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Antibacterial Activity of Some Selected Brands of Malaysian Honey

Dissertation submitted in partial fulfillment for the Degree of Bachelor of Health Sciences (Biomedicine)

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CERTIFICATE

This certify that the dissertation entitled

" Antibacterial activity of some selected brands of Malaysian honey"

is a bonafide record of research work done by

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ABBREVIATION

MIC	= Minimum inhibitory concentration
MRSA	= Methicillin resistant *Staphylococcus aureus*
VRE	= Vancomycin resistant Enterococci
USM	= Universiti Sains Malaysia
ATCC	= American type culture collection
LB	= Luria bertani
CFU/ml	= Colony forming unit/milliliter
CM	= Control medium
CI	= Control inoculum
CH	= Control Honey
CA	= Control antibiotic
E. coli	= *Escherichia coli*
S. typhi	= *Salmonella typhi*
S. sonnei	= *Shigella sonnei*
P. aeruginosa	= *Pseudomonas aeruginosa*
S. aureus	= *Staphylococcus aureus*
S. pyogenes	= *Streptococcus pyogenes*
AML	= Amoxycillin
CAZ	= Ceftazidime
CL	= Colistin
CMC	= Chloramphenicol
CXM	= Cefuroxime

R	= Resistant (No inhibition zone)
gm	= gram
mg	= milligram
mm	= millimeter
ml	= milliliter
µl	= microliter
µg	= microgram
µg/ml	= microgram/milliliter
L	= Liter
^0C	= degree celcius
N	= normality
rpm	= rotation per minute
no.	= number

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ABSTRACT

Honey is an ancient remedy for the treatment of infected wounds, which has recently been 'rediscovered' by the medical profession, particularly where conventional modern therapeutic agents are failing. Honey is made up of simple sugars and is an excellent source of energy. It is hypertonic and sterile with antibacterial activity against many pathogenic bacteria.

The present study aims to investigate the antibacterial activities of five different brands of Malaysian honey. 'Tualang', 'Hutan' and 'Gelang' honey are produced from wild bee, 'Pucuk Daun' and 'Ee Feng Gu' honey are from honeybee farm were considered for this study. Six different pathogenic bacteria, *E. coli, S. typhi, S. sonnei, P. aeruginosa, S. aureus* and *S. pyogenes* were selected as test organisms. Diluted and neat honeys were used for the antibacterial assay. Solvent extraction of honey was carried out with an aim to isolate the active principle behind the antibacterial property. MIC of the selected honey was determined.

'Tualang' honey was found to be most effective against *S. typhi.* It also showed a substantial antibacterial activity against *E. coli* and *S. pyogenes.* 'Hutan' and 'Gelang' honey does not show any antibacterial activity against the organism tested. 'Pucuk Daun' honey showed antibacterial activity *E. coli* and *S. sonnei.* 'Ee Feng Gu' was active against *E. coli, S. typhi, S. sonnei* and *S. aureus.*

Among the solvent extracts only acetone fraction showed antibacterial activity. Extracts with petroleum ether, chloroform and ethyl acetate does not show any activity.

The MIC (Minimum Inhibitory Concentration) values of 'Pucuk Daun', 'Gelang' and 'Hutan' honey were 5.0 µg/ml, 17.5 µg/ml and 28.75 µg/ml respectively against *S. sonnei.*

INTRODUCTION

The Prophet (PBUH) said:

'Honey is a remedy for every illness and the Kuran is a remedy for all illness of the mind, therefore I recommend to you both remedies, the Kuran and honey.' (Bukhari)

Malaysian Honey bee

Malaysian Honeybee is belong to Kingdom Animal, Class Insecta, Order Hymenoptera, Family Apidae, Phylum Arthropoda and Genus Apis. Genus *Apis mellifera* are the commonest found in Malaysia. Malaysia Honey bee live at Tualang Tree, the Giant Asian Honey Bees, scientific name: *Koompassia excelsa* is the tallest tree reaching heights of 150 feet and about 80 nests were observed in this tree. Each nest will contain about 30.000 giant Asian honeybees (Nature Explorer Article, 2003).

Figure 1: The 'Tualang' tree and the honey hives

HONEY

Honey is the sweet, elaborated by honeybees from the nectar and pollen collected by them from flowers. Honeys are thick preparation somewhat allied to the syrupy, differing in that honey, instead of syrup is used as a base. They are unimportant as a class of preparation today but a once time, before sugar was available, honey was the most common sweetening agent (Eva Crane, 1976).

Honeybees are very economically important insects. There are three types of honeybees are found in a hive. They are Queen, Workers and Drones. Mainly worker honeybees are responsible for the collection and production of honey (Eva Crane, 1976).

In a verse in Surah 16, An-Nahl, the Holly Kuran: Honey is a healing for mankind. This has been documented in the world's oldest medical literature. The Sumerians and the Egyptian physicians about 4000 years ago used honey to treat internal and external wound, ulcers, diseases of the eyes, lungs, skin and in particular diseases of the stomach and intestines (Maryann, 2000). The Chinese, Indian, Greeks and Romans have recorded similar practices. Hippocrates, the father of Western Medicine, used honey to treat a number of diseases. Ibne Sina, the Prince among Muslim physicians listed several beneficial uses of honey in his monumental work of "The Canon of Medicine" (Eva Crane, 1976).

Medicinal Value of Honey:

1. The antibacterial properties of honey are:

a. Osmotic effect:

Since 1937 it has been known that honey has antibacterial activity due to its high sugar concentration, 84% (being a mixture of fructose and glucose), less water, usually only 15-21% by weight. The strong interaction of these sugar molecules with water molecules leaves very few of the water molecules available for microorganisms. This "free" water is what is measured as the water activity (Khalil et al., 2001; Bose, 1982).

b. Acidity

Honey is characteristically quite acidic, its pH being between 3.2 and 4.5, which is low enough to be inhibitory to many pathogens (Efem, 1988).

c. Hydrogen Peroxide

The major antibacterial activity in honey has been found to be due to hydrogen peroxide produced enzymically in the honey. The glucose oxidase enzyme is secreted from the hypopharyngeal gland of the bee into the nectar to assist in the formation of honey from nectar (Eva Crane, 1976). The hydrogen peroxide and acidity produced by the reaction:

$$\text{Glucose} + H_2O + O_2 \rightarrow \text{gluconic acid} + H_2O_2$$

The hydrogen peroxide and acidity produced serve to preserve the honey. The hydrogen peroxide produced would be of effect as a sterilizing agent only during the ripening of honey.

Full-strength honey has a negligible level of hydrogen peroxide because this substance is short-lived in the presence of the transition metal ions and ascorbic acid in honey, which catalase its decomposition to oxygen and water. The enzyme has been found to be practically inactive in full-strength honey, it giving rise to hydrogen peroxide only when the honey is diluted. This is because the acidity produced in the action of the enzyme drops the pH to a point, which is too low for the enzyme to work any more. On dilution of honey the activity increases by factor of 2,500–50,000, thus giving a "slow-release" antiseptic at a level, which is antibacterial but not tissue-damaging (Molan 2001; Subrahmanyam et al., 2001; Postmes et al, 1993)

d. Phyto-chemical Factors.

In some honeys when treated with catalase to remove the hydrogen peroxide activity, additional non-peroxide antibacterial factors have been identified. Manuka (*Leptospermum scoparium*) honey from New Zealand has been found to have substantial levels of non-peroxide antibacterial activity (Molan and Betts, 2000; Molan, 2001). Several chemicals with antibacterial activity have been identified in honey such as pinocembrin, terpenes, benzyl alcohol, syringic acid and etc. (Waikato Honey Research Unit, 2003).

2. Wound Healing and Curative properties of honey:

Honey has been found, when applied locally, to reduce infection and promote wound healing. The wound healing properties of honey has been well documented since 2,000 years ago (Dunford, 2000). The antimicrobial properties

of honey are attributed to its hypertonicity, low pH, a thermo-labile substance called inhibine and enzymes such as catalase (Subrahmanyam, 1993).

Honey has been proven of value in treating infected surgical wounds, burns and decubitus ulcers (Subrahmanyam et al., 2001). Honey has successfully used for local application in the postoperative management of patients who had undergone radical vulvectomy for vulval carcinoma (Cavangah et al., 1970). Wound healing was accelerated and less bacterial colonization was noted by local application of honey to wounds where conventional treatment had failed (Zumla and Lulat, 1989).

A study has shown that honey is also useful in deeply infected abdominal wounds after caesarean section with effective results. The treatment is simple and inexpensive (Phuapradit and Saropala, 1992). Honey has been described as being effective when used to the local application among the infected post-surgical wounds of infants and no adverse reaction (Vardi et al., 1998). Honey as an excellent adjuvant for acceleration of wound healing and widely used for healing purpose during World War I (Bergman et al., 1983).

Manuka honey was used successfully to treat a recalcitrant wound resulting from surgical treatment of hidradenitis suppurativa. The wound had failed to heal during 3 years of treatment with conventional therapies and following four surgical procedures. But likely, after treatment with dressings impregnated with irradiated manuka honey was initiated, the patient recurrent staphylococcal infections ceased and healing was achieved within 4 months (Cooper et al, 2001).

3. Antibacterial activity of honey:

Antibacterial activity of honey (Efem, 1988), suggests that the hygroscopic properties and low pH of honey are antibacterial and that the barrier honey forms on the wound surface thus prevents bacterial penetration and colonization.

Laboratory studies have been shown that honey has a significant antibacterial activity against the major wound-infecting species including Methicillin Resistant *Staphylococcus aureus* (MRSA) (Allen, 2000; Molan, 2001). This activity is independent of the water effect. Sugar solutions and pastes have a high osmolarity and can bind water and so inhibit bacterial growth. When used on wounds, the presence of exudates dilutes sugar and paste preparations so they quickly lose their effect. This is not, however, the case with honey.

Laboratory testing has demonstrated the ability of honey to inhibit a range of wound pathogens, especially those with the potential to develop antibiotic resistance such as *Pseudomonas* (Al Jabri et al., 2003). Recently β-hemolytic streptococci, MRSA and Vancomycin-resistant enterococci (VRE) have been shown to be sensitive to honey (Kingsley, 2001; Allen et al., 2000). Honey can inhibit antibiotic-sensitive and antibiotic-resistant strains of wound pathogens *in vitro*.

Honey also very effective and powerful as an antibacterial activity against the pathogenic organisms in urine samples of urinary tract infection (UTI). The potency of honey 50% - 30% was found to be effective against pathogen in UTI (Ahmed Shawki, 2003).

REVIEW OF LITERATURE

The literature review reveals that a lot of work has been done on the antibacterial activity on the different sources of honey from the different places in the world.

Among the examples of similar project and research is shown in the paper 'Antibacterial activities of different brands of unifloral honey available at the Northern Region of Bangladesh' (Khalil et al., 2001). In this paper, they found that the tested unifloral honeys showed significant antibacterial activity against the wound infecting and enteric pathogens.

Another paper with the same tested honey, the biochemical analysis was found that the total content of sugar 77.7 – 80.3%, pH 3.30 to 4.20 and specific gravity between 1.33 to 1.36 (Khalil et al., 2001).

The study done by Nzeako and Hamdji, (2000) reported that honey has ability to kill bacteria and fungus, it's antibacterial therapeutic and antifungal agent, so honey have high broad-spectrum antimicrobial activity, even after the honey has been exposed to boiling or refrigerator temperatures.

Another paper that was helpful in the literature review is 'The potential of honey to promote oral wellness' (Molan, 2001). The large volume of literature reporting the effectiveness indicates that honey has potential for the treatment of periodontal disease, mouth ulcers, and other problems of oral health. They found honey has a good antibacterial activity against oral streptococci.

Research by Efem (1988), was reported that honey has antibacterial properties and histological properties and histological effects on the wound healing process. In one study, fifty-nine patients with wounds and ulcers most of which (80 percent) had failed to heal with conventional treatment were treated with unprocessed honey. Fifty-eight cases showed remarkable improvement following topical application of honey. Wounds that were sterile at the outside, remained sterile until healed, while infected wounds and ulcer became sterile within 1 week of topical application of honey. Honey debrided wounds rapidly, replacing sloughs with granulation tissue. It also promoted rapid epithelialization and absorption of edema from around the ulcer margins (Efem, 1988).

With the similar research (Cooper et al., 1999), reported that Manuka Honey from New Zealand was tested against *S. aureus* and proven that honey has a good antibacterial activity against the pathogens.

The only one paper from my own country and institution, the study of the effect of pure honey (source from tea plant, *Camelia sinensis* available from Cameron Highland of Peninsular Malaysia) on radiation induced mucositis. The study by Biswal et al., (2002), showed a good inhibition of bacterial growth against *P. aeruginosa, E. coli, S. pyogenes* and *S. aureus*.

Another researchers, Haffejee and Mossa (1985), had reported that, by using honey in oral rehydration solution in infants and children with gastroenteritis, honey shortens the duration of bacterial diarrhoea caused by *Salmonella* sp., *Shigella* sp., does not prolong the duration of non-bacterial diarrhoea and may be safe to be use as a substitute for glucose in an oral rehydration solution containing electrolytes.

Another helpful paper found in the review literature is 'Susceptibility of *Helicobacter pylori* to the antibacterial activity of manuka honey' (Somal et al., 1994). This study has shown that *Helicobacter pylori* the causative agent in many cases of dyspepsia and peptic ulcer can be inhibited by the antibacterial activity of honey at concentration that could be achieved in the stomach by reasonable oral dosage.

A study by Allen et al., (2001), found that honey has the potential to treat wounds infected with MRSA (Methicillin Resistant *Staphylococcus aureus* and VRE (Vancomycin Resistant *Enterococci*), both are the multiple resistant to antibiotics, the "superbugs". Since honey is of proven effectiveness in clearing wounds of infection with other bacteria and it gives many other benefits as a wound dressing material, there is good enough justification for trying it for the treatment of wounds infected with both resistant organisms.

Lacunae

So far, sufficient study was not carried out to determine the antibacterial activity of different brands of local honey.

OBJECTIVE OF THE STUDY

The objectives of this research are:

1. To determine the antibacterial activity of some selected brands of Malaysian honey.

2. To characterize the biochemical components of local honey.

So that, the local honey can stand with their own identity and standard.

MATERIALS AND METHODS

Materials:

Table 1: A different brands of Malaysian honey selected for this study

No.	Honey sample	Source
1.	'Tualang'	Purchased directly from Pok Nik madu, Kelantanese popular honey distributor.
2.	'Hutan'	
3.	'Gelang'	
4.	'Pucuk Daun'	Honey bee farm, Melaka.
5.	'Ee Feng Gu'	Honey bee farm, Cameron Highland.

Methods:

Physical characterization of different honeys

1. Appearance:

The different brands of honey have different color. Thick syrupy liquid of brownish to golden in color, translucent when fresh but frequently become opaque and granular through crystallization of dextrose.

2. Determination of pH:

Standard buffer solution:

pH 7.0 and pH 4.0 ready made buffers.

Procedure:

The electrode assembly of the pH meter was dipped into the standard buffer solution of pH 7.0 taken in a clear and dry beaker. The temperature correction knob was set to 28^0C and the fine adjustment was made by a symmetry potentially knob to pH 7.0. After wash the electrode assembly was then dipped into a solution of standard pH 4.0 and adjusted to the required pH by fine asymmetry potentially knob. The electrode assembly was raised, washed twice

with distilled water, rinsed off with honeys of the cultivars and then dipped into the honeys. The pH of the honeys was noted.

3. Determination of specific gravity:

The specific gravity of honeys was determined by Refractometer.

Procedure:

On the open daylight plate, one or two drops of the sample was placed on the prism surface. The daylight plate was closed gently so that the plate comes in contact with the prism.

The end of the daylight plate was pointed in the direction of a bright light, the eyepiece was rotated while looking through it until the image was correctly adjusted and the scale became cleanly visible.

4. Determination of moisture content:

Moisture content was determined by the conventional procedure.

Materials:

a. Porcelain container

b. Electrical balance

c. Oven

d. Dessicator

Procedure:

One (1) ml of honeys was weighed in porcelain container (which was previously cleaned and heated to about 100^0C, cooled and weighed). The container with the sample was heated in an electrical oven for about six hours at 100^0C. It was then cooled in a desiccator and weighed again.

Calculation:

Percent of moisture content (gm per 100 grams of honeys)

$$= \frac{\text{Weight of moisture obtained}}{\text{Weight of honey}} \times 100$$

Biochemical test

- Determination of lipid content of honey:

Lipid content of the different varieties of honey was determined by the method of Bligh and Dyer (1959).

Reagent:

A mixture of chloroform and ethanol (2:1 V/V).

Procedure:

One (1) gm of honey was first mixed with 10 ml of distilled water. The mixture was transferred to a separating funnel and 30 ml of chloroform-ethanol mixture was added. The mixture was mixed well. It was then kept overnight at room temperature in the dark. At the end of this period 20 ml of chloroform and 20 ml of water were further added and mixed. Generally three layers were seen. A clear lower of chloroform containing the lipid, a colored aqueous layer of layer of ethanol with all water soluble materials and a thick pasty interphase were observed.

The chloroform layer was carefully collected in a pre-weighed round bottom flask (50 ml) and then evaporated using rotary evaporator. After evaporation of the chloroform, the weight of the flask was determined again. The difference in weight provided the amount of the lipid.

Calculation:

Percent of lipid content (gm per 100 gm of honey)

$$= \frac{\text{Amount of lipid obtained}}{\text{Weight of honey}} \times 100$$

ANTIBACTERIAL SCREENING OF DIFFERENT BRANDS OF MALAYSIAN HONEY

Introduction:

Antibacterial screening was used to perform the primary selection of the compounds as therapeutic agent. In general, antibacterial screening was under taken in two phases described as follows:

1. Primary assay:

It is a qualitative assay to detect the presence or absence of the activity. The primary assay was performed *in vitro* by disc diffusion technique, Kirby-Bauer method (Fessia et al., 1988) or well diffusion method (Georgii and Korting, 1991). Disc diffusion techniques include the plate diffusion test and streak test.

The streak test permits the determination of the antibacterial effect of a test compound on several microorganisms simultaneously and is tensing suitable for the determination of the spectrum of the activity. But the plate diffusion test is commonly used.

2. Secondary assay:

It quantifies the relative potency such as minimum inhibitory concentration (MIC). The minimum concentration of antibacterial agent required to inhibit the growth of the organisms *in vivo* is referred to as minimum inhibitory concentration (MIC). It is done by serial dilution technique (Fessia et al., 1988)

Principle of disc/well diffusion method:

In the disc diffusion assay, the surface of a nutrient agar medium contained in a petri dish is uniformly inoculated with the test bacterial culture. The test solution of compounds are added to such a plate by pipette them either into circular holes cut into the agar or into previously applied glass or metal cylinders (well diffusion) or they are absorbed on to filter paper discs, which are put on the surface of the agar.

The test substances diffuse into the agar with decreasing concentration towards the periphery. In the case of positive reaction, an inhibitory zone can be observed after incubation for several hours where the concentration exceeds the MIC for that particular organism. The diameter of zone of inhibition is proportional to the logarithm of the concentration of the antibiotic. The diameter of zone of inhibition under constant experimental conditions depends on the following factors:

a. Thickness of the agar medium

b. Diffusion rate of the test compound

c. Inoculums size

d. Incubation time

e. Temperature of cultivation

f. Culture medium composition

g. Growth rate of the test organism.

Mechanism of disc/well diffusion technique:

A number of events occur simultaneously during this process:

Initially the dried disc absorbs water from the surrounding test medium and the drug becomes dissolved in it. The drug migrates through the adjacent test medium due to concentration gradient. This results in a gradual change of the drug concentration in the agar surrounding each disc.

Honey containing discs were pleased on the pre-seeded plates. The plates were kept at low temperature (4^0C) for 4 hours and then incubated at 37^0C for 12-16 hours in an incubator. A clear zone was observed where the drug was present higher than the inhibitory concentration.

Test materials used for the study:

1. Five different brands of Malaysian honeys:

- 'Tualang', 'Hutan', 'Gelang', 'Pucuk Daun' and 'Ee Feng Gu' honey.

2. Stock culture for the six pathogenic microorganisms.

Table 2: Standard Antibiotics (OXOID Ltd. England)

Antibiotics	Concentration
Amoxycillin	10 µg/disc
Chloramphenicol	30 µg/disc
Ceftazidime	30 µg/disc
Cefuroxime	30 µg/disc
Colistin	10 µg/disc
Gentamicin	10 µg/disc

Apparatus and reagents:

1. Blank filter paper discs (sterile)
2. Standard antibiotic disc
3. Plastic petri dishes (sterile)
4. Inoculating loop
5. Sterile universal bottles
6. Sterile forceps
7. Sterile swab
8. Micro-pipette
9. Bunsen burner
10. Methanol
11. Nutrient agar
12. Incubator (Memmert)
13. Refrigerator (National)
14. Autoclave (TOMMY model SS 325, Japan)
15. Incubator shaker (Innova 4080)
16. Laminar flow unit
17. Waterbath (Memmert).

Pathogenic test organisms used for the study:

Six pathogenic bacteria were selected for the test, four of them were gram negative and the rests were gram positive. Microorganisms selected for this study were collected from the Department of Medical Microbiology & Parasitology, School of Medical Sciences, Health Campus, USM.

Table 3: List of the pathogenic test organisms for this research:

No.	Gram negative bacteria	Strain number
1.	*Escherichia coli*	ATCC 25922
2.	*Salmonella typhi*	-
3.	*Shigella sonnei*	-
4.	*Pseudomonas aeruginosa*	ATCC 27853
	Gram positive bacteria	
5.	*Staphylococcus aureus*	ATCC 25923
6.	*Streptococcus pyogenes*	-

Preparation of media:

Luria Bertani (LB) broth:

10 gm Bacto-trypton (Pronadisa, CONDA, Madrid, Spain), 5 gm yeast extract (Pronadisa, CONDA, Madrid, Spain) and 10 gm Sodium chloride (MERCK, Germany) were dissolved in 800 ml of distilled water and pH was adjusted to 7.5 by pH meter) with 5N Sodium hydroxide solution. The volume was adjusted to 1000 ml with distilled water. The medium was sterilized by autoclaving at 121^0C for 15 minutes and stored at 4^0C.

Nutrient agar media (MERCK, Germany)

20 gm of nutrient agar powder was suspended in I liter of distilled water by heating in a boiling water bath or in a current of steam and autoclaved for 15 min at 121^0C.

Nutrient agar slant (MERCK, Germany)

The preparation was same as the blood agar plate but the medium was put in the sterile universal bottle and slant it. This agar slant purposely used for stock culture the grams negative organisms such as *E. coli, S. typhi, S. sonnei, P. aeruginosa* and *S. aureus.*

Nutrient broth (MERCK, Germany)

8 gm of the nutrient broth powder was suspended in 1 liter of distilled water. Warmed slightly if necessary to secure solution. Dispensed and sterilized by autoclaving at 121^0C for 15 minutes.

Blood agar (OXOID Ltd. England)

40 gm Blood agar Base was suspended in 1 Liter of distilled water. Bring to boil to dissolve completely. Mixed and sterilized by autoclaving at 121^0C for 15 minutes. Cooled to 45 - 50^0C and 7% sterile blood was added. Reconstitution and mixing was performed in a flask at least 2.5 times the volume of medium to ensure adequate aeration of the blood.

Blood agar slant

The preparation was same as the blood agar plate but the medium was kept in the sterile universal bottle and slant. This agar slant purposely used for stock the fastidious organisms such as *S. pyogenes* (*Streptococcus* group A).

Mac Conkey's Agar (OXOID Ltd. England)

51.5 gm of the Mac Conkey's powder was suspended in 1 liter of distilled water. Boiled to dissolve the medium completely. Sterilized by autoclaving at 121^{0}C for 25 minutes.

Muller Hinton Agar (OXOID Ltd. England)

38 gm of the Muller Hinton agar powder was suspend in 1 liter of distilled water. Boiled to dissolve the medium completely. Sterilized by autoclaving at 121^{0}C for 25 minutes.

Muller Hinton Blood Agar (OXOID Ltd. England)

38 gm of the Muller Hinton agar powder was suspend in 1 liter of distilled water. Boiled to dissolve the medium completely. Sterilized by autoclaving at 121^{0}C for 25 minutes. Cooled to 45 - 50^{0}C and 7% sterile blood was added. Reconstitution and mixing was performed in a flask at least 2.5 times the volume of medium to ensure adequate aeration of the blood.

Todd Hewitt broth (OXOID Ltd. England)

36.4 gm of Todd Hewitt powder was dissolved in 1 liter of distilled water. Mixed well, distributed into containers and sterilized by autoclaved at 115^{0}C for 10 minutes.

PRIMARY ASSAY:

Preparation of the fresh culture of the pathogenic bacteria:

1. *E. coli, S. typhi, S. sonnei, P. aeruginosa and S. aureus* was subcultured on Nutrient agar. *S. pyogenes* was subcultured on Blood agar and all the plates was incubated overnight at 37^0C aerobically.

2. The sterile LB broth was dispensed 5 ml each in the sterile universal bottles.

3. Finally, using sterile wire loop one colony was taken of each different pure strain of the test organisms and inoculated into each universal bottle and this process carried out under the laminar flow unit. All the inoculated universal bottles were placed on incubator shaker at 37^0C, 200 rpm for overnight (see Appendix, Figure A).

Preparation of the test plates:

1. Readymade plastic petri dishes were labelled properly.

2. 10 µl of inoculums (test organism) was take from shaker and mixed with 80 µl of sterile LB broth. This preparation was equal with Mc Farland 0.5 standard.

3. Then, 100 µl of inoculums was taken and put into each petri dishes. Using sterile swab, streaking technique was followed to ensure uniform dispersion of the organism on the surfaces of agar. Step 2 and 3 were done in the laminar flow unit. Thus the plates were ready for sensitivity test (see Appendix, Figure B)

Dilution of honey for primary assay:

For this step, the neat honey was diluted with sterile distilled water at different dilutions:

- 20%, 40%, 60% and 80%.