

CHAPTER ONE

1. Introduction

Microbial infection is major public health problem in both developed and developing countries. Due to the misuse of antibiotics "used to treat these infections" the incidence of multiple antibiotic resistance among human pathogens is increasing. All this beside the undesirable side effects of antibiotics have forced the scientists to search for new antimicrobial substances from natural sources (WHO, 2016).

Antimicrobial resistance is the ability of microorganisms to resist the effect of drugs leading to resistant infections, which may kill, can spread to other and imposes huge costs to individual and society. Misuse of antibiotics is the most important factor leading to antibiotic resistant around the world (Cassir *et al.*, 2014).

Traditional medicine is defined as the health practices, approaches, knowledge and believes incorporating plant, animal and animal based medicines, spiritual therapies, manual technique and exercises, applied singularly or in combination to prevent, diagnose and treat illnesses (WHO,2016).

Since old ancients honey has been used traditionally for the treatment of many diseases including wound infections, respiratory tract infections, urogenital tract infections and many others infections. In this study we want to prove the antibacterial activity of bees honey scientifically using reference laboratory techniques (Cheesbrough, 1991).

1.2 Rationale

Recently, modern societies face serious problems with using of the synthetic chemotherapeutic agents, in order to their multiple disadvantages such as harmful side effects, high cost and development of multi-resistant due to recurrent usage. So the traditional medicine –specially in the Middle and Far East societies- started to play an important role as a safer cheaper alternative solution.

In sudanese culture and as a part of traditional medicine Honey is used for the treatment of many infections such as respiratory tract infections, uro-genetal tract infections and wound infections, therefore it is of interest to test and prove this activity scientifically using standard microbiological techniques.

1.3 Objectives

1.3.1 General objectives

To measure antibacterial activity of honey on selected bacteria isolated from different clinical samples from Sharg Elneel Hospital.

1-3-2 Specific objectives

- To determine the antibacterial activity of honey against organisms isolated from different clinical specimens.
- To determine antibacterial activity of commonly used antibiotics against clinical isolates.
- To determine the minimum inhibitory concentration of honey.
- To compare between the activity of honey and some antibiotics against isolated organisms.

CHAPTER TWO

2. Literature Review

2.1 Antimicrobial resistance

Antimicrobial resistance is when a microbe evolves to become more or fully resistance to antimicrobials which previously could treat. Resistance arises through one of three ways: natural resistance in certain types of bacteria, genetic mutation, or by one species acquiring resistance from another. Resistance can appear spontaneously due to random mutations, or by gradual buildup over time and because of misuse of antibiotics. Microbes resist to multiple antimicrobials are called multidrug resistant (MDR) or sometimes superbugs (WHO, 2016). Antimicrobial resistant microbes are found in people, animals, food and the environment, poor infection control, inadequate sanitary conditions and inappropriate food handling encourage the spread of antimicrobial resist (D, costa *et al.*, 2011).

Resistant microbes are increasingly difficult to treat, requiring alternative medications or higher doses which may be more costly or more toxic. Without effective antimicrobial for prevention and treatment such as organ transplantation, cancer chemotherapy and major surgery become very high risk (Hoffman *et al.*, 2015). Antimicrobial resistance is on the rise with million of deaths every year and is putting the gains of the millennium development goals at risk and endangers achievement of the sustainable development goals (WHO, 2016).

Antimicrobial resistance occurs naturally over time, usually through genetic changes, however the misuse of antimicrobials is accelerating this process. Antibiotics should only be used when needed as prescribed by health professionals. The prescriber should closely adhere to the five rights of drug administration

which are, the right patient, the right drug, the right dose, the right route and the time (Cassir *et al.*, 2014).

2-2 Traditional medicine

The World Health Organization (WHO) definition of Traditional Medicine is the health practices, approaches, knowledge and believes incorporating plant, animal and animal based medicines, spiritual therapies, manual technique and exercises, applied singularly or in combination to treat, diagnose and prevent illnesses or maintain well being (WHO, 2016).

Elderly Traditional medicine is the oldest form of medicine known to Human Kind. It has been developed through observations and by trial and error. The pharmacological treatment of disease began long ago with the use of herbs. People did not know why something worked, they just knew what worked. When something worked, it was written down and considered medical knowledge that was passed on from generation to generation (Fahd and Toufic,1996).

Presently The (WHO) estimates that 4 billion people (80% of the World population) use traditional medicine for primary health care (WHO, 2016). Traditional medicine remains the foundation of modern Pharmacology. Animal and plant derived substances have been used as the basis for a large proportion of mainstream medicines; salicylic acid, Aspirin precursor, originally derived from meadow sweet plant. Quinine derived from cinchona bark. The opium poppy yields morphine (WHO, 2016).

2.3 Honey

2.3.1 Bees

Bees are flying insects closely related to wasps and ants, and are known for their role in pollination and for producing honey and beeswax. Bees are amonophyletic lineage within the superfamily Apoidea, presently classified by the unranked taxon name Anthophila. There are nearly 20,000 known species of bees in seven to nine

recognized families. Although many are undescribed and the actual number is probably higher (Danforth *et al.*, 2006).

2.3.1.1 Scientific classification

Table 1. Scientific classification of bees

Kingdom	Animalia
Phylum	Arthropoda
Class	Insecta
Order	Hymenoptera
Suborder	Apocrita
Superfamily	Apoidea
Series	Anthrophila

(Michael *et al.*, 2009)

2.3.2 Honey production

Firstly, the foraging bees collect nectar from flowers using tube like structure called proboscis, in the bees stomach the nectar metabolized by certain enzymes such as amylase and glucose oxidase. The metabolized nectar then dropped into the beeswax comb and finally converted into thickened honey after being evaporated by the bees' wings (Subramanian *et al.*, 2007).

2.3.3 Chemical composition

Table 2. Chemical composition of honey

Nutrients	Amount in 100g of honey
Water	17.1g
Carbohydrates (total)	82.4g
Fructose	38.5g
Glucose	31.0g
Maltose	7.2g
Sucrose	1.5g
Proteins, amino acids, vitamins and minerals	0.5g
Energy	304Kcal
Vitamins	Amount in 100g of honey
Thiamine	0.006mg
Riboflavin	0.06mg
Niacin	0.36mg

Pantothenic acid	0.11mg
Pyridoxine(B ₆)	0.32mg
Ascorbic acid(C)	2.2-2.4mg
Minerals	Amount in 100g of honey
Calcium	4.4-9.2mg
Copper	0.003-.01mg
Iron	0.06-1.5mg
Magnesium	1.2-3.5mg
Mangnese	0.02-0.4mg
Phosphorus	1.9-6.3mg
Potassium	13.2-16.8mg
Sodium	0.0-7.6mg
Zinc	0.03-0.4mg

(Subramanian *et al.*, 2007).

Many researches to date have addressed honey antibacterial properties and its effect on many infections. The following Laboratory studies and clinical trials have shown that *honey is* effective broad-spectrum antibacterial agent. Since 1992 a study showed the antibacterial activity of *honey* on wound-infecting species of bacteria. The results proved the high efficiency of *honey* to inhibit all bacteria tested (Willix *et al.*, 1992). While in 1999 a study on the antibacterial activity of honey against *Staphylococcus aureus* isolated from wounds showed that the minimum inhibitory concentrations of honey tested were all between 2%-3% for mauka honey (from New Zealand), and between 3%-4% for the pasture honey (Cooper *et al.*,1999). Also in 1999 another study was done on the anti microbial activity of honey on 20 strains of *Pseudomonas* isolated from infected wounds. The minimum inhibitory concentrations were 5.5%-8.7% (v\v) for mauka honey and 5.8%-9.0% for pasture honey (Cooper and molan,1999). On the other hand in 2000 a study under the title of the inhibitory activity of honey against food borne pathogens as influenced by the presence of hydrogen peroxide and the level of antioxidant power, this study proved the inhibitory action of honey against *Escherichia coli* O157:H7, *Salmonella typhimurium*, *Shigella sonnie*, *Listeria monocytogenes*, *Stapylococcus aureus* and *Bacillus cereus* (Peter *et al.*, 2000). In 2001 the antimicrobial activity of honey on bacteria isolated from wounds was also studied showing that 100% of organisms isolated failed to grow at concentration 30% of honey in MH medium.(Subrahmanyam *et al.*, 2001). In 2004 both invitro and invivo investigations of the antimicrobial activity of honey on the pathogenic bacterial infection of surgical wounds and conjunctiva was made. The results of the invitro investigation proved that 100% of isolated bacteria was inhibited by tested honey, while the local application of tested honey on surgical wounds and infected conjunctiva of experimental mices reduced the redness swelling, time for complete

resolution of lesions, pus discharge and time for complete eradication of bacterial infection (Noori and Alwali, 2004). Also in 2004 the bactericidal activity of honey against pathogenic bacteria was studied; showing that 93% of bacteria were inhibited by tested honey (Patricia *et al.*, 2004). A study in 2005 on the antibacterial activity of honey against coagulase negative *Staphylococci* showed that honey were inhibitory at dilutions down to 3.6% for pasture honey and 3.4% for mauka honey (French *et al.*, 2005). The activity of honey on *Helicobacter pylori* was studied in 2006 proving that all honey tested had inhibitory action against *Helicobacter pylori* (Basil *et al.*, 2006). Also In 2007 an invitro study of the effectiveness of honey dressing for healing pressure ulcers showed that after 5 weeks of treatment by honey, patients were completely healed (Yupucu Gunes *et al.*, 2007). In 2007 a study was made on the antimicrobial activity of different floral sources of honey against bacterial isolation, the results showed that 92.5% of bacterial isolates was inhibited by honey (Hyungjaer *et al.*, 2007). While in 2009 a study under the title of the effectiveness of honey on *Staphylococcus aureus* and *Pseudomonas auroginosa* biofilms showed that 100% of the isolates were effectively inhibited by honey (Alandejani *et al.*, 2009). While in 2010 another study on the antibacterial properties of honey and its effect in wound management was made; resulting in that both Gram-positive and Gram-negative bacteria isolated were completely inhibited by the honey tested (Nur Azida *et al.*, 2010).

CHAPTER THREE

3. Materials and Methods

3.1.Methods

3.1.1 Study design

This study was a cross sectional hospital based study.

3.1.2 Study area

The study was conducted in Sharg Elneel Hospital in Khartoum state.

3.1.3 Study duration

The study was conducted during March to July 2016.

3.1.4 Study population

Different clinical pathogens isolated from patients of different ages groups and gender attending Sharg Alneel hospital during study duration.

3.1.5 Inclusion criteria

Most commonly isolated pathogenes were included.

3.1.6 Exclusion criteria

All rarely isolated pathogenes, slow growing organisms and fastidious organisms were excluded.

3.1.7 Sample size

One hundred of different clinical bacterial isolates that already isolated from specimens received during study duration were tested.

3.1.8 Isolation and identification of clinical isolates

3.1.8.1 Culture

Different types of culture media (CLED agar, Blood agar, Macconckey Agar and Chocolate blood Agar) were used for identification and isolation of clinical isolates (Appendix 2).

3.1.8.2 Inoculation and incubation

Different clinical specimens received were inoculated and incubated according to WHO SOPs.

3.1.8.3 Gram stain

1- Three drops of sterile normal saline were added in clean dry slides using sterilized wire loop.

2-From pure culture of the tested organisms one colony was touched by sterilized wire loop (for each slide), and mixed with normal saline and spreaded evenly on an area of about 15-20 mm.

3- The dried smears were fixed by heating (using the flame).

4- The fixed smears were covered with crystal violet stain (Appendix 2) for 30 minutes.

5- Smears rapidly washed off with clean water.

6- The smears were covered with lugol's iodine for 30-60minutes then washed.

7-Rapid de-colorization were done (few seconds) with acetone alcohol, then washed.

8- The smears were covered with neutral red for 2 minutes then washed.

9-Then smears dried, drop of immersion oil added and the smears examined microscopically using X100 (Cheesbrough, 1991).

3.1.8.4 Biochemical tests

A. Gram positive bacteria:

I. Catalase

-Requirements:

Hydrogen peroxide (Appendix 3) and wooden stick.

-Method:

2-3ml of the hydrogen peroxide solution was poured into tube test, using sterile wooden stick a good growth of the tested organism was removed and immersed in the hydrogen peroxide solution, immediate appearance of air bubbles was observed (Cheesbrough, 1991).

II. Coagulase test

-Requirements:

Undiluted human plasma, slide and physiological saline.

-Method:

A drop of physiological saline was added in each end of a slide, a colony of the test organism was emulsified in each drop to make two thick suspensions and then a drop of plasma was added to one of the suspension and mixed gently by rotating. clumping of the organism within 10 seconds was observed (Monica Cheesbrough, 1991).

III. Deoxyribonuclease (DNase) test

-Requirements:

DNase agar plate (Appendix 3), 1ml of hydrochloric acid (1% HCL).

-Method:

The tested organism was cultured on a medium which contain DNA. After overnight incubation, the colonies were tested for DNase production by flooding the plate with a weak hydrochloric acid solution. The acid precipitates un-

hydrolyzed DNA. DNase producing colonies are therefore surrounded by clear areas indicating DNA hydrolysis (Cheesbrough , 1991).

B. Gram negative bacteria:

I. Oxidase test

-Requirements:

Filter paper impregnated with oxidase reagent (Appendix 3), clean slide and wooden stick.

-Method:

A peice of filter paper was placed on a clean glass slide and three to four drops of freshly prepared oxidase reagent were added using sterile Pasteur pipette, wooden stick was used to pick a colony of the test organism and placed on the filter paper (Cheesbrough, 1991).

II. Indole test

-Requirements:

Sterile peptone water in small test tube, Kovac's reagent (Appendix 3), wire loop and Pasteur pipette.

-Procedure:

The tested colony was inoculated in sterile peptone water using sterile wire loop and then incubated at 37°C aerobically overnight. Few drops of Kovac's reagent were added to the medium using Pasteur pipette (Cheesbrough, 1991).

III. Citrate utilization test

-Requirements:

Simmon's citrate slope agar medium (Appendix 3) and straight loop.

-Procedure:

A small part of the tested colony was picked off using sterile straight loop and inoculated on the surface of the slope of the medium in a zigzag manner, and then incubated at 37⁰ C aerobically overnight (Cheesbrough, 1991).

IV. Urease test

-Requirements:

Christensen's urea agar medium (Appendix 3) and straight loop.

-Procedure:

the tested colony was inoculated on the surface of the slope medium by sterile straight loop in zigzagging manner and then incubated overnight at 37⁰C aerobically (Cheesbrough, 1991).

V. Motility testing

-Required:

Semisolid media and straight loop.

-Method:

The tested colony was taken by a sterile straight loop, and inoculated by stabbing the media, then incubated aerobically at 37⁰C overnight (Cheesbrough, 1991).

VI. KIA(kligler iron agar)

-Required:

KIA medium (Appendix 3) in a slope position and a straight loop.

-Method:

A small part of the tested colony was picked off using a straight loop and inoculated in KIA medium, first stabbing the butt, then streaking the slope in zigzag pattern, and then incubate at 37⁰C aerobically overnight (Cheesbrough, 1991).

3.2.9 Antibacterial sensitivity tests

3.2.9.1 Antibacterial activity of selected antibiotics

The isolated clinical organisms were tested for their susceptibility to different routinely used antibacterial agents including ciprofloxacin, gentamicin and ceftriaxone from Himedia (India) using Kirby-Bauer diffusion method according to Podschun and Ullmann (1998).

3.2.9.2 Testing antibacterial activity of honey

1. Preparation of serial dilutions of honey

Honey was obtained from Kingdom Company of Honey and Bee Product in Khartoum. This honey was considered as stock then four double folded serial dilutions performed using sterile distilled water to determine the minimum inhibitory concentration (MIC). And dilutions were performed as follows:

100% honey undiluted honey sample.

50% dilution (v/v) 10 ml of stock honey dissolved in 10ml of sterile distilled water.

25% dilution (v/v) 10 ml of 1/2 diluted *honey* dissolved in 10 ml of sterile distilled water.

12.5% dilution (v/v) 10 ml of 1/4 diluted *honey* dissolved in 10 ml of sterile distilled water.

6.25% dilution (v/v) 10 ml of 1/8 diluted *honey* dissolved in 10 ml of sterile distilled water.

2. Preparation of bacterial suspension

Ten ml of normal saline were placed in the test tubes and sterilized in autoclave at 121⁰C for 15 minute, a loop full of purified bacteria were inoculated in sterile normal saline and compare with 0.5 McFarland standard (Cheesbrough, 1991).

3. Modified diffusion technique (Cup plate method)

The diffusion method was adopted with some minor modification to assess the antimicrobial activity of the prepared honey dilutions. Two ml of bacterial suspension were taken with sterile disposable syringe and added to twenty ml of molten Muller Hinton media and mixed, then allow the media to set and solidify for few minutes, wells were made using sterile cork borer of 5 mm diameter.

Alternated cups were filled with 0.5 ml of honey dilutions using sterile disposable syringes .Allowed to diffuse at room temperature for 30 min then the plate incubated in incubator in upright position at 37⁰C for 18 hours, (Hamza *et al.*, 2015).

The diameters of the resultant growth inhibition zones were measured and interpreted according to Cruickshank, (1975) in the terms sensitive, resistant and intermediate as:

Sensitive: zones more than 18mm

Intermediate: zones (14-18) mm

Resistant: zones less than 14 mm

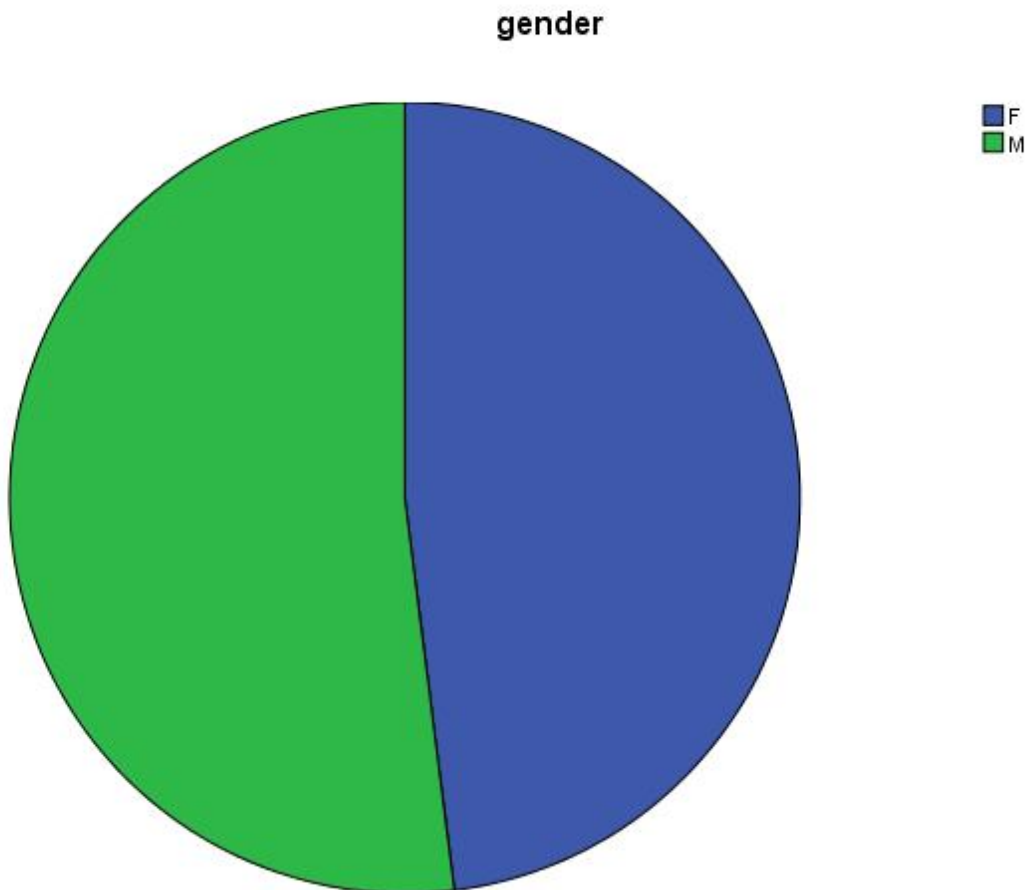
CHAPTER FOUR

4. RESULTS

4.1 Gender frequency

Among the studied population (100 patients) there were 53(53%) males and 47(47%) females as shown in figure1.

Figure1. Gender frequency among study population



4.2 Sample type frequency:

Among 100 different clinical samples tested there were 62 urine samples, 28 wound swabs, 6 tissue and only 4 aspirations (Table 4).

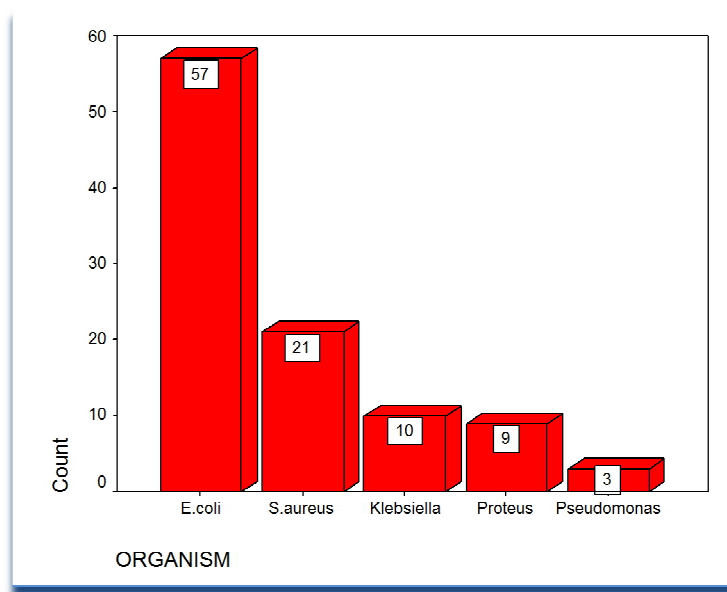
Table3. Sample type frequency

Sample type	Number	Frequency
Urine	62	62%
Wound swab	28	28%
Tissue	6	6%
Aspiration	4	4%
Total	100	100%

4.3 Clinical isolates frequency

In this study 100 clinical isolates were tested as follows; 57(57%) *Escherichia coli*, 21(21%) *Staphylococcus aureus*, 10(10%) *Klebsiella pneumoniae*, 5(5%) *Proteus mirabilis*, 4(4%) *Proteus vulgaris* and 3(3%) *Pseudomonas aeruginosa* (Table 5).

Figure2. frequency of clinical isolates



4.4 Susceptibility of clinical isolates to selected antibiotics

Isolated organisms were tested for their susceptibility to three commonly used antibiotics including ciprofloxacin, gentamicin and ceftriaxone and the results obtained interpreted in the terms of sensitive (S), resistant(R) and intermediate (I) according to manufacture guidelines.

4.4.1 Ciprofloxacin

There were 48 organisms sensitive to ciprofloxacin, while 50 were resistant and only 2 organisms were intermediate. As shown in table 6.

4.4.2 Gentamicin

There were 54 organisms sensitive to gentamicin and 46 were resistant. As shown in table 6.

4.4.3 Ceftriaxone

There were only 16 organisms sensitive to ceftriaxone, while the majority 76 were resistant and 8 organisms were intermediate. As shown in table 6.

Table4. Susceptibility of clinical isolates to selected antibiotics

Antibiotics	S	R	I	Total
CIP	48	50	2	100
GEN	54	46	0	100
CTR	16	76	8	100

Key words:

CIP: ciprofloxacin

GEN: gentamicin

CTR: ceftriaxone

S: sensitive

R: resistant

I: intermediate

4.5 Susceptibility of clinical isolates to Honey

4.5.1 100% honey

There were 70 (70%) organisms sensitive to stock *honey* and only 30 (30%) were resistant as shown in table 5.

4.5.2 50% (v/v) honey

As same as stock *honey* there were also 70 (70%) organisms sensitive to stock *honey* and 30 (30%) were resistant as shown in table 5.

4.5.3 25% (v/v) honey

There were 59 organisms sensitive to this dilution, while 38 were resistant and 3 organisms were intermediate (Table 5).

4.5.4 12.5% (v/v) honey

There were only 11 organisms sensitive to this dilution, while 88 were resistant and only 1 organism were intermediate (Table 5).

4.5.5 1/6.25% (v/v) honey

All tested clinical isolates were resistant to this dilution of honey as shown in table 5.

Table5. Susceptibility of clinical isolates to honey

Dilution (v/v)	S	R	I	Total
100%	70	30	0	100
50%	70	30	0	100
25%	59	38	3	100
12.5%	11	88	1	100
6.25%	0	100	0	100

Key words:

S: sensitive

R: resistant

I: intermediate

(v/v): volume of honey per volume of distilled water

4.5.6 MIC of honey

According to results shown above, 12.5% honey can be considered as the minimum concentration of honey required to inhibit the bacterial growth.

4.5.7 Percentage of inhibition among different clinical isolates

Table 6 below shows 75% of *Staphylococcus aureus* isolated were inhibited by honey (figure 6), while 72 % of *Escherichia coli* (figure 3), 50% of *Proteus vulgaris*, 40% of both *Proteus mirabilis* and *Klebsiella pneumonia* (figure 5-4) and 33% of *Pseudomonas aeruginosa* were inhibited (figure7).

Table6. Percentage of inhibition of different clinical isolates to one or more of honey dilutions:

Organism	% of inhibition
<i>Staphylococcus aureus</i>	75%
<i>Escherichia coli</i>	72%
<i>Proteus vukgaris</i>	50%
<i>Proteus mirabilis</i>	40%
<i>Klebsiella pneumonia</i>	40%
<i>Pseudomonas aeruginosa</i>	33%

CHAPTER FIVE

Discussion

Microbial infection is major public health problem in the developing countries. Due to the misuse of antibiotics the incidence of multiple antibiotic resistance among human pathogens is increasing. All this beside the undesirable side effects of antibiotics have forced the scientists to search for new antimicrobial substances from natural sources (WHO, 2008). In the same line this study was carried out to evaluate the antibacterial activity of honey against bacteria isolated from different clinical specimens including urine, wound swabs, tissues and aspirations. In this study urine was the most frequent sample representing 62% of all specimens, followed by wound swabs (28%), tissues (6%) and aspiration (4%).

The results showed that gentamicin was the most effective antibiotic inhibiting 54% of organisms followed by ciprofloxacin inhibited 48% of organisms, while the worst antibiotic was ceftriaxone inhibiting only 16% of all organisms. This may be due to availability of both ciprofloxacin and ceftriaxone as tablets making them more frequently used specially on self medication than Gentamicin which is available only as injection (Hamza *et al.*, 2015).

Honey was tested as stock and four double folded serial dilutions. The results showed that both 100% honey and 50% (v/v) honey inhibited 70% of organisms, meaning that honey was more effective than the three tested antibiotics ciprofloxacin, gentamicin and ceftriaxone in the inhibition of organisms. These findings agreed with the results reported by Allen *et al.*, (1991), Willix *et al.*, (1992), Dimitrova *et al.*, (2007), Basauldo *et al.*, (2007) and Devarajan and Venugobal, (2011) . They showed that undiluted honey was also able to inhibit the

growth of *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Proteus spp*, and *Pseudomonas aeruginosa*.

On the other hand 25% (v/v) honey inhibited the growth of 59% of clinical isolates. It is important to mention that in this study 1/4 diluted honey inhibited had almost the same activity of gentamicin which inhibited 54% of clinical isolates. This result agreed with Iurlina and Fritz (2005) and Hamza *et al.*, (2015) whom reported that honey at this dilution had efficient antibacterial activity against bacteria tested.

While 12.5% (v/v) honey inhibited only 11% of organisms. This was similar Nagaraj *et al.*, (2012) who reported that honey at this dilution had an antibacterial effect on the tested bacteria (*Escherichia coli*, *Staphylococcus aureus* and *salmonella spp.*). Actually in this study 1/8 diluted honey had almost the same activity of ceftriaxone antibiotic which 6.25 % (v/v) of isolates .Since all organisms were resistant to the fourth dilution of honey (1/16), the minimum inhibitory concentration (MIC) was 12.5% (v/v) honey.

Regarding organisms honey was most effective against isolated *Staphylococcus aureus*, inhibiting 75% of isolates. Favorable results also obtained against 72% of isolated *Escherichia coli* isolates. 50% and 40% of *Proteus mirabilis* and *Proteus vulgaris* isolates were inhibited respectively. While 40% of *Klebsiella pneumoniae* were inhibited and the less activity of honey was shown against *Pseudomonas aeruginosa*, inhibiting 33% of isolates. This different on sensitivity may be due to the osmotic effect, the effect of pH, the sensitivity of these organisms to hydrogen peroxide, different organism's growth rate and different nutritional requirements (Hamza *et al.*, 2015).

From these results we can conclude that honey has broad activity against both gram positive and gram negative bacteria, therefore honey can be regarded as a broad spectrum antibacterial agent.

Conclusion

1. We concluded that honey can be regarded as a broad spectrum antibacterial agent; since its broad activity against both gram positive and gram negative bacteria.
2. Honey can be used with different concentrations to treat different types of infections.
3. Honey is more efficient against *Staphylococcus aureus*.
4. *Pseudomonas aeruginosa* is the most resistant organism to honey.
5. Ceftriaxone resistant rate is very high.

Recommendations

1. Further advanced Techniques to determine the active components responsible for the antimicrobial activity.(for example using chromatography that is powerful way to extract the active gradients of honey).
2. Confirmatory *in vivo* investigations to evaluate the antimicrobial activity of honey which need collaboration between many health and medical sectors.
3. Determination of the Minimum Inhibitory Concentration (MIC) using tube dilution method.

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APPENDIX 1

Color plates

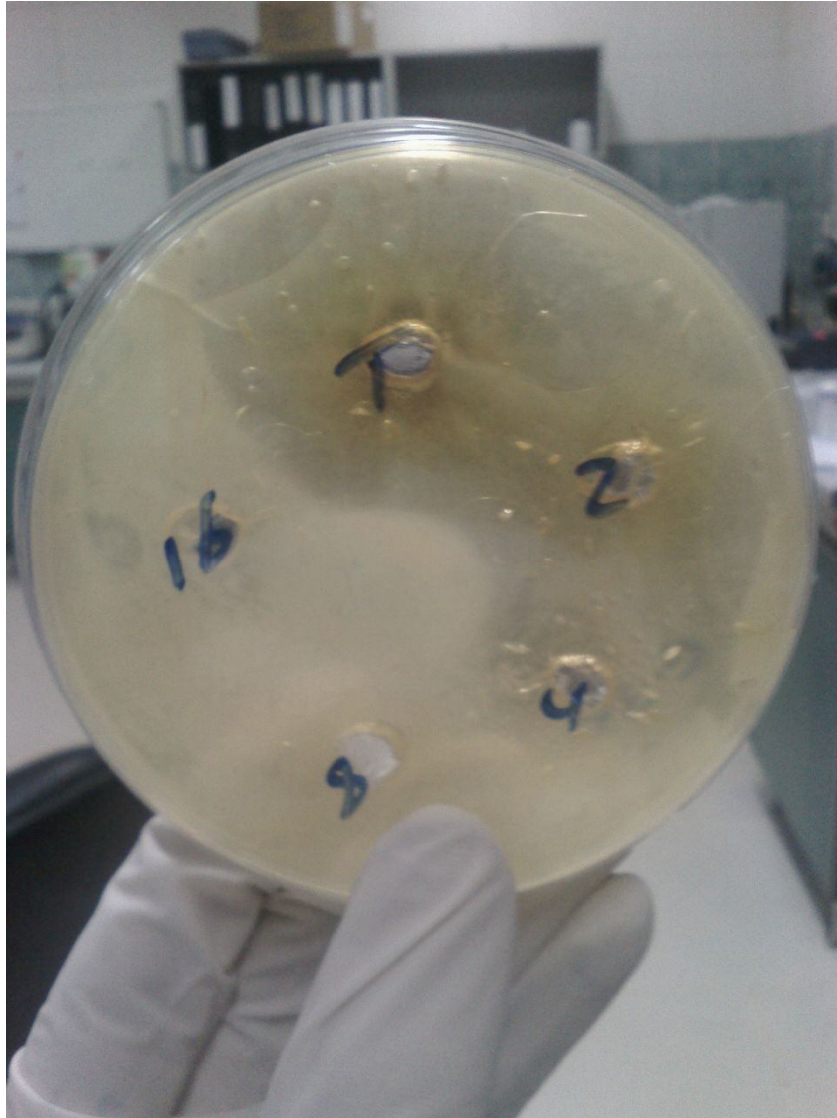


Figure3 : The inhibition zone of honey against *Escherichia coli*

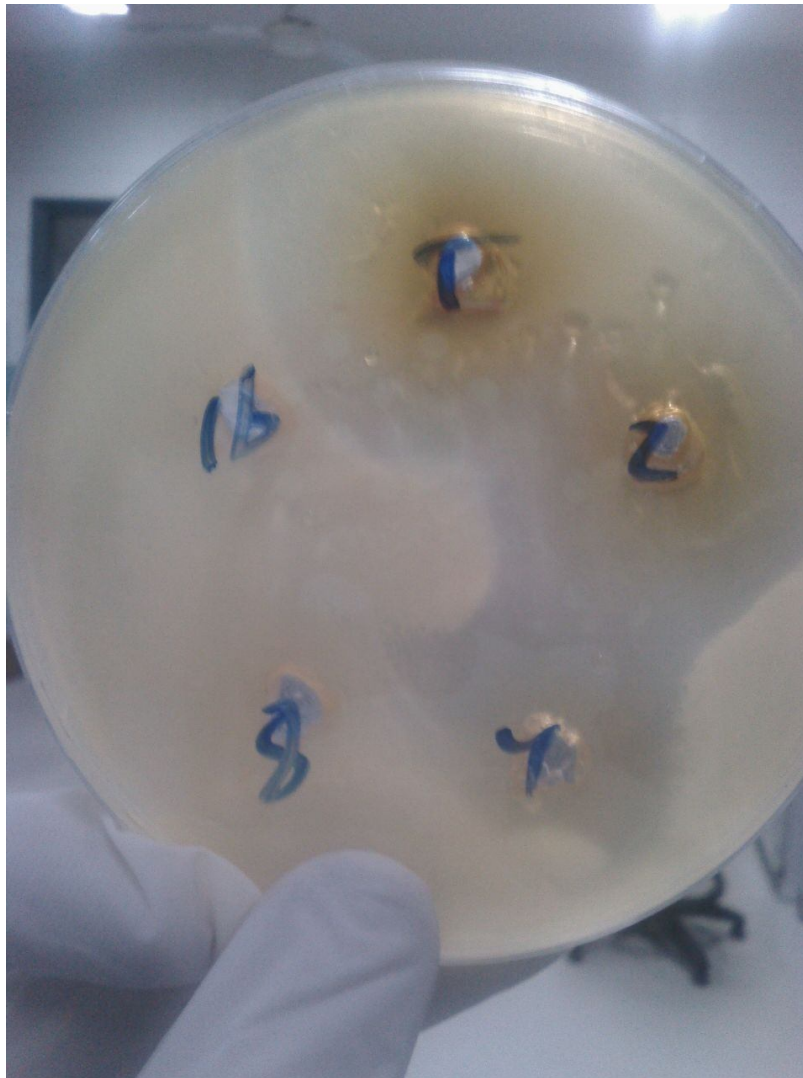


Figure 4: The inhibition zone of honey against *Klebsiella pneumoniae*



Figure 5: The inhibition zone of honey against *Proteus spp*,

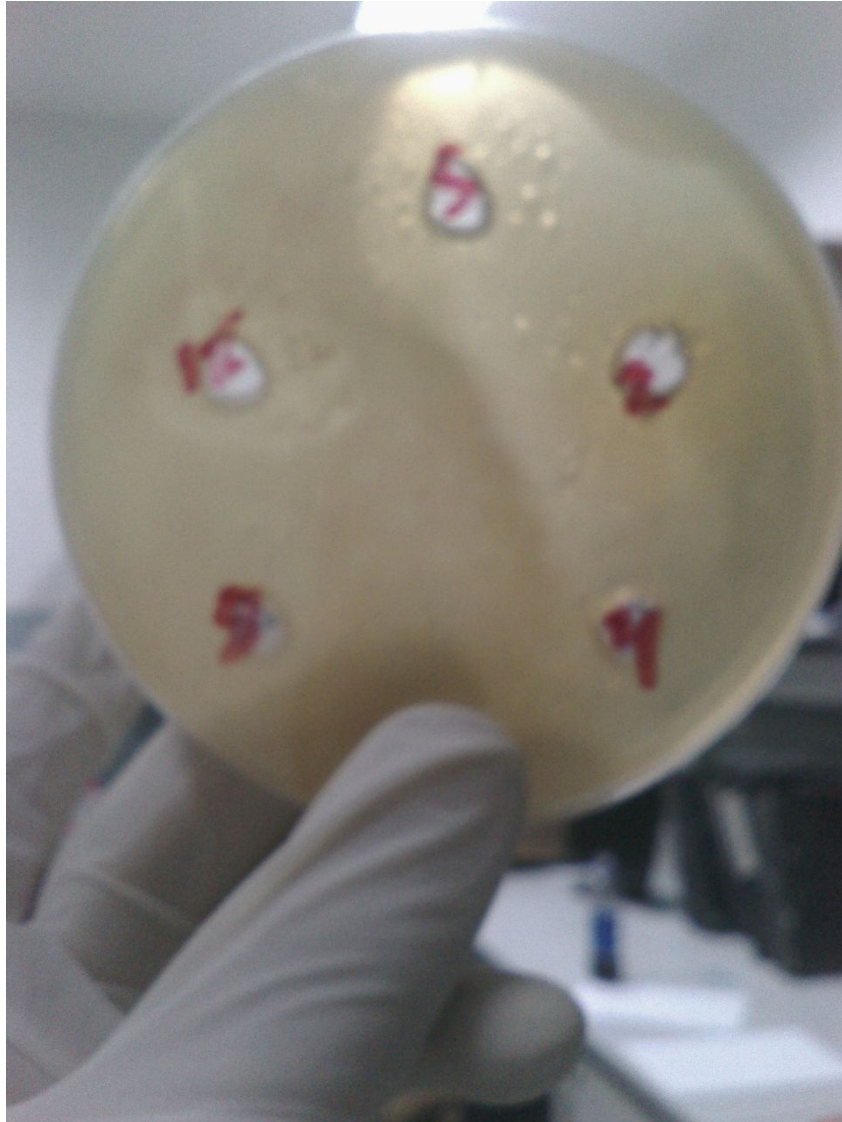


Figure 6: The inhibition zone of honey against *Staphylococcus aureus*

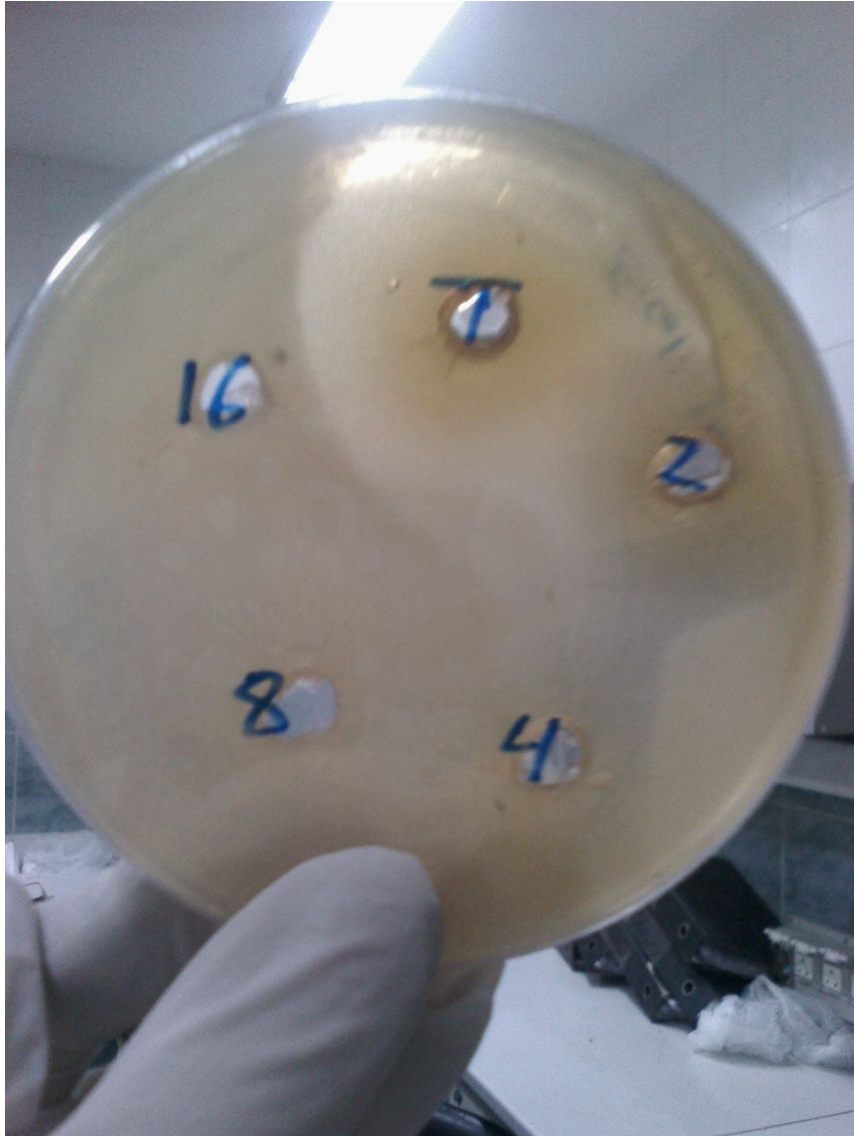


Figure 7: The inhibition zone of honey against *Pseudomonas aeruginosa*

APPENDIX 2

Materials

A. Equipments

- 1-Autoclave
- 2-Incubator
- 3-Hot air oven
- 4-Refrigerator
- 5-Sensitive balance
- 6-Light microscope with oil immersion lenses
- 7-Wire loops with handles
- 8-Straight loops with handles
- 9-cork borer (0.5 cm in diameter)
- 10-Bunsen burner
- 11-Rack
- 12-syringes

B. Glassware

- 1-Petri dishes
- 2-Flasks with different size
- 3-Measuring cylinder
- 4-Beakers
- 5-Sterile containers (bijou bottles)
- 6-Test tubes

C. Disposable materials

1-Disposable syringes

2-Wooden applicators30

3-swabs

APPENDIX 3

A.Reagents:

1. Crystal violet Grams stain

To make 1 liter

Crystal violet.....	20 g
Ammonium oxalate	9 g
Ethanol or methanol , absolute	95ml
Distilled water	to 1 liter

Procedure:

- 1.Weight the crystal violet on a piece of clean paper. Transferred to a brown bottle pre marked to hold one litter
2. Add the absolute ethanol or methanol and mix until the dye is completely dissolved.
3. Weight the ammonium oxalate and dissolve in about 200 ml of distilled water. Add the stain, make up to one litter with distilled water and mixed well.
4. Label the bottle and store it at room temperature. The stain is stable for several months

2. Kovac's reagent:

Content:

to prepare 20ml:

4-dimethylaminobenzaldehyde.....	1g
Isoamylalcohol (3-methyl-1-butanol).....	15ml
Concentrated hydrochloric acid.....	5ml

Procedure:

Weight the dimethyaminobenzaldehyade,dissolve in the isoamylalcohol. Add concentrated hydrochloric acid and mix well. Transfer to a clean brown bottle and stored at 2-8C.

3.Lugol's iodine solution

To make one litter

Potassium iodine solution20 g
Iodine 10 g
Distilled waterto 1 litter

Procedure:

1. Weight the potassium iodine , and transfer to brown bottle pre marked to hold 1 litter .
2. Add about quarter of the volume of water, and mix until the potassium iodine solution is completely dissolved .
3. Weight the iodine, and add to potassium iodide solution. Mix until the iodine is dissolved .
4. Make up to 1 litter distilled water, mix well. Label the bottle and marked toxic.
Store at dark place,

4.Oxidase reagent:

Prepare fresh before use.

To make 10 ml:

Tetramethyle-*p*-phenylenediamine dihydrochloride0.1 g
Distilled water10mL

Procedure:

Dissolve the chemical in water . The reagent is not stable .

5.Turbidity Standard Equivalent to 0.5 Mc. Farland (Barium sulphate):

To make 1% v/v

- Concentrated sulphuric acid 1ml
- Dihydrate barium chloride (BaCl₂.H₂o)..... 0.5g
- Distilled water.....150ml

Procedure:

- 1.Prepare 1%v/v solution of sulphuric acid by adding 1ml of concentrated sulphuric acid to 99ml of distilled water, mix.
- 2.. Prepare 1% w/v solution of barium chloride by dissolving 0.5g of dehydrates barium chloride in 50ml of distilled water.Add 0.6ml of barium chloride to 99.4ml of sulphuric acid solution and mix well.

B.Preparation of media:

1. Blood agar base

Blood agar base is recommended as base to which blood may be added for use in the isolation and cultivation of fastidious pathogenic microorganisms.

Compositions

Ingredients	Gms/L
Beef heart, infusion (beef extract).....	5000
Tryptose	10
Sodium chloride	5
Final pH	7.3

Directions

Suspend 40 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterile by Autoclave at 15 ibs pressure (121°C) for 15 min.

Cool to 50°C and aseptically add 5% v/v sterile defibrinated blood. Mix well and pour into sterile petridishes.

2.DNAse Agar

Composition:

Typical formula	g/l
Contents:	
Tryptose.....	20
Deoxyribonucleic acid.....	2
Sodium chloride.....	5
Agar.....	12
PH.....	7.2 0.2

Direction:

Suspend 3.9g in 1 liter of distilled water. Bring to boil to dissolve completely. Sterilize by autoclaving at 121C for 15 minutes. Cool to 50C, and pour into sterile Petri dishes. Dry the surface of the medium before inoculation.

3.Kliger Iron Agar (KIA)

KIA reactions are based on the fermentation of lactose and glucose (dextrose) and the production of hydrogen sulphide.

Compositions

Ingredients	Gms/L
Peptic digest of animal tissue.....	15
Yeast extract.....	3
Beef extract.....	3
Peptose peptone	5
Dextrose	1

Lactose	10
Ferrous sulphate	0.20
Sodium chloride	5
Sodium thiosulphate.....	.0.3
Phenol red.....	0.042
Agar.....	15
Final pH(at 25°C).....	7.4

Directions

Suspend 57.5 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterile by Autoclave at 15 lbs pressure (121°C) for 15 min. mix and pour. set as slope with butt.

4.Mac Conkey Agar medium

Mac Conkey Agar medium is a differential medium to distinguish between bacteria by neutral red indicator which changes colour when acid is produced following fermentation of lactose sugar.

Composition

Ingredients	Gms/L
Peptic digest of animal tissue.....	17
Protease peptone.....	3
Lactose.....	10
Bile salts	1.5
Sodium chloride	5
Neutral red.....	.03
Agar.....	15
Final pH(at 25°C).....	7.2

Directions

Suspend 51.53 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterile by Autoclave at 15 lbs pressure (121°C) for 15 min. mix and pour.

5.Muller Hinton agar

Muller Hinton agar is used for testing susceptibility of common and rapidly growing bacteria using antimicrobial disc, it manufactured to contain low level of thymine, thymidine, calcium and magnesium.

Compositions

Ingredients	Gms/L
Casein acid hydrolysate	17
Beef heart infusion	2
Starch soluble	1.5
Agar.....	17
Final pH(at 25°C).....	7.3

Directions

Suspend 38 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterile by Autoclave at 15 lbs pressure (121°C) for 15 min. mix and pour.

6.Nutrient agar

Nutrient agar is used for cultivation of less fastidious organisms, can be enriched with blood or other biological fluids.

Compositions

Ingredients	Gms/L
Peptone	10

Beef extract	10
Sodium chloride	5
Yeast xtract.....	1.5
Agar.....	15
Final pH(at 25°C).....	7.3

Directions

Suspend 28 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterile by Autoclave at 15 ibs pressure (121°C) for 15 min. mix and pour.

7.Peptone water

Used for culturing organisms to proceed indole test in the presence of Kovac’s or Ehrlich’s reagent that reacts with the indole to produce a red coloured compound.

Compositions

Ingredients	Gms/L
Peptic digest of animal tissue.....	10
Sodium chloride	5
Final pH(at 25°C).....	7.2

Directions

Suspend 15 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterile by Autoclave at 15 ibs pressure (121°C) for 15 min. mix and pour.

8.Simmons citrate Agar

This test is used 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.

Compositions

Ingredients	Gms/L
Magnesium sulphate	0.20
Ammonium dihydrogen phosphate	1
Dipotassium phosphate	1
Sodium citrate	2
Sodium chloride	5
Bromothymol blue	0.08
Agar.....	15
Final pH(at 25°C).....	6.8

Directions

Suspend 24.28 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterile by Autoclave at 15 lbs pressure (121°C) for 15 min. mix and pour. set as slope .

9.Urea Agar Base (Christensen)

Testing for Urease enzyme activity is important in differentiating enterobacteria. Especially for *proteus spp*

Compositions

Ingredients	Gms/L
Peptic digest of animal tissue.....	1
Dextrose	1
Disodium phosphate	1.20
Monopotassium phosphate	0.80
Sodium chloride	5
Phenol red.....	0.012
Agar.....	15

Final pH(at 25°C)..... 6.8

Directions

Suspend 24 grams in 950 ml distilled water. Heat to boiling to dissolve the medium completely. Sterile by Autoclave at 10 lbs pressure (115°C) for 20 min. Cool to 50°C and aseptically add 50 ml of sterile 40% of urea solution (FD048) and mix.