=Sputum, TS=Throat Swab.

MIC and MBC Test (µg/ml) of Lemon juice and Honey mixture against *Haemophilus influenzae*

Sample No.	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
	10:50		20:50		30:50		40:50		50:50	
SP 267	30.0	50.0	25.0	40.0	30.0	50.0	30.0	45.0	17.5	25.0
SP 1270	30.0	45.0	30.0	45.0	25.0	40.0	17.5	25.0	17.5	25.0

Key: MIC=Minimum Inhibitory Concentration, MBC= Minimum Bactericidal Concentration, SP=Sputum.

MIC and MBC Test (µg/ml) of Lemon juice and Honey mixture against *Pseudomonas aeruginosa*

Sample No.	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
	10:50		**20:50**		**30:50**		**40:50**		**50:50**	
ES 1243	25.0	45.0	25.0	50	35.0	50.0	25.0	45.0	15.0	22.5

ES 225	30.0	50.0	25.0	40	15.0	25.0	17.5	25.0	15.0	22.5
ES 235	25.0	40.0	22.5	35	17.5	25.0	17.5	25.0	15.0	22.5
ES 350	30.0	45.0	25.0	50	15.0	25.0	15.0	25.0	15.0	22.5
ES 876	17.5	25.0	15.0	25	17.5	25.0	17.5	25.0	25.0	40.0
ES 877	35.0	50.0	25.0	40	15.0	25.0	17.5	25.0	25.0	50.0
ES 895	20.0	35.0	20.0	40	17.5	25.0	17.5	25.0	25.0	45.0
SP 1203	30.0	50.0	25.0	50	30.0	50.0	15.0	25.0	15.0	22.5
SP 1204	25.0	40.0	25.0	45	17.5	25.0	15.0	25.0	17.5	25.0
SP 1205	25.0	50.0	25.0	50	30.0	45.0	25.0	40.0	25.0	40.0
SP 259	25.0	40.0	20.0	35	17.5	25.0	17.5	25.0	25.0	40.0
SP 897	22.5	35.0	25.0	45	15.0	25.0	17.5	25.0	17.5	25.0

Key: MIC=Minimum Inhibitory Concentration, MBC= Minimum Bactericidal Concentration, ES=Ear Swab, SP=Sputum.

MIC and MBC Test (µg/ml) of Lemon juice and Honey mixture against *Streptococcus pneumoniae*

Sample No.	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
	10:50		20:50		30:50		40:50		50:50	
SP 228	70.0	100	50.0	80.0	50.0	100	40.0	70.0	25.0	60.0
SP 232	35.0	50.0	30.0	50.0	17.5	25.0	17.5	25.0	17.5	25.0
SP 285	50.0	70.0	45.0	70.0	20.0	35.0	17.5	25.0	17.5	25.0
SP 803	50.0	90.0	50.0	80.0	50.0	100	40.0	70.0	25.0	50.0
SP 903	35.0	50.0	30.0	50.0	17.5	25.0	17.5	25.0	17.5	25.0
TS 328	30.0	50.0	35.0	50.0	17.5	25.0	17.5	25.0	17.5	25.0
TS 901	35.0	50.0	30.0	50.0	17.5	25.0	17.5	25.0	17.5	25.0

Key: MIC=Minimum Inhibitory Concentration, MBC= Minimum Bactericidal Concentration, SP=Sputum, TS=Throat Swab.

MIC and MBC Test (µg/ml) of Lemon juice and Honey mixture against *Streptococcus pyogenes*

Sample No.	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
	10:50		20:50		30:50		40:50		50:50	
SP 265	30.0	50.0	30.0	50.0	17.5	25.0	17.5	25.0	17.5	25.0
SP 1349	25.0	50.0	25.0	50.0	35.0	50.0	25.0	40.0	17.5	25.0
SP 275	17.5	25.0	17.5	25.0	17.5	25.0	17.5	25.0	17.5	25.0
SP 321	50.0	70.0	35.0	50.0	30.0	45.0	25.0	40.0	25.0	40.0
SP 266	50.0	80.0	35.0	50.0	30.0	45.0	25.0	45.0	17.5	25.0
SP 269	25.0	40.0	25.0	40.0	25.0	40.0	25.0	40.0	17.5	25.0

Key: MIC=Minimum Inhibitory Concentration, MBC= Minimum Bactericidal Concentration, SP=Sputum.

MIC and MBC Test (µg/ml) of Standard Antibiotics against *Klebsiella pneumoniae*

Sample No.	MIC CN	MBC	MIC CRO	MBC	MIC AMC	MBC	MIC LEV	MBC	MIC AZM	MBC
ES 848	100	NB	15.6	31.3	3.91	125	15.6	125	NI	NB
SP 1348	100	NB	1.95	3.91	NI	NB	7.81	125	NI	NB
SP 1386	3.13	50.0	15.6	62.5	NI	NB	0.98	7.81	NI	NB
SP 1715	1.56	3.13	15.6	31.3	15.6	62.5	0.98	15.6	3.13	50.0
SP 224	25.0	50.0	15.6	62.5	3.91	125	3.91	7.81	NI	NB
SP 229	6.25	50.0	3.91	15.6	NI	NB	3.91	31.3	NI	NB
SP 270	1.56	12.5	15.6	62.5	3.91	15.6	0.98	3.91	NI	NB
SP 340	1.56	25.0	15.6	31.3	3.91	62.5	7.81	62.5	NI	NB
SP 867	1.56	6.25	15.6	62.5	NI	NB	0.98	15.6	NI	NB
SP 870	100	NB	1.95	3.91	15.6	125	0.98	31.3	NI	NB
SP 871	100	NB	15.6	62.5	NI	NB	7.81	31.3	NI	NB
SP 892	100	NB	15.6	31.3	125	NB	0.98	31.3	NI	NB
SP 894	25.0	100	15.6	62.5	NI	NB	NI	NB	NI	NB
TS 1234	100	NB	7.81	31.3	NI	NB	15.6	125	NI	NB
TS 320	1.56	12.5	125	NB	NI	NB	7.81	62.5	NI	NB

Key: CN=Gentamicin, CRO=Ceftriaxone, AMC=Amoxicillin-Clavulanic acid, LEV=Levofloxacin, AZM=Azithromycin, MIC=Minimum Inhibitory Concentration, MBC=Minimum Bactericidal Concentration, NI=No Inhibition and NB=Not Bactericidal

MIC and MBC Test (µg/ml) of Standard Antibiotics against *Staphylococcus aureus*

Sample No.	MIC CN	MBC	MIC CRO	MBC	MIC AMC	MBC	MIC LEV	MBC	MIC AZM	MBC
NS 256	1.56	3.13	15.6	62.5	62.5	125	0.98	31.3	0.78	50.0
NS 893	100	NB	62.5	>125	NI	NB	0.98	3.91	25.0	>50
SP 1096	25.0	100	NI	NB	NI	NB	31.3	125	NI	NB
SP 1308	NI	NB	1.95	7.81	31.5	125	0.98	62.5	NI	NB
SP 1347	100	NB	1.95	3.91	125	NB	0.98	3.91	0.78	25.0
SP 1382	1.56	3.13	15.6	62.5	7.81	31.3	0.98	3.91	25.0	>50
SP 260	1.56	25.0	7.81	15.6	NI	NB	0.98	7.81	1.56	50.0
SP 277	1.56	6.25	1.95	3.91	125	NB	0.98	3.91	NI	NB
SP 278	1.56	3.13	3.91	15.6	3.91	62.5	0.98	3.91	0.78	6.25
SP 307	1.56	6.25	3.91	31.3	62.5	125	0.98	7.81	0.78	6.25
SP 334	1.56	3.13	7.81	15.6	3.91	15.6	0.98	3.91	0.78	12.5
TS 1097	1.56	3.13	15.6	62.5	15.6	125	0.98	62.5	12.5	>50
TS 227	1.56	3.13	3.91	7.81	3.91	7.81	7.81	31.3	0.78	3.13
TS 312	6.25	12.5	1.95	7.81	7.81	62.5	62.5	>125	NI	NB

Key: CN=Gentamicin, CRO=Ceftriaxone, AMC=Amoxicillin-Clavulanic acid, LEV=Levofloxacin, AZM=Azithromycin, MIC=Minimum Inhibitory Concentration, MBC=Minimum Bactericidal Concentration, NI=No Inhibition and NB=Not Bactericidal

MIC and MBC Test (µg/ml) of Standard Antibiotics against *Haemophilus influenzae*

Sample No.	MIC CN	MBC	MIC CRO	MBC	MIC AMC	MBC	MIC LEV	MBC	MIC AZM	MBC
SP 267	1.56	12.5	62.5	>125	NI	NB	0.98	62.5	NI	NB
SP 1270	1.56	6.25	62.5	>125	NI	NB	0.98	31.3	NI	NB

Key: CN=Gentamicin, CRO=Ceftriaxone, AMC=Amoxicillin-Clavulanic acid, LEV=Levofloxacin, AZM=Azithromycin, MIC=Minimum Inhibitory Concentration, MBC=Minimum Bactericidal Concentration, NI=No Inhibition and NB=Not Bactericidal.

MIC and MBC Test (µg/ml) of Standard Antibiotics against P*seudomonas aeruginosa.*

Sample No.	MIC CN	MBC	MIC CRO	MBC	MIC AMC	MBC	MIC LEV	MBC	MIC AZM	MBC
ES 1243	1.56	3.13	125	NB	NI	NB	3.91	62.5	NI	NB
ES 225	NI	NB	15.6	125	NI	NB	7.81	125	NI	NB
ES 235	1.56	12.5	NI	NB	NI	NB	0.98	7.81	50.0	>50
ES 350	1.56	12.5	31.3	125	125	NB	0.98	15.6	NI	NB
ES 876	1.56	25.0	31.3	125	NI	NB	0.98	7.81	NI	NB
ES 877	1.56	25.0	15.6	125	NI	NB	0.98	7.81	25.0	50.0
ES 895	1.56	25.0	31.3	125	NI	NB	0.98	62.5	50.0	>50
SP 1203	1.56	12.5	125	NB	NI	NB	0.98	7.81	NI	NB
SP 1204	1.56	25.0	62.5	>125	NI	NB	0.98	7.81	NI	NB
SP 1205	1.56	12.5	7.81	62.5	NI	NB	3.91	15.6	50.0	>50
SP 259	1.56	6.25	125	NB	NI	NB	0.98	7.81	12.5	50.0
SP 897	1.56	6.25	31.3	125	NI	NB	0.98	31.3	50.0	>50

Key: CN=Gentamicin, CRO=Ceftriaxone, AMC=Amoxicillin-Clavulanic acid, LEV=Levofloxacin, AZM=Azithromycin, MIC=Minimum Inhibitory Concentration, MBC=Minimum Bactericidal Concentration, NI=No Inhibition and NB=Not Bactericidal

MIC and MBC Test (μg/ml) of Standard Antibiotics against *Streptococcus pneumoniae*

Sample No.	MIC CN	MBC	MIC CRO	MBC	MIC AMC	MBC	MIC LEV	MBC	MIC AZM	MBC
SP 228	25.0	NB	7.81	125	31.5	125	0.98	62,5	NI	NB
SP 232	25.0	NB	1.95	7.81	62.5	>125	0.98	62.5	NI	NB
SP 285	3.13	6.25	1.95	31.3	62.5	>125	62.5	>125	50.0	>50
SP 803	3.13	25.0	125	NB	NI	NB	3.91	125	NI	NB
SP 903	NI	NB	15.6	62.5	62.5	>125	3.91	125	NI	NB
TS 328	3.13	25.0	62.5	>125	NI	NB	3.91	125	NI	NB
TS 901	NI	NB	1.95	15.6	31.5	125	0.98	62.5	NI	NB

Key: CN=Gentamicin, CRO=Ceftriaxone, AMC=Amoxicillin-Clavulanic acid, LEV=Levofloxacin, AZM=Azithromycin, MIC=Minimum Inhibitory Concentration, MBC=Minimum Bactericidal Concentration, NI=No Inhibition and NB=Not Bactericidal.

MIC and MBC Test (µg/ml) of Standard Antibiotics against *Streptococcus pyogenes*

Sample No.	MIC CN	MBC	MIC CRO	MBC	MIC AMC	MBC	MIC LEV	MBC	MIC AZM	MBC
SP 265	NI	NB	31.3	125	NI	NB	62.5	>125	NI	NB
SP 1349	NI	NB	62.5	>125	62.5	>125	7.81	125	62.5	>125
SP 275	NI	NB	15.6	125	125	NB	15.6	125	125	NB
SP 321	NI	NB	31.3	125	NI	NB	7.81	125	NI	NB
SP 266	1.56	6.25	31.3	125	NI	NB	0.98	62.5	NI	NB
SP 269	NI	NB	31.3	125	125	NB	7.81	125	125	NB

Key: CN=Gentamicin, CRO=Ceftriaxone, AMC=Amoxicillin-Clavulanic acid, LEV=Levofloxacin, AZM=Azithromycin, MIC=Minimum Inhibitory Concentration, MBC=Minimum Bactericidal Concentration, NI=No Inhibition and NB=Not Bactericidal.

Appendix III: Comparing the mean zones of inhibition of Honey and Honey/Lemon juice mixture

Comparing the mean zones of inhibition of Honey and the mixture of Honey and Lemon juice against *Klebsiella pneumoniae*.

(I) Groups_1	(J) Groups_1	Mean±SEM	P-value	LOS
Honey (100%) (19.333±0.374)	H:L; 10:50	20.867±0.696	0.365	NS
	H:L; 20:50	21.000±0.586	0.260	NS
	H:L; 30:50	21.933±0.331	0.007	SF
	H:L; 40:50	21.533±0.467	0.043	SF
	H:L; 50:50	22.800±0.571	0.000	SF
Honey (50%) (16.000±0.378)	H:L; 10:50	20.867±0.696	0.000	SF
	H:L; 20:50	21.000±0.586	0.000	SF
	H:L; 30:50	21.933±0.331	0.000	SF
	H:L; 40:50	21.533±0.467	0.000	SF
	H:L; 50:50	22.800±0.571	0.000	SF

Honey (25%) (11.733±0.442)	H:L; 10:50	20.867±0.696	0.000	SF
	H:L; 20:50	21.000±0.586	0.000	SF
	H:L; 30:50	21.933±0.331	0.000	SF
	H:L; 40:50	21.533±0.467	0.000	SF
	H:L; 50:50	22.800±0.571	0.000	SF

Key: LOS=Level of significance; NS=Not significantly different; SF=Significantly different; H=Honey and L=Lemon juice.

Comparing the mean zones of inhibition of Honey and the mixture of Honey and Lemon juice against *Staphylococcus aureus*.

(I) Group_2	(J) Group_2	Mean±SEM	P-value	LOS

Honey (100%) (19.429±0.402)	H:L; 10:50	22.071±0.529	0.019	SF
	H:L; 20:50	22.143±0.501	0.014	SF
	H:L; 30:50	22.643±0.830	0.002	SF
	H:L; 40:50	22.429±0.511	0.004	SF
	H:L; 50:50	24.500±0.489	0.000	SF
Honey (50%) (15.714±0.438)	H:L; 10:50	22.071±0.529	0.000	SF
	H:L; 20:50	22.143±0.501	0.000	SF
	H:L; 30:50	22.643±0.830	0.000	SF
	H:L; 40:50	22.429±0.511	0.000	SF
	H:L; 50:50	24.500±0.489	0.000	SF
Honey (25%) (12.500±0.542)	H:L; 10:50	22.071±0.529	0.000	SF
	H:L; 20:50	22.143±0.501	0.000	SF
	H:L; 30:50	22.643±0.830	0.000	SF
	H:L; 40:50	22.429±0.511	0.000	SF
	H:L; 50:50	24.500±0.489	0.000	SF

Key: LOS=Level of significance; NS=Not significantly different; SF=Significantly different; H=Honey and L=Lemon juice.

Comparing the mean zones of inhibition of Honey and the mixture of Honey and Lemon juice against *Haemophilus influenzae*.

(I) Group_3	(J) Group_3	Mean±SEM	P-value	LOS
Honey (100%) (20.000±0.000)	H:L; 10:50	20.500±0.500	0.543	NS
	H:L; 20:50	23.000±2.000	0.691	NS
	H:L; 30:50	21.000±1.000	0.998	NS
	H:L; 40:50	24.000±2.000	0.407	NS
	H:L; 50:50	25.500±1.500	0.147	NS
Honey (50%) (16.000±1.000)	H:L; 10:50	20.500±0.500	0.035	SF
	H:L; 20:50	23.000±2.000	0.050	SF
	H:L; 30:50	21.000±1.000	0.210	NS
	H:L; 40:50	24.000±2.000	0.025	SF
	H:L; 50:50	25.500±1.500	0.009	SF
Honey (25%) (10.000±0.000)	H:L; 10:50	20.500±0.500	0.001	SF
	H:L; 20:50	23.000±2.000	0.001	SF
	H:L; 30:50	21.000±1.000	0.004	SF
	H:L; 40:50	24.000±2.000	0.001	SF
	H:L; 50:50	25.500±1.500	0.000	SF

Key: LOS=Level of significance; NS=Not significantly different; SF=Significantly different; H=Honey and L=Lemon juice.

Comparing the mean zones of inhibition of Honey and the mixture of Honey and Lemon juice against *Pseudomonas aeruginosa*.

(I) Group_4	(J) Group_4	Mean±SEM	P-value	LOS
Honey (100%) (22.167±1.325)	H:L; 10:50	20.500±0.314	0.678	NS
	H:L; 20:50	21.750±0.329	1.000	NS
	H:L; 30:50	21.417±0.621	0.994	NS

	H:L; 40:50	21.833±0.366	1.000	NS
	H:L; 50:50	23.667±0.376	0.782	NS
Honey (50%) (18.167±0.991)	H:L; 10:50	20.500±0.314	0.254	NS
	H:L; 20:50	21.750±0.329	0.009	SF
	H:L; 30:50	21.417±0.621	0.026	SF
	H:L; 40:50	21.833±0.366	0.007	SF
	H:L; 50:50	23.667±0.376	0.000	SF
Honey (25%) (12.583±0.417)	H:L; 10:50	20.500±0.314	0.000	SF
	H:L; 20:50	21.750±0.329	0.000	SF
	H:L; 30:50	21.417±0.621	0.000	SF
	H:L; 40:50	21.833±0.366	0.000	SF
	H:L; 50:50	23.667±0.376	0.000	SF

Key: LOS=Level of significance; NS=Not significantly different; SF=Significantly different; H=Honey and L=Lemon juice.

Comparing the mean zones of inhibition of Honey and the mixture of Honey and Lemon juice against *Streptococcus pneumoniae*.

(I) Group_5	(J) Group_5	Mean±SEM	P-value	LOS
Honey (100%) (19.714±0.808)	H:L; 10:50	23.143±0.769	0.020	SF
	H:L; 20:50	23.286±0.522	0.014	SF
	H:L; 30:50	22.143±0.553	0.226	NS
	H:L; 40:50	23.143±0.800	0.020	SF
	H:L; 50:50	24.571±0.783	0.000	SF
Honey (50%) (15.429±0.528)	H:L; 10:50	23.143±0.769	0.000	SF
	H:L; 20:50	23.286±0.522	0.000	SF
	H:L; 30:50	22.143±0.553	0.000	SF
	H:L; 40:50	23.143±0.800	0.000	SF
	H:L; 50:50	24.571±0.783	0.000	SF
Honey (25%) (11.286±0.680)	H:L; 10:50	23.143±0.769	0.000	SF
	H:L; 20:50	23.286±0.522	0.000	SF
	H:L; 30:50	22.143±0.553	0.000	SF
	H:L; 40:50	23.143±0.800	0.000	SF
	H:L; 50:50	24.571±0.783	0.000	SF

Key: LOS=Level of significance; NS=Not significantly different; SF=Significantly different; H=Honey and L=Lemon juice.

Comparing the mean zones of inhibition of Honey and the mixture of Honey and Lemon juice against *Streptococcus pyogenes*.

(I) Group_6	(J) Group_6	Mean±SEM	P-value	LOS
Honey (100%) (19.167±0.543)	H:L; 10:50	23.333±0.955	0.008	SF
	H:L; 20:50	23.833±0.872	0.002	SF
	H:L; 30:50	21.333±0.715	0.483	NS
	H:L; 40:50	23.833±0.703	0.002	SF
	H:L; 50:50	25.833±1.014	0.000	SF
Honey (50%) (15.833±0.477)	H:L; 10:50	23.333±0.955	0.000	SF
	H:L; 20:50	23.833±0.872	0.000	SF

	H:L; 30:50	21.333±0.715	0.000	SF
	H:L; 40:50	23.833±0.703	0.000	SF
	H:L; 50:50	25.833±1.014	0.000	SF
Honey (25%) (11.500±0.619)	H:L; 10:50	23.333±0.955	0.000	SF
	H:L; 20:50	23.833±0.872	0.000	SF
	H:L; 30:50	21.333±0.715	0.000	SF
	H:L; 40:50	23.833±0.703	0.000	SF
	H:L; 50:50	25.833±1.014	0.000	SF

Key: LOS=Level of significance; NS=Not significantly different; SF=Significantly different; H=Honey and L=Lemon juice.

Appendix IV: Comparing the mean zones of inhibition of Lemon juice and Honey/Lemon mixture

Comparing the mean zones of inhibition of Lemon juice and the mixture of Honey and Lemon juice against *Klebsiella pneumoniae*.

(I) Group_7	(J) Group_7	Mean±SEM	P-value	LOS
Lemon (100%) (21.333±0.374)	H:L; 10:50	20.867±0.696	0.998	NS
	H:L; 20:50	21.000±0.586	1.000	NS
	H:L; 30:50	21.933±0.331	0.989	NS
	H:L; 40:50	21.533±0.467	1.000	NS
	H:L; 50:50	22.800±0.571	0.421	NS
Lemon (50%) (18.333±0.374)	H:L; 10:50	20.867±0.696	0.010	SF
	H:L; 20:50	21.000±0.586	0.005	SF
	H:L; 30:50	21.933±0.331	0.000	SF
	H:L; 40:50	21.533±0.467	0.000	SF
	H:L; 50:50	22.800±0.571	0.000	SF
Lemon (25%) (15.133±0.435)	H:L; 10:50	20.867±0.696	0.000	SF
	H:L; 20:50	21.000±0.586	0.000	SF
	H:L; 30:50	21.933±0.331	0.000	SF
	H:L; 40:50	21.533±0.467	0.000	SF
	H:L; 50:50	22.800±0.571	0.000	SF

Key: LOS=Level of significance; NS=Not significantly different; SF=Significantly different; H=Honey and L=Lemon juice.

Comparing the mean zones of inhibition of Lemon juice and the mixture of Honey and Lemon juice against *Staphylococcus aureus*.

(I) Group_8	(J) Group_8	Mean±SEM	P-value	LOS
Lemon (100%) (20.071±0.848)	H:L; 10:50	22.071±0.529	0.428	NS
	H:L; 20:50	22.143±0.501	0.381	NS
	H:L; 30:50	22.643±0.830	0.138	NS
	H:L; 40:50	22.429±0.511	0.223	NS
	H:L; 50:50	24.500±0.489	0.000	SF
Lemon (50%) (17.500±0.918)	H:L; 10:50	22.071±0.529	0.000	SF
	H:L; 20:50	22.143±0.501	0.000	SF
	H:L; 30:50	22.643±0.830	0.000	SF
	H:L; 40:50	22.429±0.511	0.000	SF
	H:L; 50:50	24.500±0.489	0.000	SF
Lemon (25%)	H:L; 10:50	22.071±0.529	0.000	SF

(13.714±0.615)	H:L; 20:50	22.143±0.501	0.000	SF
	H:L; 30:50	22.643±0.830	0.000	SF
	H:L; 40:50	22.429±0.511	0.000	SF
	H:L; 50:50	24.500±0.489	0.000	SF

Key: LOS=Level of significance; NS=Not significantly different; SF=Significantly different; H=Honey and L=Lemon juice.

Comparing the mean zones of inhibition of Lemon juice and the mixture of Honey and Lemon juice against *Haemophilus influenzae*.

(I) Group_9	(J) Group_9	Mean±SEM	P-value

				LOS
Lemon (100%) (20.500±1.500)	H:L; 10:50	20.500±0.500	0.766	NS
	H:L; 20:50	23.000±2.000	0.880	NS
	H:L; 30:50	21.000±1.000	1.000	NS
	H:L; 40:50	24.000±2.000	0.633	NS
	H:L; 50:50	25.500±1.500	0.283	NS
Lemon (50%) (17.500±0.500)	H:L; 10:50	20.500±0.500	0.150	NS
	H:L; 20:50	23.000±2.000	0.207	NS
	H:L; 30:50	21.000±1.000	0.633	NS
	H:L; 40:50	24.000±2.000	0.108	NS
	H:L; 50:50	25.500±1.500	0.040	SF
Lemon (25%) (16.000±1.000)	H:L; 10:50	20.500±0.500	0.056	SF
	H:L; 20:50	23.000±2.000	0.077	NS
	H:L; 30:50	21.000±1.000	0.283	NS
	H:L; 40:50	24.000±2.000	0.040	SF
	H:L; 50:50	25.500±1.500	0.016	SF

Key: LOS=Level of significance; NS=Not significantly different; SF=Significantly different; H=Honey and L=Lemon juice.

Comparing the mean zones of inhibition of Lemon juice and the mixture of Honey and Lemon juice against *Pseudomonas aeruginosa*.

(I) Group_10	(J) Group_10	Mean±SEM	P-value	LOS
Lemon (100%) (18.667±0.466)	H:L; 10:50	20.500±0.314	0.046	SF
	H:L; 20:50	21.750±0.329	0.000	SF
	H:L; 30:50	21.417±0.621	0.000	SF
	H:L; 40:50	21.833±0.366	0.000	SF
	H:L; 50:50	23.667±0.376	0.000	SF
Lemon (50%) (16.250±0.429)	H:L; 10:50	20.500±0.314	0.000	SF
	H:L; 20:50	21.750±0.329	0.000	SF
	H:L; 30:50	21.417±0.621	0.000	SF
	H:L; 40:50	21.833±0.366	0.000	SF
	H:L; 50:50	23.667±0.376	0.000	SF
Lemon (25%) (12.667±0.310)	H:L; 10:50	20.500±0.314	0.000	SF
	H:L; 20:50	21.750±0.329	0.000	SF
	H:L; 30:50	21.417±0.621	0.000	SF
	H:L; 40:50	21.833±0.366	0.000	SF
		23.667±0.376	0.000	SF

	H:L; 50:50			

Key: LOS=Level of significance; NS=Not significantly different; SF=Significantly different; H=Honey and L=Lemon juice.

Comparing the mean zones of inhibition of Lemon juice and the mixture of Honey and Lemon juice against *Streptococcus pneumoniae*.

(I) Group_11	(J) Group_11	Mean±SEM	P-value	LOS
Lemon (100%) (23.000±1.195)	H:L; 10:50	23.143±0.769	1.000	NS
	H:L; 20:50	23.286±0.522	1.000	NS

	H:L; 30:50	22.143±0.553	0.988	NS
	H:L; 40:50	23.143±0.800	1.000	NS
	H:L; 50:50	24.571±0.783	0.761	NS
Lemon (50%) (18.286±0.184)	H:L; 10:50	23.143±0.769	0.000	SF
	H:L; 20:50	23.286±0.522	0.000	SF
	H:L; 30:50	22.143±0.553	0.007	SF
	H:L; 40:50	23.143±0.800	0.000	SF
	H:L; 50:50	24.571±0.783	0.000	SF
Lemon (25%) (15.429±0.297)	H:L; 10:50	23.143±0.769	0.000	SF
	H:L; 20:50	23.286±0.522	0.000	SF
	H:L; 30:50	22.143±0.553	0.000	SF
	H:L; 40:50	23.143±0.800	0.000	SF
	H:L; 50:50	24.571±0.783	0.000	SF

Key: LOS=Level of significance; NS=Not significantly different; SF=Significantly different; H=Honey and L=Lemon juice.

Comparing the mean zones of inhibition of Lemon juice and the mixture of Honey and Lemon juice against *Streptococcus pyogenes*.

(I) Group_12	(J) Group_12	Mean±SEM	P-value	LOS
Lemon (100%) (20.667±0.558)	H:L; 10:50	23.333±0.955	0.233	NS
	H:L; 20:50	23.833±0.872	0.090	NS
	H:L; 30:50	21.333±0.715	0.998	NS
	H:L; 40:50	23.833±0.703	0.090	NS
	H:L; 50:50	25.833±1.014	0.001	SF
Lemon (50%) (17.833±0.543)	H:L; 10:50	23.333±0.955	0.000	SF
	H:L; 20:50	23.833±0.872	0.000	SF
	H:L; 30:50	21.333±0.715	0.043	SF
	H:L; 40:50	23.833±0.703	0.000	SF
	H:L; 50:50	25.833±1.014	0.000	SF
Lemon (25%) (15.333±0.558)	H:L; 10:50	23.333±0.955	0.000	SF
	H:L; 20:50	23.833±0.872	0.000	SF
	H:L; 30:50	21.333±0.715	0.000	SF
	H:L; 40:50	23.833±0.703	0.000	SF
	H:L; 50:50	25.833±1.014	0.000	SF

Key: LOS=Level of significance; NS=Not significantly different; SF=Significantly different; H=Honey and L=Lemon juice.

Appendix V: **Data on Samples Collected**

S/No.	Age	Sex	Sample source	Sample No.
1	31	F	SPUTUM	SP 300
2	42	M	SPUTUM	SP 307
3	7	M	SPUTUM	SP 321
4	14	F	THROAT SWAB	TS 312
5	51	M	THROAT SWAB	TS 311

6	43	F	SPUTUM	SP 340
7	3	F	THROAT SWAB	TS 320
8	28	F	SPUTUM	SP 224
9	22	M	THROAT SWAB	TS 227
10	9	M	SPUTUM	SP 223

Key: SP=Sputum, TS=Throat Swab, F=Female, M=Male.

Data on Samples Collected

S/No.	Age	Sex	Sample source	Sample No.
11	21	M	SPUTUM	SP 229
12	18	M	SPUTUM	SP 871
13	37	M	SPUTUM	SP 867

14	20	F	SPUTUM	SP 228
15	5	F	SPUTUM	SP 870
16	36	F	SPUTUM	SP 868
17	38	M	SPUTUM	SP 883
18	27	F	EAR SWAB	ES 876
19	25	M	EAR SWAB	ES 235
20	2	M	EAR SWAB	ES 225

Key: ES=Ear Swab, SP=Sputum, F=Female, M=Male.

Data on Samples Collected

S/No.	Age	Sex	Sample source	Sample No.
21	32	M	SPUTUM	SP 803
22	17	M	EAR SWAB	ES 877
23	13	M	SPUTUM	SP 231

24	39	F	SPUTUM	SP 232
25	29	M	NASAL S.	NS 893
26	21	M	SPUTUM	SP 241
27	6	F	SPUTUM	SP 890
28	48	F	SPUTUM	SP 888
29	17	F	EAR SWAB	ES 248
30	12	F	SPUTUM	SP 246

Key: ES=Ear Swab, NS=Nasal Secretion, SP=Sputum, F=Female, M=Male.

Data on Samples Collected

S/No.	Age	Sex	Sample source	Sample No.
31	25	M	THROAT SWAB	TS 245
32	22	F	SPUTUM	SP 892
33	34	M	SPUTUM	SP 249

34	51	M	SPUTUM	SP 891
35	12	M	THROAT SWAB	TS 241
36	19	M	THROAT SWAB	TS 1097
37	35	M	SPUTUM	SP 255
38	16	F	SPUTUM	SP 1096
39	38	M	EAR SWAB	ES 895
40	33	F	SPUTUM	SP 334

Key: ES=Ear Swab, SP=Sputum, TS=Throat Swab, F=Female, M=Male.

Data on Samples Collected

S/No.	Age	Sex	Sample source	Sample No.
41	10	M	THROAT SWAB	TS 339
42	28	F	SPUTUM	SP 258

43	13	F	EAR SWAB	ES 350
44	26	F	NASAL S.	NS 256
45	48	F	NASAL S.	NS 340
46	19	M	EAR SWAB	ES 165
47	45	F	SPUTUM	SP 898
48	26	M	SPUTUM	SP 259
49	17	M	SPUTUM	SP 899
50	11	F	SPUTUM	SP 894

Key: ES=Ear Swab, NS=Nasal Secretion, SP=Sputum, TS=Throat Swab, F=Female, M=Male.

Data on Samples Collected

S/No.	Age	Sex	Sample source	Sample No.
51	23	F	SPUTUM	SP 896

52	20	F	SPUTUM	SP 266
53	12	M	SPUTUM	SP 265
54	50	F	SPUTUM	SP 268
55	24	M	SPUTUM	SP 897
56	29	M	SPUTUM	SP 203
57	31	M	SPUTUM	SP 267
58	27	M	SPUTUM	SP 261
59	14	M	SPUTUM	SP 269
60	19	F	SPUTUM	SP 260

Key: SP=Sputum, F=Female, M=Male.

Data on Samples Collected

S/No.	Age	Sex	Sample source	Sample No.

61	27	F	SPUTUM	SP 270
62	6	F	SPUTUM	SP 276
63	29	F	SPUTUM	SP 278
64	15	F	SPUTUM	SP 900
65	10	F	SPUTUM	SP 902
66	16	M	THROAT SWAB	TS 901
67	42	M	THROAT SWAB	TS 235
68	39	M	THROAT SWAB	TS 273
69	32	M	SPUTUM	SP 279
70	35	F	THROAT SWAB	TS 274

Key: SP=Sputum, TS=Throat Swab, F=Female, M=Male.

Data on Samples Collected

S/No.	Age	Sex	Sample source	Sample No.
71	2	M	SPUTUM	SP 277
72	31	M	SPUTUM	S 903
73	14	M	SPUTUM	SP 271
74	28	M	SPUTUM	SP 233
75	53	M	SPUTUM	SP 275
76	37	F	SPUTUM	SP 272
77	48	F	SPUTUM	SP 1204
78	25	M	SPUTUM	SP 1205
79	18	M	EAR SWAB	ES 1209
80	45	M	SPUTUM	SP 1203

Key: ES=Ear Swab, SP=Sputum, F=Female, M=Male.

Data on Samples Collected

S/No.	Age	Sex	Sample source	Sample No.
81	40	F	THROAT SWAB	TS 1234
82	10	M	EAR SWAB	ES 1243
83	26	M	THROAT SWAB	TS 1235
84	31	F	SPUTUM	SP 1270
85	37	F	SPUTUM	SP 1300
86	14	M	SPUTUM	SP 1307
87	28	F	SPUTUM	SP 1308
88	25	M	SPUTUM	S 1309
89	27	M	EAR SWAB	ES 1319
90	16	M	SPUTUM	SP 1349

Key: ES=Ear Swab, SP=Sputum, TS=Throat Swab, F=Female, M=Male.

Data on Samples Collected

S/No.	Age	Sex	Sample source	Sample No.
91	33	M	EAR SWAB	ES 1351
92	41	F	SPUTUM	SP 1348
93	30	M	THROAT SWAB	TS 1353
94	17	M	SPUTUM	SP 1347
95	28	F	SPUTUM	SP 1382
96	22	F	SPUTUM	SP 1386
97	50	F	SPUTUM	SP 1390
98	35	F	SPUTUM	SP 1454
99	49	M	SPUTUM	SP 1455
100	23	F	SPUTUM	SP 1562

Key: ES=Ear Swab, SP=Sputum, TS=Throat Swab, F=Female, M=Male.

Data on Samples Collected

S/No.	Age	Sex	Sample source	Sample No.
101	27	M	SPUTUM	SP 1563
102	32	M	THROAT SWAB	TS 1579
103	39	F	THROAT SWAB	TS 1574
104	41	M	SPUTUM	SP 1599
105	12	F	THROAT SWAB	TS 1616
106	27	M	THROAT SWAB	TS 1167
107	25	M	SPUTUM	SP 723
108	34	F	THROAT SWAB	TS 332
109	11	M	THROAT SWAB	TS 345
110	38	F	SPUTUM	SP 346

Key: SP=Sputum, TS=Throat Swab, F=Female, M=Male.

Data on Samples Collected

S/No.	Age	Sex	Sample source	Sample No.
111	32	M	SPUTUM	SP 343
112	13	M	THROAT SWAB	TS 349
113	18	F	THROAT SWAB	TS 352
114	5	F	EAR SWAB	ES 848
115	23	M	SPUTUM	SP 687
116	17	F	SPUTUM	SP 1714
117	29	M	SPUTUM	SP 1715
118	20	M	SPUTUM	SP 291
119	16	F	EAR SWAB	ES 350
120	34	M	THROAT SWAB	TS 939

Key: ES=Ear Swab, SP=Sputum, TS=Throat Swab, F=Female, M=Male.

Data on Samples Collected

S/No.	Age	Sex	Sample source	Sample No.
121	26	F	SPUTUM	SP 344
122	29	F	SPUTUM	SP 1634
123	22	F	THROAT SWAB	TS 328
124	30	F	SPUTUM	SP 1278
125	52	M	THROAT SWAB	TS 1671
126	19	M	SPUTUM	SP 285

Key: SP=Sputum, TS=Throat Swab, F=Female, M=Male.

Appendix VI: Ethical clearance

UNIVERSITY HEALTH SERVICES

MAIN CAMPUS CLINIC
AHMADU BELLO UNIVERSITY
ZARIA, NIGERIA.

Vice Chancellor: Professor Abdullahi Mustapha, *Bsc, (Hons) Pharm (A.B.U.), Ph.d (London) FPSN*
Director: Dr. M. A. Usman; *MBBS (ABU), FWACS (on)*

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Our Ref:

UNIVERSITY HEALTH SERVICES STUDY PROTOCOL REVIEW COMMITTE

PERMISSION LETTER

PRINCIPAL INVESTIGATOR: MSHELIA B. MARI (PHARM-SC/6309/2010-2011)

ADDRESS OF PRINCIPAL INVESTIGATOR: DEPARTMENT OF PHARMACEUTICS & PHARMACEUTICAL MICROBIOLOGY, AHMADU BELLO UNIVERSITY, ZARIA

PROTOCOL TITLE: ASSESSING THE ANTIBACTERIAL EFFICACY OF HONEY & LEMON AGAINST BACTERIAL ISOLATES FROM URTI.

STATUS: APPROVED

The University Health Services Study Protocol Review Committee has reviewed your protocol tiled: "ASSESSING THE ANTIBACTERIAL EFFICACY OF HONEY & LEMON AGAINST BACTERIAL ISOLATES FROM UPPER RESPIRATORY TRACT INFECTIONS"

The study involved the use natural content of diet in the Nigerian culture.
This has already been carried out by you and from our records; there was no reported adverse effect on the patients who participated in the study

The project is set up to study the antibacterial activities of honey and lemon. The outcome may be useful in the development of strategies to improve the health care of our patient and also give us insight to their relevance in the Alternative Health care practices.

It is our believe that you follow the National Code for Health Research Ethics; this requires you to comply with all institutional guidelines, rules and regulations and with the tenets of the Code including ensuring that all adverse events are report promptly to the appropriate authority. No changes are permitted in the research without prior approval by the University Health Services except in circumstances outlined in the Code..

04/03/2014

Director
University Health services

23/03/2014

Interim Committee Chairman