PHYSICOCHEMICAL PROPERTIES AND *IN-VITRO* INHIBITORY EFFECTS OF STINGLESS BEE (*Trigona* spp.) HONEY AGAINST *Escherichia coli*

By

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ABSTRACT

PHYSICOCHEMICAL PROPERTIES AND *IN-VITRO* INHIBITORY EFFECTS OF STINGLESS BEE (*Trigona* spp.) HONEY AGAINST *Escherichia coli*

Jocelyn Goh Shi Jing

The re-evaluation of therapeutic use of honey is due to the emergence of multi-drug resistant bacteria nowadays. Hence, the aims of this study were to evaluate as well as to compare the physicochemical properties and *in-vitro* antibacterial activities of stingless bee honey among different Trigona species and processing time. In this study, the honey samples tested against *Escherichia coli* (ATCC 25922 and ATCC 35218) were *T. itama* honey which processed at 41˚C for 8 hours, *T. itama* honey which processed at 41˚C for 15 to 20 hours and *T. apicalis* honey which processed at 41˚C for 15 to 20 hours. From agar well-diffusion assay, undiluted Trigona honey samples were shown to exhibit the greatest inhibitory effect with the largest zone of inhibition. Endotoxin assay revealed that the bactericidal effect of honey was time dependent whereby the endotoxin levels in 24-hour samples were significantly higher than 0-hour samples. The morphological changes also were observed on *E. coli* after treated with Trigona honey under scanning electron microscope. Based on the physicochemical analyses, *T. apicalis* honey (15-20 hrs, 41˚C) was shown to have significantly higher total phenolic content than *T. itama* honey (15-20 hrs, 41˚C). Meanwhile, *T. itama* honey (15-20 hrs, 41˚C) had significantly higher total sugar content and significantly lower moisture content as compared to other honey samples. However, no significant

difference was observed in hydrogen peroxide level and pH value between honey samples. In short, present findings demonstrated that *T. apicalis* honey exhibited the most potent antibacterial effect that contributed by its physicochemical properties such as higher acidity, total phenolic content and hydrogen peroxide level. Besides, longer processing time was suggested to affect the antibacterial effectiveness of honey by reducing the acidity and hydrogen peroxide level.

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Lastly, grateful acknowledgement is expressed to my beloved family members and friends for their love, motivation and undying support which push me farther than what I expect to go.

DECLARATION

I hereby declare that the project report is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

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JOCELYN GOH SHI JING

APPROVAL SHEET

This project report entitled "**PHYSICOCHEMICAL PROPERTIES AND *IN-VITRO* INHIBITORY EFFECTS OF STINGLESS BEE (*Trigona* spp.) HONEY AGAINST *Escherichia coli***" was prepared by JOCELYN GOH SHI JING and submitted as partial fulfilment of the requirement for the degree of Bachelor of Science (Hons) Biomedical Science at Universiti Tunku Abdul Rahman.

Approved by:

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(Mr. Ng Wen Jie) Date: 1st September 2016

Supervisor

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PERMISSION SHEET

It is hereby certified that **JOCELYN GOH SHI JING** (ID No: **13ADB00054**) has completed this final year project entitled "**PHYSICOCHEMICAL PROPERTIES AND *IN-VITRO* INHIBITORY EFFECTS OF STINGLESS BEE (*Trigona* spp.) HONEY AGAINST *Escherichia coli***" under the supervision of Mr. Ng Wen Jie from the Department of Biomedical Science, Faculty of Science, and Dr. Ee Kah Yaw from the Department of Agricultural and Food Science, Faculty of Science.

I hereby give permission to the University to upload the softcopy of my final year project in pdf format into the UTAR Institutional Repository, which may be accessible to the UTAR community and public.

Yours truly,

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(JOCELYN GOH SHI JING)

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
CFU/ml	Colony-forming unit per milliliter
˚C	Degree celcius
dH_2O	Distilled water
et al.	*Et alii*
GAE	Gallic acid equivalent
g	Gram
g/100 g	Gram per hundred gram
g/ml	Gram per milliter
HCl	Hydrochloric acid
H_2O_2	Hydrogen peroxide
HMF	Hydroxymethylfurfural
Kg	Kilogram
L	Liter
µg	Microgram
µg/ml	Microgram per milliter
µl	Microliter
µmol/L	Micromole per liter
mg	Milligram
ml	Milliliter
mm	Millimeter
MHA	Mueller Hinton agar
Na_2CO_3	Sodium carbonate

NaCl	Sodium chloride
NaOH	Sodium hydroxide
OD	Optical density
O_2	Oxygen
%	Percentage
PBS	Phosphate buffered solution
±	Plus minus
pH	Power of hydrogen
SEM	Scanning electron microscope
spp.	Species
v/v	Volume per volume
w/v	Weight per volume

CHAPTER 1

INTRODUCTION

Therapy with bee products which is also known as apitherapy has been practiced since ancient times. Honey has been used as a traditional medicine in healing wounds as well as to treat cardiovascular, liver and gastrointestinal diseases (Al-Jabri, 2005; Ezz El-Arab, et al., 2006). However, with the discovery and the advent of antibiotics, the apitherapy faded into the recesses of history. Along with the increasing reports of bacterial resistance towards the antibiotics, there has been a resurgence of interest in the clinical application of honey (Levy and Marshall, 2004).

Despite being used as a remedy since a long time ago, the prophylactic medicinal value of honey has been revealed due to its antibacterial activity only in a century back (Molan, 2001). The antibacterial activities of honey are highly dependent on its unique physicochemical properties. Natural honey contains several chemical compounds that contribute to its antibacterial activities, for instance hydrogen peroxide and phytochemicals. Hydrogen peroxide was recognized as the main antibacterial component in honey in the 1960s (Adcock, 1962; White, Subers and Schepartz, 1963; Molan, 1992). Most honey was found to be peroxide dependent which can inhibit bacteria colonization and indirectly preventing the infectious disease. Conversely, non-peroxide constituents such as phytochemical factors including phenolic compounds also play an important role in antibacterial actions of honey (Molan, 2001).

Aside from that, the antibacterial activities of honey are tightly linked to the acidity and osmotic effect of the honey. Acidity of honey is associated with the presence of gluconic acid formed from the glucose oxidase reaction which responsible to inhibit the growth of pathogens. On the other hand, osmotic effect of honey is attributable to the high sugar content and low moisture content of honey. Owing to the high osmolarity of honey, water is drawn away from microbes thereby halting their growth (Molan, 2001).

Bacteria tend to develop resistance towards antibiotics as the drugs are engineered to have a specific effect on the bacteria as for example, degrading their cell wall; but once the bacteria mutate their way around the effect, the effectiveness of drug will be reduced or eliminated (Eaton, 2014). *Escherichia coli* is known to be one of the bacteria that develop resistance towards several antibiotics by producing extended spectrum β-lactamase (Public Health England, 2014). Unlike antibiotics, honey can inhibit the growth of bacteria on multiple levels, making it more difficult for bacteria to develop resistance (American Chemical Society, 2014).

Other than honeybee, stingless bee is another group of bees that able to produce considerable amount of honey. The antibacterial activities and physicochemical properties of stingless bee honey are rather different from the honeybee honey. Besides, stingless bee honey is considered in folk medicine to be more effective than honeybee honey in treating common diseases (Garedew, Schmolz and Lamprecht, 2003). However, Malaysian stingless bee

honey that originated from *Trigona* spp. stingless bee is still not well studied as compared to honeybee honey.

Hence, the objectives of this study were:

1. To evaluate the *in-vitro* antibacterial activities of stingless bee honey against *Escherichia coli*.
2. To determine the physicochemical properties of stingless bee honey.
3. To compare the antibacterial activities and physicochemical properties of stingless bee honey among different species and processing time.

CHAPTER 2

LITERATURE REVIEW

2.1 Honey

2.1.1 Production of Honey

Honey is a natural sweet substance which is derived from floral nectar and subsequently processed by the bees. The foraging worker bees first ingest the nectar and store it in their honey sacs within their digestive systems to be carried back to their hive. Meanwhile, the foragers will secrete specific substances such as enzymes from their glands which are then mixed with the nectar in their honey sacs. After that, the foragers will regurgitate the content from their honey sac and pass to the housebees in the hive through trophallaxis. Upon receiving the nectar, more enzymes will be secreted by the housebees and added into the nectar before placing it into the honeycomb cells for ripening into honey. Once the nectar turns into honey, the honeycomb cells will be capped with bee wax (Stone, 2005; Tsutsumi and Oishi, 2010).

Bees usually produce more honey than their colonies need, thus, beekeepers can remove the excess honey for commercialization. Firstly, the beekeepers will collect the honeycomb frames and scrape off the wax cap often with a heated knife. Next, the honeycomb will be placed into an extractor and subjected to centrifugal force to spin the honey out from the comb. Later, the honey can be collected at the bottom due to gravitational pulls. Thereafter, the honey is strained to remove big particles and the remaining bee wax. Some beekeepers will carry out additional heat treatment in order to hasten the

straining process and simultaneously pasteurize the honey. During pasteurization, honey is first heated to 77˚C and then cooled to 54˚C. For large commercial operations, pasteurization is usually performed with the purpose of preventing fermentation and crystallization of honey. However, this process will cause the honey to lose some of its beneficial health and medicinal properties due to alteration of the chemical composition during heating (Tsutsumi and Oishi, 2010; National Honey Board, 2014). Subsequently, dehydration of honey is usually carried out to reduce the moisture content of honey to a certain level. This is also to prevent fermentation thereby extending the shelf life of honey (Gill, et al., 2015).

2.1.2 Composition of Honey

Honey is usually consumed by people for its high nutritional values in addition to its beneficial health promoting effects. It has been reported to contain at least 200 substances and most of them are essential in our diet (Tornuk, et al., 2013; Escuredo, et al., 2014). Sugar is the major constituent in honey and it accounts for 95-99% of honey dry matter. Two main types of sugar that can be found in honey are fructose and glucose which represent 85-95% of total sugar. Other sugars such as maltose, sucrose, pannose, melezitose, etc are only present in a small amount (Oskouei and Najafi, 2013). The sugars which are present in the honey are responsible for some qualities including energy value, viscosity, hygroscopicity as well as crystallization (Kamal and Klein, 2011).

On the other hand, water is the second main component in honey whereby it constitutes approximately 17% of the honey. The water content of honey is

inversely proportionate to the total sugar content of honey. Furthermore, about 0.57% of honey is made up of organic acids which are derived from sugars in the honey. These organic acids contribute largely to the acidity of honey and hence, honey usually has low pH (Oskouei and Najafi, 2013). The predominant acid in honey is gluconic acid which is formed together with hydrogen peroxide through glucose oxidation catalyzed by glucose oxidase (Karabagias, et al., 2014). Other than glucose oxidase, other enzymes like invertase, amylase, catalase, etc are also important in the formation of honey (Oskouei and Najafi, 2013).

Apart from that, honey also contains mineral compounds such as potassium, calcium, magnesium, sodium, sulphur, phosphorus, iron, copper, zinc and manganese in small quantities (0.1% to 1.0%) (Oskouei and Najafi, 2013). These compounds are important for the fundamental function in biological system including maintaining normal physiological response as well as inducing overall metabolism (Alqarni, Owayss and Mahmoud, 2012). Moreover, vitamin B and vitamin C are also can be found in honey in a minute amount (Oskouei and Najafi, 2013). On top of that, honey contains phenolic compounds which include both flavanoids (flavones, flavanols, anthocyanidin, chalcones) and non-flavanoids (phenolic acid). These phenolic compounds play a significant role in antioxidant activity in addition to antimicrobial activity (Andersen and Markham, 2006).

2.1.3 Medical Uses of Honey

The usage of honey can be dated back to at least 8000 years ago, as evidenced by the Stone Age paintings. Besides serving as a food or sweetener, honey was prescribed by the physicians of many ancient races to treat various ailments. According to ancient India ayuruvedic medicine, honey was used for the treatment of eye diseases, cough, vomiting, diarrhea and healing wounds. Moreover, honey was also used as an agonist whereby it was mixed with some medicines to enhance their effects. On the other hand, honey was known as the most popular drug in ancient Egypt. Most of their medicine contained honey in addition to wine and milk. Honey could be used for wound healing as written in Smith papyrus (an ancient Egyptian medical text). In ancient Greece, honey was prescribed to treat acute fevers, baldness, sore throat, eye diseases as well as prevention and treatment of scars (Oskouei and Najafi, 2013; Bogdanov, 2016).

For the past few decades, many studies have been carried out mainly based on the antibacterial activity of honey that was first reported in 1892 by van Ketal (Dustmann, 1979). According to the research done by Meda and colleagues, honey can be used to treat urinary tract infections (UTI) as some bacteria such as *Escherichia coli*, *Proteus* species and *Enterococcus faecalis* were found susceptible to the antibacterial activity of honey (Meda, et al., 2004). Other studies showed that honey can be used in the treatment for gastrointestinal diseases such as diarrhea and gastroenteritis which caused by bacteria. In the case of bacterial gastroenteritis, the duration of diarrhea was claimed to be

shorten when the patients were subjected to replacement fluid that contain honey at 5% (v/v) concentration instead of sugar (Haffejee and Moosa, 1985).

Aside from that, honey has been studied for its wound healing properties. It has been revealed that various types of wounds such as abscess, amputation, ulcers, burns etc are found to be responsive to honey therapy. The effectiveness of honey in wound healing can be demonstrated in a case of knee amputation of a young boy. In this case, it has been reported that the lesion which was seriously infected by *Pseudomonas aeruginosa* and *Staphylococcus aureus* was successfully healed in just 10 weeks after the application of the dressing pads with sterilized Manuka honey (Dunford, Cooper and Molan, 2000). Additionally, there was also a study of the honey effect on Fournier's gangrene which is a rapidly spreading infection. Honey has been reported to cause speedy recovery in the wound and eventually lead to a decrease in mortality (Gurdal, et al., 2003). According to some researchers, the effect of honey on wound healing is mainly due to its antibacterial effect as well as the ability to activate immune response against infection by stimulating the release of inflammatory cytokines from monocytic cells (Tonks, et al., 2001; Sampath, et al., 2010). Nowadays, there are several medical-grade honey products available in the market as shown in Figure 2.1.

Figure 2.1: Medical-grade honey products from Medihoney™ (Norgesplaster, n.d.).

On top of that, honey has been reported to have antifungal actions. There were studies which claimed that pure honey was able to inhibit the growth of fungi, for example species of *Aspergillus* and *Penicillium* as well as their toxin production (Brady, Molan and Harfoot, 1997; Sampath, et al., 2010). According to Obaseiki-Ebor and Afonya (1984), candidiasis which caused by *Candida albicans* can be treated by honey as the fungus is susceptible to honey. Other than antibacterial and antifungal effects, natural honey was also tested to have antiviral effects as well. Based on Al-Waili (2004), topical honey application was shown to be safer and more effective in managing the recurrent lesions from labial and genital herpes as compared to acyclovir cream. In addition, it has been reported that honey was also able to inhibit rubella virus activity according to Al-Waili (2004).

2.2 Stingless Bees

2.2.1 Taxonomy and Distribution

Like honeybees, stingless bees belong to the order Hymenoptera under family Apidae and subfamily Apinae. However, stingless bees differ from honeybees wherein they are categorized in tribe Meliponini. The tribe Meliponini can be further divided into two main genera which are *Trigona*, the largest group and *Melipona* (Rahman, et al., 2015).

Stingless bees are widely distributed throughout tropical and subtropical regions such as Central and South America, Africa, Asia and northern Australia (Boorn, et al., 2010). To date, there are more than 500 described species in 32 genera but more new species of stingless bee are estimated to be identified every year (Michener, 2013). Specifically for Malaysia, as shown in Figure 2.2, 32 species have been recorded currently and most of them belong to *Trigona* spp. according to the study done by Norowi and colleagues (2010). On the other hand, it has been indicated that meliponiculture or commonly known as stingless bee farming which involved *Trigona* spp. is gaining popularity in Malaysia principally due to their role in pollination as well as producing substantial amount of honey with high medicinal values. According to Kelly and colleagues (2014), *Trigona itama* and *Trigona thoracica* are the two most important species which are in managed.

Number	Species	(Schwarz, 1939)	Specimens in MARDI insect Museum	(Osawa and Tsubaki, 2003)
1	*Trigona itama*	X	X	
2	*T. erythrogastra*	X	X	
3	*T. canifrons*	X	X	X
4	*T. fimbriata*	X	X	X
5	*T. thoracica*	X	X	
6	*T. fuscobalteata*	X	X	
7	*T. iridipennis*	X	X	
8	*T. geissleri*	X	X	X
9	*T. atripes*	X	X	X
10	*T. atripes var collina*	X	X	
11	*T. atripes var fuscibasis*	X	X	
12	*T. apicalis var smith*	X	X	
13	*T. apicalis var melanoleuca*	X	X	
14	*T. apicalis var peninsularis*	X	X	X
15	*T. scintillans*	X	X	
16	*T. pendleburyi*	X	X	
17	*T. nitiventris*	X		
18	*T. ventralis*	X	X	
19	*T. terminata var smith*	X	X	
20	*T. terminata var latabalteata*	X		
21	*T. minor sakagami*	X	X	
22	*T. rufibasalia*	X	X	
23	*T. moorei schwarz*	X	X	
24	*T. pagdeniformis*		X	X
25	*T. minangkabau*		X	X
26	*T. leeviceps*		X	X
27	*T. nitidirentris*			X
28	*T. reepeni*		X	
29	*T. pagdeni*		X	
30	*T. melina*		X	
31	*T. nitidiventris*		X	
32	*T. klossi*		X	

Figure 2.2: Species of stingless bees recorded in Malaysia (Norowi, et al., 2010).

2.2.2 Characteristics and Features

Stingless bees are highly evolved eusocial insects and live in perennial colonies which composed of few hundreds to several thousands of bees. The colony is indicated to have an organized system of division of labour (Rahman, et al., 2015). As compared to honeybees, stingless bees generally are smaller in size, ranges from 2 mm to 14 mm. However, the largest stingless bee is comparable to a honeybee (Sommeijer, et al., 2003). The morphology of

stingless bees is rather different from honeybees. Firstly, stingless bees do not possess functional sting (Figure 2.3) as the name implied and hence, they do not sting. However, they have strong mandibular musculature which allows them to attack intruders by inflicting mild bite. Some of the stingless bees may emit caustic liquid from their mouth and cause intense skin irritation. Secondly, the wax glands of the stingless bees are located dorsally whereas honeybees have ventral position of the wax glands. Thirdly, stingless bees are known to have reduced wing venation which may in part relate to the dwarfism (Sommeijer, et al., 2003; Hermani, 2012; Rahman, et al., 2015).

(a) (b)

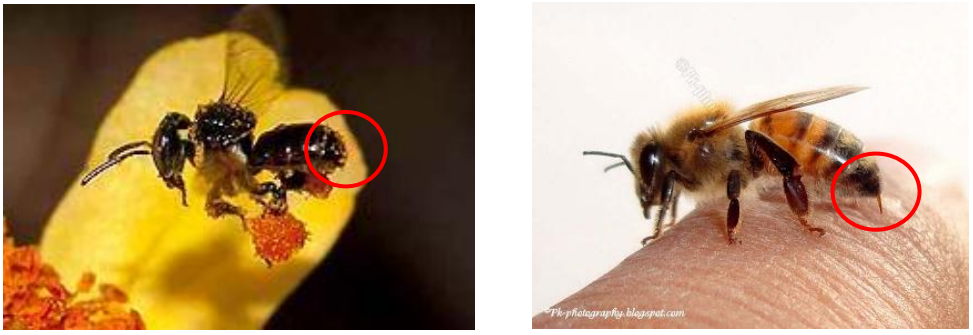

Figure 2.3: Comparison of sting between (a) stingless bee (Aussiebee, 2012) and (b) honeybee (Birdy Official, n.d.).

Most of the stingless bees build their nests in cavities such as hollow trunks, underground cavities or wall cavities. However, there are a few species that build their nests in exposed positions (Sommeijer, et al., 2003). The nests of stingless bees are usually made of wax which is secreted by the metasomal terga of the bees plus the resin and gums collected by them. Some species may add mud, vegetative material, faeces and other materials for certain part of the construct as well (Micherner, 2007). Inside the stingless bees nest, the brood combs and the food storage are clearly separated. Unlike honeybees which

always build vertical hanging wax combs, the brood cells of stingless bees can be arranged in horizontal combs (Figure 2.4) or may be built in clusters depending on the species. The compact stack of brood combs is surrounded by involucrum which is made up of waxy sheets. The main function of this involucrum is to insulate and protect the brood cells (Jurenka, 2015). On the other hand, the storage pots (Figure 2.4) which store the pollen and honey are built outside the involucrum. The height of these storage pots can be ranged from 5 to 40 mm high (Bradbear, 2009). Stingless bee nest is often sealed off from the rest of cavities by batumen plates and connected to the outside via a resinous entrance tube as shown in Figure 2.5 (Jurenka, 2015).

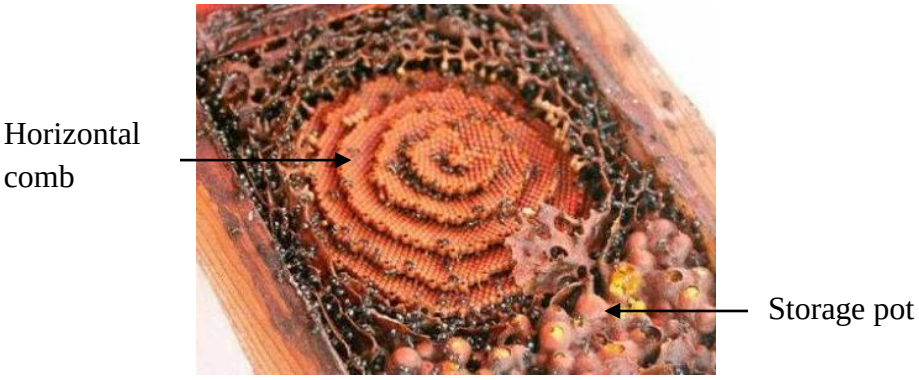

Figure 2.4: Hive of stingless bee (Adoptabeehive, 2014).

Figure 2.5: Entrance tube of the stingless bee nest (Jurenka, 2015).

2.3 Stingless Bee Honey

2.3.1 Antibacterial Activities

Based on Ewnetu, Lemma and Birhane (2013), stingless bee honey such as Tazma honey produced a larger zone of inhibition on both *Escherichia coli* and *Staphylococcus aureus* in a well-diffusion assay as compared to *Apis mellifera* honey. Besides, they also showed that Tazma honey can inhibit higher percentage of test microorganisms than *Apis mellifera* honey. By having the same concentration, Tazma honey was able to inhibit 80% of a particular test organism whereas *Apis mellifera* honey was only able to inhibit 40% of it.

A study by Andualem (2014) from Ethiopia also showed that the inhibitory effects of Trigona honey (stingless bee honey) against pathogenic bacteria were significantly greater than honeybee honey as well as artificial honey. In this study, it has been reported that Trigona honey had the greatest zone of inhibition among the three types of honey. Moreover, Trigona honey was reported to have the lowest minimum inhibitory concentration (MIC) value against the pathogenic bacteria for example *Listeria monocytogenes*, *Escherichia coli*, *Staphylococcus aureus*, *Shigella flexneri*, *Proteus vulgaris*, *Streptococcus pneumoniae, Shigella dysenterae* and *Salmonella* spp.. Most of these pathogens are usually associated with pneumonia, gastroenteritis, urinary tract and wound infections (Andualem, 2014).

According to Chanchao (2009), the antibacterial activity of *Trigona laeviceps* (stingless bee) honey was reported to be dose-dependent as observed from the

well-diffusion assay whereby the diameter of inhibition zone increased in higher concentrations of the honey. On the other hand, Boorn, et al. (2010) reported that the antibacterial activity of stingless bee honey was time-dependent as well. By using the time-kill assay, it has been indicated that there was a significant decrease in the viability of bacteria such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* from zero hour to one hour after the treatment of bacteria with honey.

2.3.2 Physicochemical Properties

The antibacterial activity of honey is highly related to the physicochemical properties of honey such as acidity, moisture content, total sugar content, total phenolic content as well as hydrogen peroxide level. These properties may be varied among different honeys due to the differences in the honey composition which is highly dependent on the types of flower, geographical regions, climate, bee species, processing and storage time. According to many researchers, the physicochemical properties of stingless bee honey are quite different from the honeybee honey (Garedew, Schmolz and Lamprecht, 2003; Chuttong, et al., 2015).

2.3.2.1 Acidity

Generally, honey is acidic due to the presence of organic acids, particularly gluconic acid that is formed from glucose oxidase reaction. The pH value of honey ranges from 3.2 to 4.5 which is low enough to inhibit the growth of most bacteria as their optimal growth pH lies between 7.2 and 7.4 (Vandamme, et al., 2013). Based on Garedew, Schmolz and Lamprecht (2003), stingless bee

honey including Trigona honey is more acidic and with strong sour taste as compared to honeybee honey. Besides contributing to antibacterial activity, acidity of Trigona honey also contributes to wound healing by lowering the wound pH which later resulting in reduce of protease activity, increase fibroblast activity and increase oxygen release (Gethin, Cowman and Conroy, 2008).

2.3.2.2 Moisture Content and Total Sugar Content

The moisture content of honey is usually measured in order to determine the amount of water that present as it is an important parameter to indicate the quality of honey. The moisture content of honey is normally low and should be maintained within the limit (≤ 20%) as recommended by the international quality regulations (Khalil, et al., 2012). If not, fermentation by osmotolerant yeast may occur which will deteriorate the quality of honey. The fermented honey may taste sour due to the formation of acetic acid when the ethyl alcohol is oxidized in the presence of oxygen (Chirife, Zamora and Motto, 2006). Moisture content of honey is largely dependent on botanical origin of the honey, the level of maturity achieved in the hive, processing techniques and storage conditions (Silva, et al., 2016).

Moisture content of honey is associated to the total sugar content of honey. Low moisture content and high total sugar content make honey a potential antibacterial agent. Both of these factors result in hyperosmolarity in the honey which is able to prevent the growth of microbes. As water is important

for most of the bacteria to survive, withdrawal of water due to the osmotic effect can lead to the death of bacteria (Molan, 1992).

However, stingless bee honey is often characterized by its higher moisture content with an average of 31% (Roubik, 1983). This may be linked to the humid tropical environment where it is more difficult for the stingless bees to extract water from the nectar. Although stingless bee honey has higher moisture content, this honey has been shown to have similar shelf life as the well-dehydrated honeybee honey. It was hypothesized that the honey contains enzymes and other substances which were added by stingless bees during the processing of nectar into honey that result in the antibiotic and preservative activity (Bijlsma, et al., 2006).

2.3.2.3 Total Phenolic Content

Phenolic compounds are the secondary metabolites of plants and mainly synthesized for protection against oxidative damage as well as bacterial aggression (Nitiema, et al., 2012). The phytochemicals are transferred to honey via the nectar that the bees collect. Basically, these compounds contain an aromatic ring with one or more hydroxyl groups as they derived from phenylalanine and tyrosine (Costa, et al., 2015).

Based on several studies, phenolic compounds have been reported to exhibit antibacterial activity. In a study conducted by Borges, et al. (2013), phenolic acids such as gallic acid and ferulic acid have been indicated to cause disruption of cell membrane for both Gram-positive and Gram-negative

bacteria through several mechanisms. These compounds can cause hydrophobicity changes on cell surface, decrease of negative surface charge and formation of pores in cell membrane which eventually lead to the leakage of cytoplasmic content.

On top of that, phenolic compounds in honey are also known to possess antioxidant activity. They may function as antioxidants by carrying out free radical scavenging and hydrogen-donation activities (Costa, et al., 2015). Furthermore, phenolic compounds such as flavanoid can become a substrate for radicals whereby it is oxidized by the radicals to form a more stable form of radicals (Kucuk, et al., 2007).

In Malaysia, stingless bee honey (Trigona honey) has been reported to have higher total phenolic content than the *Apis* spp. honeys such as Tualang, Gelam, Pineapple and Borneo honey (Kek, et al., 2014). This property may contribute largely to the antibacterial as well as the antioxidant activities of stingless bee honey.

2.3.2.4 Hydrogen Peroxide Level

Based on a research carried out by Garedew and colleagues (2003), stingless bee honey was shown to possess stronger total antibacterial activities against some bacterial species than non-peroxide honey (honey after treated with catalase to remove hydrogen peroxide). This indicates that hydrogen peroxide is one of the predominant factors that contribute to the antibacterial activities of stingless bee honey.

In honey, hydrogen peroxide (H_2O_2) is produced along with gluconic acid by the conversion of glucose which is catalyzed by glucose oxidase under aerobic condition (Tao, et al., 2009). The chemical reaction is demonstrated as the equations below:

$$\text{Glucose} + O_2 \longrightarrow \text{glucono-}\delta\text{-lactone} + H_2O_2$$

$$\text{Glucono-}\delta\text{-lactone} + H_2O \longrightarrow \text{gluconic acid} + H_2O_2$$

The presumed function of hydrogen peroxide is to prevent the spoilage of unripe honey by microbes when the concentration of sugar is still very low. During the ripening of honey, the production of hydrogen peroxide will be at minimum or not at all as the glucose oxidase is inactivated at this stage. However, this enzyme will regain activity on a moderate dilution of honey (30-50%) (Kwakman and Zaat, 2012).

According to Brudzynski (2006), there is a significant correlation between the level of hydrogen peroxide in honey and the inhibitory degree on bacterial growth. In this study, it was observed that the bacteria were unable to respond normally to the proliferative signals and their growth is inhibited when exposed to honey that contains high level of hydrogen peroxide. Moreover, they also proved that hydrogen peroxide can lead to bacterial DNA degradation. However, the researchers showed that the extent of effect of hydrogen peroxide in honey was strongly influenced by the bacterial sensitivity to oxidative stress. *E. coli* is one of the bacteria that was proven to be sensitive to the oxidative action of hydrogen peroxide in honey.

2.4 *Escherichia coli*

2.4.1 Infections

Basically, *E. coli* can be divided into commensal and pathogenic types. The commensal strains are referring to the normal flora that exists in a symbiotic state and provides resistance against pathogenic microbes. On the other hand, the pathogenic *E. coli* is referring to the strains that can cause diseases. Three major infections which can be caused by pathogenic *E. coli* are diarrhoeagenic disease, urinary tract infection and meningitis (Katouli, 2010).

E. coli that causes diarrhoeagenic disease is also known as diarrhoeagenic *E. coli*. These *E. coli* strains can be further divided into six pathotypes which are enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), diffusely adherent *E. coli* (DAEC) and lastly shiga toxin-producing *E. coli* (STEC) which usually cause outbreak (Centers for Disease Control and Prevention, 2015).

Uropathogenic *E. coli* (UPEC) is the *E. coli* strain that causes urinary tract infection (UTI) which have certain virulence genes that differentiate them from other strains. These strains are usually originated from the feces and cause UTI by first entering the urinary tract through colonization of vaginal introitus and the periuretheral area. They may then ascend towards the bladder and cause cystitis or further ascend to the kidney and cause pyelonephritis which eventually leads to kidney failure and death if no treatment is given (Katouli, 2010).

Pathogenic *E. coli* will also cause meningitis particularly in neonates as well as immunosuppressive children and adults. Unlike other *E. coli* strains, meningitis-associated *E. coli* have acquired the virulence genes such as K1 capsule polysaccharide and S-frimbriae that enable them to cross the blood brain barrier. It is also indicated that the K1 capsule can be used to protect the bacterium from being phagocytosed (Katouli, 2010).

2.4.2 Extended Spectrum Beta-lactamase (ESBL)

Resistance of pathogenic organisms to multiple drugs especially antibiotics has posted a challenge to the treatment of infectious diseases. This phenomenon is primarily due to the misuse of antibiotics (Shaikh, et al., 2015).

Beta-lactam antibacterial agent is the most common drug in which the bacteria develop resistance to as the antibiotic is often used to treat bacterial infections. Persistent exposure of bacteria to large number of beta-lactams will induce the constant production and mutation of β-lactamases in these bacteria, leading to the formation of extended-spectrum beta lactamases (ESBLs). These ESBL-producing bacteria are usually resistant to multiple β-lactam antibiotics and even the newly developed ones. (Paterson and Bonomo, 2005; Pitout and Laupland, 2008).

E. coli is one of the common bacterium that possesses ESBLs. It has been reported that in many parts of the world, 10-40% of *E. coli* strains express ESBLs and expected to increase as time goes by (Rupp and Fey, 2003). The

types of ESBL that can be found in *E. coli* are TEM, CTX and OXA and each of them acts on different beta-lactam antibiotics (Shaikh, et al., 2015).

2.4.3 Endotoxin

Basically, the Gram-negative bacterial cell wall is made up of inner membrane, peptidoglycan and outer membrane. Endotoxin (also termed as lipopolysaccharide) is found on the outer membrane of Gram-negative bacteria and comprised of O-antigen, core oligosaccharide and lipid A as shown in Figure 2.6 (Totora, Funke, and Case, 2010). Normally, the O-antigen and core oligosaccharide are not vital for the survival of bacteria, however, they play a significant role in providing bacterial resistance against various antimicrobial agents including detergents and the complement membrane attack complex of the host (Vaara, 1993). On the other hand, the hydrogen-bond donors and acceptors in the lipid A molecule aid in maintaining the integrity of bacterial outer membrane by preventing the additional lateral interactions between phospholipids. In addition, by having six to seven saturated acyl chains, lipid A also serves to reduce the fluidity of outer membrane. As a result, the tight lateral interactions between endotoxin plus the low membrane fluidity provide a permeability barrier in the outer membrane that only allows the passage of low molecular weight and hydrophilic molecules. This is essential for the bacteria in order to prevent the penetration of toxic molecules into the cells. Lipid A has been recognized as the target for most of the antibiotics and anti-inflammatory agents because it is both important for bacterial survival and a potent inflammatory mediator (Russell and Herwald, 2005).

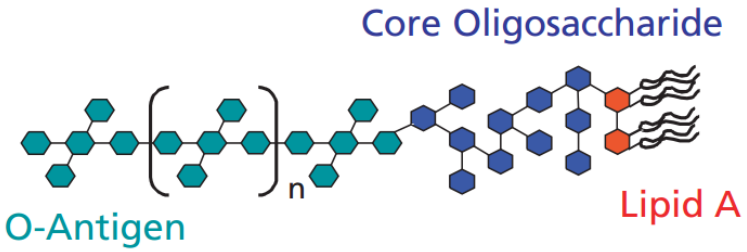

Figure 2.6: Schematic of bacterial endotoxin (Cutter and Goodarzi, 2016).

Endotoxin of Gram-negative bacteria will be released either from disruption and death of the bacterial cells, or during vigorous growth of the bacteria. The presence of low amount of endotoxin in the body will induce local inflammation due to the body immune response against the bacteria. However, if the infection persists or due to the release of high amount of endotoxin which is often associated with the antibiotic treatment, the massive host response will subsequently cause diffuse endothelial injury, tissue hypoperfusion, disseminated intravascular coagulation (DIC) and refractory shock. Patient who suffers from this will usually have fever and hypotension and may die in a more severe state (Prins, et al., 1994; Opal, 2010).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Honey Samples

Three Trigona honey samples were obtained from a bee farm which is located at Bahau, Negeri Sembilan. Two of the honey samples were originated from the same stingless bee species namely *Trigona itama*. One of the *Trigona itama* honey samples was treated at 41˚C for 8 hours while the other was processed for 15 to 20 hours at the same temperature. Another honey sample that originated from *Trigona apicalis* was also treated at 41˚C for 15 to 20 hours. All the processed honey samples were stored in dark at room temperature prior analyses as the peroxide activity of honey is sensitive to heat and light (Bogdanov, 1997).

3.1.2 Bacterial Samples

The selected bacterial samples as test microorganisms were Gram-negative *Escherichia coli* (ATCC 25922 and ATCC 35218). *E. coli* ATCC 25922 was identified as β-lactamase negative strain whereas *E. coli* ATCC 35218 was known to be β-lactam antibiotic resistant strain that characterized with the presence of plasmid-encoded TEM-1 β-lactamase (Clinical and Laboratory Standards Institute, 2014). The bacterial samples were cultured and maintained with MacConkey agar which is a selective and differential medium for Enterobacteriaceae (Totora, Funke and Case, 2010).

CHAPTER 4

RESULTS

4.1 Antibacterial Assessments

4.1.1 Agar Well-diffusion Assay

Based on Table 4.1, in general, the zone of inhibition increased along with the increasing concentrations of honey and the biggest zone of inhibition was observed in the undiluted honey (100%, v/v). The formation of inhibition zone started at 20% (v/v) honey concentration for *Trigona apicalis* honey (15-20 hrs, 41˚C) indicating that the honey sample was more potent against *Escherichia coli*. Other than that, *T. apicalis* honey (15-20 hrs, 41˚C) also showed the greatest inhibitory efficacy with the largest zone of inhibition than the other two honey samples. By comparing the inhibitory effects of undiluted Trigona honey samples (Table 4.2), *T. itama* honey (8 hrs, 41˚C) exhibited significantly bigger inhibition zone than *T. itama* honey (15-20 hrs, 41˚C); besides, undiluted *T. apicalis* honey (15-20 hrs, 41˚C) was also shown to exhibit bigger inhibition zone than *T. itama* honey (15-20 hrs, 41˚C) significantly.

CHAPTER 5

DISCUSSION

5.1 Antibacterial Assessments

Based on the result in agar well-diffusion assay, all the tested Trigona honey samples were shown to have inhibitory effect against *Escherichia coli*. This result is in concordance to other studies which had reported that Trigona honey was able to inhibit the growth of many species of bacteria including *Escherichia coli* (Garadew, Schmolz and Lamprecht, 2003; Boorn, et al., 2010; Ewnetu, Lemma and Birhane, 2013; Eswaran, Priya and Bhargava, 2015).

On the other hand, the result of this study also demonstrated that the zone of inhibition was directly proportional to the concentration of honey indicating that the antibacterial effect of honey was much greater in undiluted honey. This result is in agreement with the research carried out by Chanchao (2009) who studied the antimicrobial activity of *Trigona laeviceps* (stingless bee) honey from Thailand. Furthermore, a study that was carried out by Eswaran and colleagues (2015) also showed that the greatest inhibitory effect of Trigona honey from India on *E. coli* was in its undiluted form. The possible explanation for this outcome is due to the antibacterial properties such as acidity, osmolarity and phytochemical compounds are well preserved in undiluted honey (Molan 2001; Badawy, et al., 2004). However, this result is contradicted to a study done by Ng, Chin and Khoo (2014) whereby they showed that the antibacterial effect of Apis (honeybee) honey against biofilm mass was greater in lower concentration than the undiluted one. This was

CHAPTER 6

CONCLUSION

In brief, all Trigona honey samples tested were reported to have inhibitory effect on *Escherichia coli* and exhibited the greatest antibacterial effect in the undiluted form, 100% (v/v) concentration. Besides, the bactericidal activity of Trigona honey was proven to be time dependent as demonstrated in the endotoxin assay whereby more bacteria were killed when treated with honey for a longer time. Based on both agar well-diffusion and endotoxin assays, it was also verified that *Trigona apicalis* honey (15-20 hrs, 41˚C) had the highest antibacterial potency against *E. coli*. Moreover, *T. apicalis* honey was shown to cause morphological changes on *E. coli* under scanning electron microscopic examination. The effectiveness of *T. apicalis* honey in antibacterial activities may relate to its physicochemical properties such as higher acidity, total phenolic content and hydrogen peroxide level than *T. itama* honey which processed with the similar method. On the other hand, longer honey processing time was suggested to influence the antibacterial effectiveness as the honey had lower acidity and hydrogen peroxide level, although the moisture content was lower while the total sugar content and phenolic content were greater.

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Appendix A

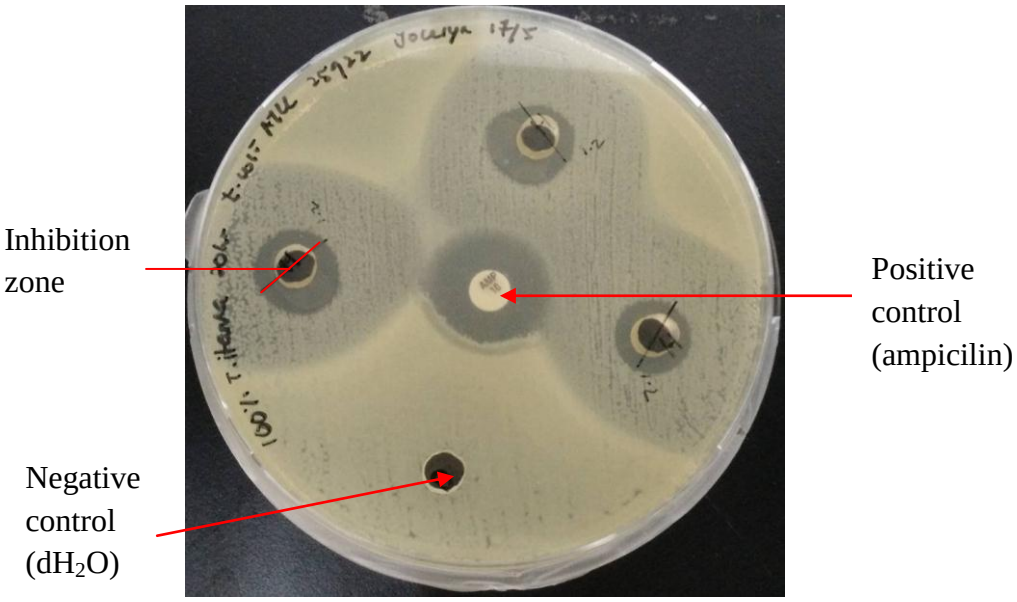

Figure 1.1: Example of the result from agar-well diffusion assay.

Appendix C

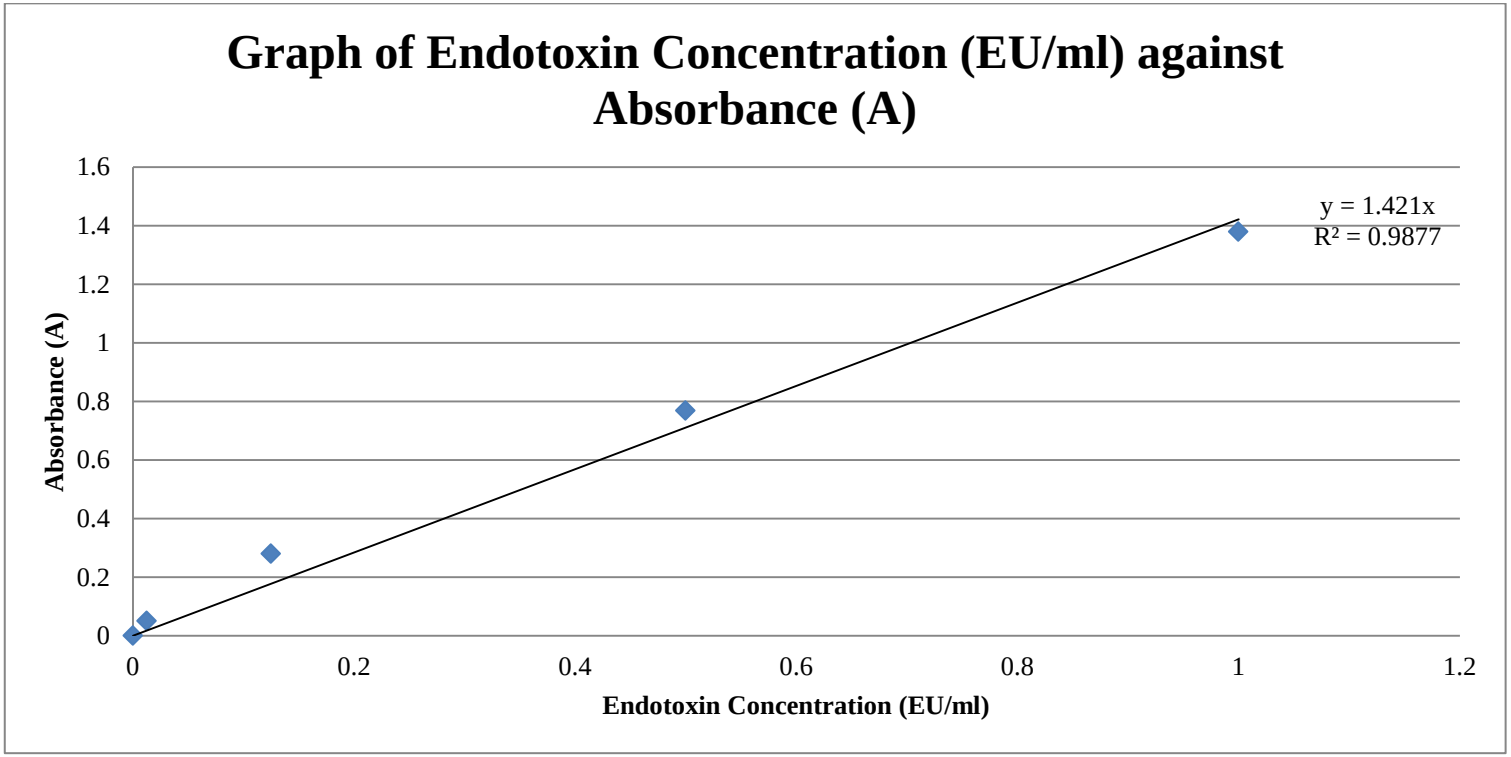

Figure 1.1: Standard curve for endotoxin assay.

Appendix E

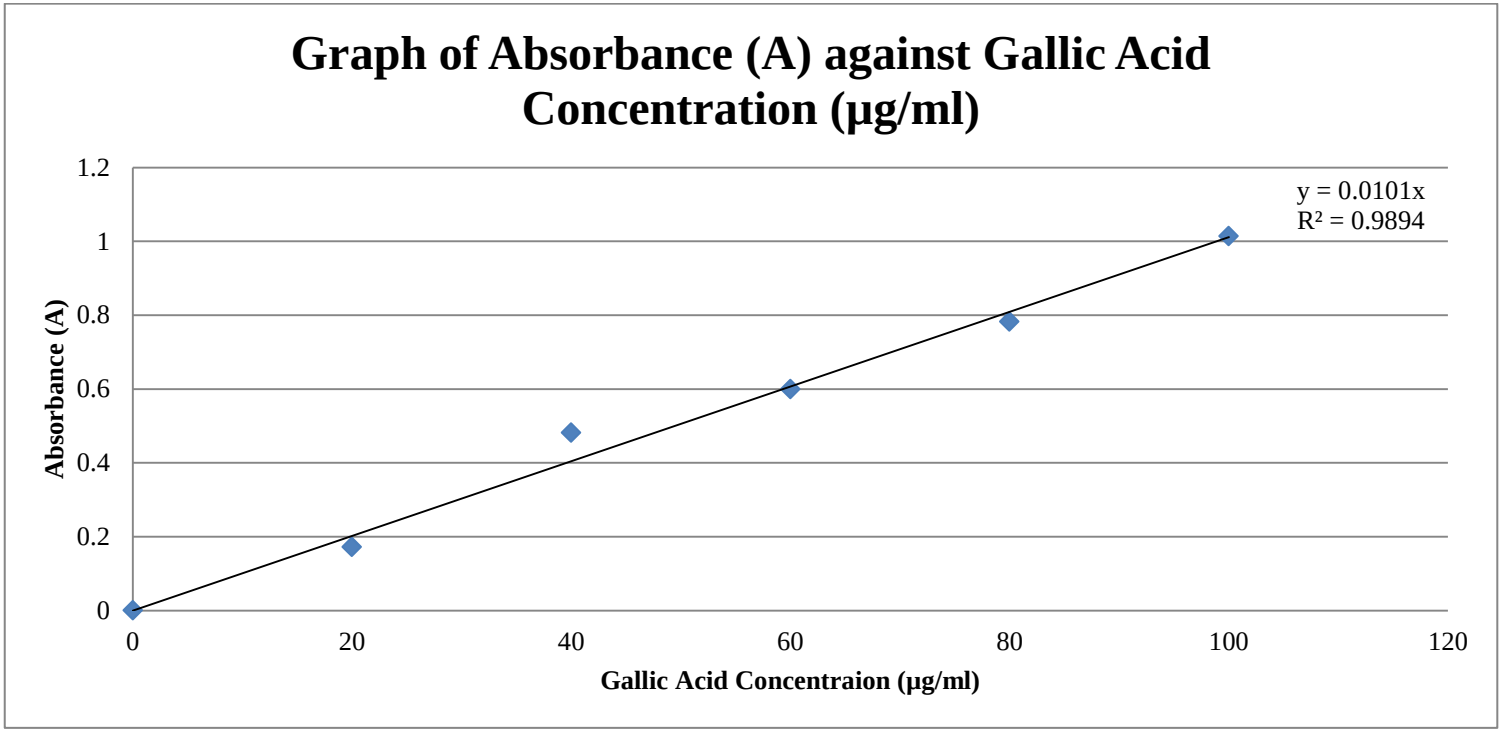

Figure 1.3: Gallic acid standard curve.