Physicochemical Properties and Biological Activities of Thai Originated Honeys

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A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Functional Food and Nutrition

Prince of Songkla University

2018

Thesis Title	Physicochemical Properties and Biological Activities of Thai Originated Honeys
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บทคัดย่อ

น้ำผึ้งจากดอกไม้แต่ละชนิด จะมีลักษณะทางเคมีกายภาพและฤทธิ์ทางชีวภาพที่แตกต่างกันโดยเฉพาะกลุ่มของสารต้านอนุมูลอิสระ งานวิจัยนี้จึงมีวัตถุประสงค์เพื่อศึกษาองค์ประกอบทางเคมีกายภาพและฤทธิ์ทางชีวภาพของน้ำผึ้งที่ผลิตจากแหล่งดอกไม้ที่แตกต่างกัน คือ น้ำผึ้งลำไย น้ำผึ้งเงาะและน้ำผึ้งยางพารา ผลการวิเคราะห์องค์ประกอบทางเคมีกายภาพของน้ำผึ้งทั้ง 3 ชนิด ได้แก่ ความชื้น สี ค่าการนำไฟฟ้า พีเอช ความหนืด ปริมาณน้ำตาลและปริมาณโพรลีน พบว่าน้ำผึ้งทั้งสามชนิดมีพีเอชอยู่ในช่วง 4.04 – 4.44 ค่าสีอยู่ในช่วง 22 – 44 พีฟันด์สเกล ความชื้นอยู่ในช่วงร้อยละ 14.84 – 17.80 ความหนืดอยู่ในช่วง 2,755.67 – 3,220.23 เซนติพ้อยและค่าการนำไฟฟ้าอยู่ในช่วง 0.352 - 0.518 มิลลิซีเมนต์ต่อเซนติเมตรซึ่งค่าที่วัดได้จากผลการทดลองอยู่ในช่วงเกณฑ์มาตรฐานของน้ำผึ้งจากธรรมชาติ น้ำผึ้งลำไยมีพีเอชมากที่สุดคือ 4.44, มีค่าสีมากที่สุดคือ 44 พีฟันด์สเกล, ความชื้นร้อยละ 17.80 และคามหนืด 3220.33 เซนติพ้อย น้ำผึ้งยางพารามีค่าการนำไฟฟ้ามากที่สุดคือ 0.518 มิลลิซีเมนต์ต่อเซนติเมตร การวิเคราะห์ปริมาณน้ำตาลพบว่า น้ำผึ้งเงาะมีปริมาณน้ำตาลรีดิวซิ่งมากที่สุดคือ ร้อยละ 73.21 น้ำผึ้งลำไยมีปริมาณโอลิโกแซคคาไรด์ (ชนิดราฟฟิโนส) มากที่สุดคือ ร้อยละ 2.78 และมีปริมาณโพรลีนมากที่สุด คือ 785.75 มิลลิกรัมต่อกิโลกรัมน้ำผึ้ง การวิเคราะห์เอนไซม์ในน้ำผึ้งประกอบด้วยไดแอสเทสและอินเวอร์เทสพบว่าน้ำผึ้งมีค่าของเอนไซม์ทั้งสองชนิดอยู่ในช่วง 6-12 โกธสเกลและ 36.87 – 147.02 หน่วยต่อกิโลกรัมน้ำผึ้ง ปริมาณสารประกอบฟีนอลิกและฟลาโวนอยด์ในน้ำผึ้งพบว่าน้ำผึ้งลำไยมีปริมาณมากกว่าน้ำผึ้งอีกสองชนิด โดยน้ำผึ้งยางพารามีสารประกอบฟีนอกลิกและฟลาโวนอยด์ต่ำที่สุด การวิเคราะห์สมบัติการต้านอนุมูลอิสระ โดยวิธี DPPH, ABTS, FRAP และ Ferrous reducing พบว่า น้ำผึ้งลำไยมีคุณสมบัติในการต้านอนุมูลอิสระสูงสุด และน้ำผึ้งลำไยยังมีฤทธิ์ในการต้านเบาหวานดีที่สุดอีกด้วย การศึกษาคุณสมบัติความเป็นพรีไบโอติกของโอลิโกแซคคาไรด์ในน้ำผึ้งลำไยโดยการหมักในระบบจำลองในลำไส้ใหญ่มนุษย์ พบว่าโอลิโกแซคคาไรด์ในน้ำผึ้งสามารถกระตุ้นการเจริญเติบโตของ *Bifidobacteria* spp. และ *Lactobacillus* spp. ได้อย่างมีนัยสำคัญใกล้เคียงกับฟรุคโตโอลิโกแซคคาไรด์พรีไบโอติกทางการค้า และพบว่าโอลิโกแซคคาไรด์ในน้ำผึ้งยังสามารถเพิ่มปริมาณกรดไขมันสายสั้น เช่น อะซิติก โพรพิโอนิก บิวทาริก และแลกติกได้เช่นกัน

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Academic Year	2018

ABSTRACT

Honey harvested from different sources has different physicochemical and biological characteristics, biologically active compounds present in honeys, especially the group of antioxidants. This research studied on honeys produced from three different sources including para rubber, rambutan and longan. The results of physical and chemical analysis including moisture content, color, electrical conductivity, pH, viscosity, proline content and sugar content were found that they are in ranged of 14.84-17.80, 22-44 Pfundscale, 0.325-0.518 mS/cm, 4.04-4.27, 2,755.67-3,220.33, 116.25-785.75 and 71.38-73.21%, respectively. The obtained values from experiment were in the standard range of natural honey. Rambutan honey has the highest sugar content of 73.21% and longan honey has the highest content of oligosaccharides of 2.78% as well as highest proline content of 787.75 mg/kg. Analysis of phenolic and flavonoid compounds were found that longan honey contained more phenolic and flavonoid compound than others two honeys. Analysis of enzyme activities in honey including diastase and invertase the results showed that both enzymes are in the ranged of 6-12 Gothe Scale and 36.87-147.02 Unit/Kg honey, respectively. Analysis of antioxidant activities, the 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) scavenging activity, ferric reducing antioxidant power (FRAP), and ferrous chelating showed that longan honey showed better antioxidant capacity than rubber and rambutan honeys. Longan honey had highest antidiabetic activity. Prebiotic property of oligosaccharide in honey was studied by *in vitro* batch fermentation. The oligosaccharides was separated by activated charcoal filtration. The result showed that oligosaccharides

presented in longan honey able to significantly stimulated microorganisms growth which were *Bifidobacteria spp*. and *Lactobacillus spp.* similar to that result of fructooligosaccharides fermentation oligosaccharide in longan honey also able to increase SCFA such as butyric acid, acetic acid and propionic acid

ACKNOWLEDGEMENT

I would like to thank my advisor, Dr. Nualpun Sirinupong from Interdiscripnary Graduate School of Nutraceutical and Functional Food, Prince of Songkla University, for the successful completion of this research. It would not have been possible without the reassurance, driving force and attention, including ministrations from my advisor. I also would like to thank for her invaluable suggestion and lessons for my academic knowledge.

I would like to thank my co-advisor, Asst. Prof. Dr. Santad Wichienchot from Interdiscripnary Graduate School of Nutraceutical and Functional Food, Prince of Songkla University, for helpful guidance and assistance during my study. I also would like to thank for his deliberation and suggestion for my academic knowledge.

I am also grateful to thank my examining committees, Prof. Dr. Supayang Voravuthikunchai from Department of microbiology, Faculty of Science, Prince of Songkla University and Assoc. Prof. Dr. Jitbanjong Tangpong from School of Allied Health Sciences, Walailak University for their kindness, comments and helpful suggestion.

I would like to thank my family. They don’t have word to indicate my emotion towards my parents for their understanding the problem during study, support throughout my life and always being there for me during my study.

Many thanks to all teachers, lecturers, staffs and all my friend at Major of Functional Food and Nutrition, Interdisciplinary School of Nutraceutical and Functional Food, Faculty of Agro-Industry, Prince of Songkla University for their kindness help and creating a healthy learning atmosphere.

Orawee Foomaneechot

CONTENTS

CONTENTS (Continued)

CONTENTS (Continued)

CONTENTS (Continued)

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

A_{510}	UV absorbance at specified wavelength, in this example, 510 nanometers
FOS	Fructooligosaccharide
g	Gram
hr	Hour
kg	Kilogram
mL	Milliliter
M_W	Molecular weight
nm	Nanometer
PBS	Phosphate buffered saline
SDS	Sodium dodecyl sulfate (detergent)
µg	Microgram
UV	Ultraviolet light
V_t	Total volume

CHAPTER 1

INTRODUCTION AND REVIEW OF LITERATURE

1.1 Introduction

Honey is the major product of honeybees which has important nutritional value and provides significant contributions. Quality control of honey is important to determine its suitability for processing and to meet the demand of the market. Honey shall not have foreign taste, begun to ferment, heated to the extent of destroying its natural enzymes and a substance that endanger human health (Official Journal of the European Communities, 2002). However, the particular content rely on many elements such as the nectar composition of the source plant, bee's species, the region, environmental and seasonal conditions, agricultural process and remedy of honey during harvest and collect (Iglesias *et al*., 2012). According to Basualdo *et al*. (2007), the physical and chemical properties fully effect to the biological activities of honey. It has been used not only in foods and beverages as a sweetener and favoring but also in medicine since the initial humans. The act of this product in the treatment of burns, gastrointestinal disorders, respiratory illnesses, infected and chronic wounds, skin ulcers and cancer has been studied recently by many researchers (Castaldo and Capasso, 2002; Orhan *et al*., 2003; Ramalhosa *et al*., 2011).

Honey is complex supersaturated sweet natural liquid produced by honey bees from the nectar of plants. The substances collected by bees were combined with their own specific substances then deposited, dehydrated, stored and left in honeycombs to ripen and mature. Around 200 substance have been reported in this complex natural liquid but the composition especially its secondary metabolites and quality of honey may be influenced by some external factors such as environmental and seasonal factors, processing, handling and storage (Gheldof *et al*., 2002: Saxena *et al*., 2010; Khalil *et al*., 2012). The substances including in honey are sugars (main components: fructose 38%, glucose 31%), proteins, moisture (10-20%), vitamins (ascorbic acid, niacin, etc.), mineral salts (potassium, calcium, sodium,

phosphorus, etc.), organic acids (acetic acid and gluconic acid, etc.), 5-hydroxymethylfurfural (HMF), enzymes (phosphatases, glucose oxidase, invertase and catalase), flavonoids, phenolic acids and volatile compounds. EC Directive 2011/110 specified the criteria to ensure the quality of honey by contents analysis, are electrical conductivity, moisture, ash, free acidity, sugars (reducing and non-reducing), HMF and diastase activity (White, 1975: Council Directive of the European Unioun, 2012: Nayik and Nanda, 2015).

The biochemical constituent, enzymes are approximately nearly to the nutritive contented and honey freshness. Nevertheless enzymes are existing in minute number, they have expressive result on the charisteristics of honey. This is the reason enzymes would significant influence the protein components, free amino acid form and acidity of honey. Generally, enzymes found in honey are excreted from bee salivary, especially amylases, oxidases and catalases (Gheldof *et al*., 2002). These enzymes would inculcated complex sugars within simple sugars like fructose and glucose. The simple sugars ability to more catalyzed within alcohol and acetic acid below an useful quantity of moisture content at the suitable temperature circumstances because of fermentation. Also influencing the pH value, the activity of enzymes ability variation the flavor and aroma of honey later fermentation. Amino acid and proline has been revealed as the greatest sufficient amino acid in honey (Rebane and Herodes, 2008). It was confirmed from determination of sugar-fed honey that proline arise from the salivary excretion of honey bees while nectar alteration (Iglesias *et al*., 2004).

Because of enzymes are current in small element, more studies are expected to point on sugar content and pollen for analysis of honey source (Mateo and Bosch-Reig, 1997; Weston and Brocklebank, 1999; Da Costa Leite *et al*., 2000). Including macronutrient, the content of monosaccharides and disaccharides and their proportion could be helped to analyzed the degree of honey maturity. Normally, ripen honey have lower disaccharides as maltose and sucrose constituents than those from honey harvested at an previous stage. By reason of many of disaccharides have been transformed within monosaccharides by the activity of enzymes. Thus, predominant sugars and their proportion are essential criterion for honey characterization.

Honey has retained a location of significance in conventional medicine for life (Jeffrey and Echazarreta, 1996; Patricia *et al*., 2004, 2013). Various years, honey has been an important participant as an antioxidant and it has been revealed that honey can be benefit as a hepato protective and cardio protective agent (EL-Kholy *et al*., 2009; EI Denshary *et al*., 2012; Erejuwa *et al*., 2012). Further, honey has careful reaction against gastrointestinal disease (Salem, 1981; El-Arab *et al*., 2006).

Honey is among the first wound healers possible in the natural. The aged-old Chinese, Eygyptians, Assyrian, Romans and Greek apply various category of honey to heal wounds and ailment of the intestine. Honey expend obvious antibacterial results against various microorganisms, containing *Salmonella* spp., *Helicobacter pylori, Shigella* spp. and *Escherichia coli* (Al Somal *et al*., 1994; McGovern *et al*., 1999). Furthermore, honey is release to have anti-inflammatory (Kassim *et al*., 2010; Nasuti *et al*., 2006) and anti-cancer capacities against breast, cervical (Fauzi *et al*., 2011) and prostate cancers (Samarghandian *et al*., 2011) includind osteosarcoma (Ghashm *et al*., 2010). In addition, honey is generally benefit as an anti-diabetic (Erejuwa *et al*., 2012) and hypolipidemic agent (Adnan *et al*., 2011) and to ameliorate thyroid disturbances (Adewoye and Omolekulo, 2014).

As known that, there is a wide variety of honeys quality with different taste, aroma, color, and substance composition, depending on their botanical origin. The aim of this work was to evaluate the quality of blossom and honeydew honeys originated in Thailand from the point of view of physicochemical properties and biological activities. The results of these experiments are indicative of the properties or characteristics of honeys produced in Thailand, which is expected to help preventing the contamination of natural honeys, which would be beneficial for Thai apiculture and beekeeper.

1.2 Review of literature

1.2.1 History of honey

Honey is produced by honey bees, especially by the species of *Apis mellifera* (Havsteen, 2002) as blossom honey by secreting nectars of flowers honeydew honey is a type of honey made from honeydew secreted by plant-sucking insects such as aphids or young leave of plants (Adebiyi *et al*., 2004). Bees first convert the flower nectar into honey by a process of regurgitation and evaporation, then store it as a primary food source in wax honeycombs inside the beehive with the clear, golden amber color. Honey flavor will vary based on the specie of flower from which the nectar was harvested. Honey can then be harvested from the hives for human consumption. According to historical evidence, there is evidence of the harvesting of wild honey, dating back 10,000 years. However, by 2400 BC, the art of beekeeping was well established in Egypt at least, and used honey as a natural food source and is also as ingredients in various food preparations (Adebiyi *et al*., 2004; Durrani *et al*., 2011; Eleazu *et al*., 2013; Allsop and Miller., 1996). Honey is accepted as a food source and medicine by both modern and ancient generations, traditions and civilizations (Allsop and Miller., 1996). The religion of Islam recommended the use of honey, and a separate chapter was denoted in their holy book, Holy Quran for honey (The Holy Qur'an, 1990). The Buddha considered honey to be one of the five essential medicines and food. According to Food and Agriculture Organization, among the honey producing countries, China is in the top and followed by Turkey, United States of America, Russian federation, India, Mexico, Brazil, Ukraine, and New Zealand (Jedege, 2018). Honey is produced in large quantities worldwide.

1.2.2 Nectar and honey

Nearly all honey obtains from nectar, a coming from phloem sap that is certain from individual cell groups called nectaries. Nectaries are indication floral if they appear as part of a flower or extra floral if they appear else where on a plant. Nectar is an aqueous solution of sugars, amino and other acids, proteins, lipids,

minerals, and other constituents which some sugar shown in figure 1.1. The precise constituents of nectar change excessively, upon on the plant varieties and the natural circumstanced. Such as, the total sugar contented could be extent from 5 to 80 percent of the nectar. The groups of sugars could be also differ; common sugars. In many types of nectar, sucrose is the major or whole sugar, as well as in few nectars fructose, glucose, and sucrose are existing in approximately alike portion. Such as, in the families *Lamiacea* (mints) and Ranunculacea (buttercups and clematis) sucrose is the main sugar. Almost nectars have so small sucrose but variable amount of fructose and glucose when it is in the families *Brassicaceae* (mustard and cabbage) and *Asteraceae* (asters, daisies, and sunflowers) (Baker, 1983). Only hardly are another sugar found. Raffinose, galactose, and sorbitol have been found in some plant nectars, but they are not generally found. Phloem sap includes most of sucrose, and then a chemical revision must appear to make fructose and glucose in the nectar. These chemical reversed are resolved by transglucosidases and transfructosidases that are found in the nectaries.

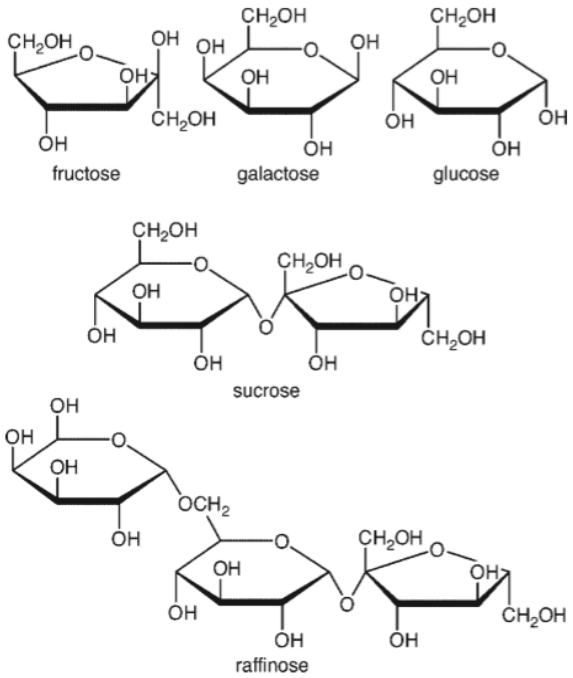

Figure 1.1 Structure of some common sugars found in nectar and honey

Source: David, 2007

While the process of this procedure, the nectar is blended with the excretions of two glands, the hypopharyngeal and the salivary. While a worker bee drinks nectar from a flower, a maximal of about 25 mg of nectar is collected at the top of the esophagus in a multiply area called the honey stomach or honey sac. Two enzymes contain invertase and diastase, these enzymes which break down greater saccharides, especially sucrose, into monosaccharides. While the excretions of the second glands, which include enzymes, which start to chemical change the nectar. Glucose oxidase is further existing. Then, chemical transformation of the nectar into honey starts nearly direcly (White, 1992). Bees keep an involed anatomy that is benefit to accumulated nectar from plants.

The nectar can further be passed to other bee's house for added transform. In beehive, the forager bees discharge the nectar and change it to a bee's house. In act so, many enzyme-consist of excretions are blende with the nectar. The bee's house drinks the nectar completed her organs and can eject and redrink the nectar increased times up a period of 15–20 minutes. Finally, the nectar droplet is collect within the honeycomb (Figure 1.2).

Bees carry on to addition nectar to distinctive honeycomb cells as far as the chamber is full, after which the bees will cap the cell with newly produced beeswax. The certain change method from salivated nectar to honey takes 1–3 days. Bees are steadily blows on the honeycomb with their wings, accelering water evaporation. The maturing of nectar within honey is a synthesis of two procedures: the conversion of sucrose into fructose and glucose and the dispersal of leaving water. Honey is approximately 80% of the total nectar must be vapolize, so below 20% water. In actully, overload humidity can be damaging to honey producing, and beehives are usually little heading to avoid combine of water internal the hive. (White, 1992). The process starts when field bees collect quantities of nectar from flowering plants and return to the hive. This nectar is stored in the honey sac where invertase, an enzyme, is addition to the nectar. Invertase enables the nectar, primarily a sucrose solution, to be converted to a mainly levulose and dextrose solution. The nectar collected by field bees is stored in wax cells in the hive where it is converted into honey. During this process the moisture content is reduced to 14–21%. When the honey is ripe, bees cap the cells with beeswax. The higher the humidity or colder the

climate, the more difficult it is for house bees to reduce the moisture content. Honey with a moisture content over 21% is likely to ferment and spoil (Somerville, 2012).

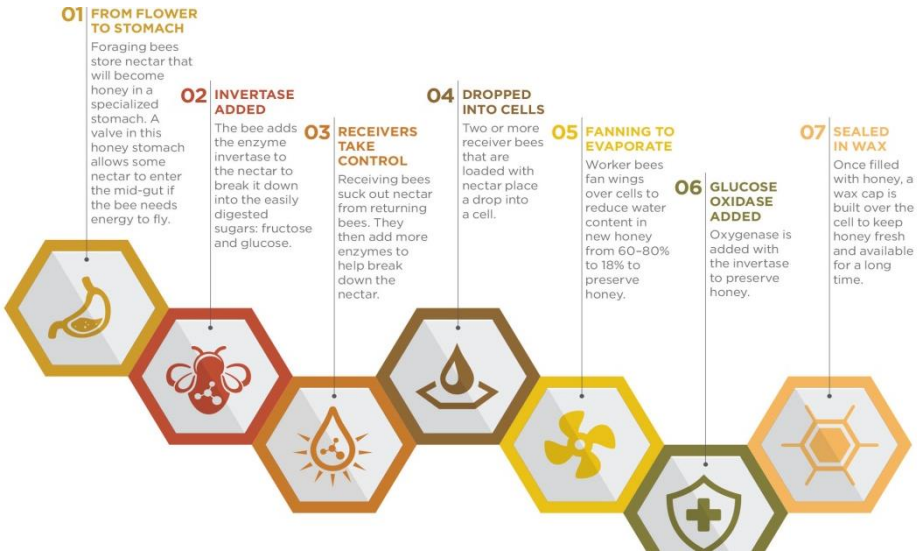

Figure 1.2 Process of honey producing from nectar

Source: https://ppp.purdue.edu/resources/ppp-publications/the-complex-life-of-the-honey-bee/

Honey is the natural sweet substance produced by honey bees from the nectar of plants or from secretions of living parts of plants or excretions of plant sucking insects on the living parts of plants, which the bees collect, transform by combining with specific substances of their own, deposit, dehydrate, store and leave in the honey comb to ripen and mature (Codex, 1981). Blossom honey or nectar honey is the honey which comes from nectars of plants. It is a flowering plant that produces nectar as part of its reproductive strategy. These plants create nectar, which attract pollinating insects and sometimes other animals such as birds. Honeydew honey is the honey which comes mainly from excretions of plant sucking insects (*Hemiptera*) on the living parts of plants or secretions of living parts of plants.

1.2.3 Physical properties

The physical presence of honey diverge with the process of abstraction, processing, packaging and storage (Crane, 1975; Gonzalez-Paramas *et al*., 2000; Ajibola, 2015). Nevertheless, there are main characteristics correlated with this natural product regardless of the process complicated in development (White and Doner, 1980; Decaix, 1976; Ajibola, 2015). These contain water content, crude content and water absorption. Honey includes around 16% water and around 80% suspended fragment (White and Doner., 1980; Ajibola, 2013). Freshly exacted honey is a viscosty liquid food, and it viscous depends on the diverse honey compositions (Crane, 1975). Hence, the viscous it largely affected by the content of honey, mostly its water content. The capacity of honey to absorb and hold water from the climate is explain as hygroscopicity. Honey will take in moisture from air at a relative humidity of around 60% (Decaix, 1976). Other part effecting the physical characteristics of honey is surface tension, which effected by the colloidal element in the honey, a consideration of the honey's geographical source. The surface tension and highly viscous of honey due to the foaming properties of honey (Olaitan *et al*., 2007).

The another physical characteristics and features of honey contain, smell and color (Bogdanov, 2015; White and Doner, 1980). Honey has a swet smell and taste, and is observed as sweetener, due to its includes an exact sweet sugar, fructose commonly blended correlatively with other sugar, glucose (Doner, 1977; Ajibola, 2013). The color of liquid honey differ from clear and colorless, yellow, amber to dark amber or black. The color differ with honey's source, storage circumstanxes and age, but clarity or transparency gather on the quantity of suspended fractions as pollen (Bogdanov, 2015; Gonzalez-Paramas *et al*., 2000; Olaitan *et al*., 2007). Another honey color are bright yellow (sunflower), reddish undertones (chest nut), greyish (eucalyptus) and greenish (honeydew).

1.2.4 Chemical composition

Honey is a food that consist of around 200 element (Escuredo *et al*., 2013)., and contain major of sugars, water, and another position as protein (enzymes), organic acids, vitamins (particularly vitamin B6, thiamine, niacin, riboflavin and pantothenic acid), minerals (containing zinc, sodium, potassium, phosphorus, manganese, magnesium, iron, copper and calcium), pigments, phenolic contents, full diversity of volatile composition, and solid fragment recived from honey collecting (Alqarni *et al*., 2012; Ciulu *et al*., 2011; Pontes *et al*., 2007). Average substances of honey shown in Table 1.1.

Table 1.1 Average composition of honey (Nutritional value/100 g).

Components	Average
Carbohydrates	82.400 g
Total sugar	82.120 g
-Fructose	38.500 g
-Glucose	31.000 g
-Maltose	1.440 g
-Sucrose	0.890 g
-Galactose	3.100 g
-Raffinose	0.08 g
Dietary fiber	0.200 g
Fat	0 g
Protein	0.300 g
Water	17.100 g
Riboflavin (Vit. B_2)	0.038 mg
Niacin (Vit. B_3)	0.121 mg
Pantothenic acid (Vit. B_5)	0.068 mg

Source: adapted from USDA (2016)

1.2.4.1 Sugars

The sugars represent in honey are important for charisteristics such as energy value, viscous and hygroscopic. Monosaccharides present around 75% of the sugars establish in honey, contain 10-15% disaccharides and slight quantity of another sugars. (Kamai and Klien, 2011).

The amount of sugar is important to the geographical origin of honey, source of flowers that benefit from bees, botanical and impressive sources in the region, storage and processing (Escuredo *et al*., 2014; Tornuk *et al*., 2013). Most of all fructose honey, is the large amounts of carbohydrate, excepted in few honeys, rape (*Brassica napus*) and dandelion (*Taraxacum officinate*), where part of glucose may be over than the portion of fructose (Escuredo *et al*., 2014), mainly have a fast crystallization. The combination of fructose and glucose, as the ratio between them, is a valuable factor for the classification of monofloral honeys (kaskoniene *et al*., 2010).

The sugar content of honey has been examined by scientifics through the world. In these contents, numerousness sugars were recognize, as fructose, glucose, sucrose, erlose, raffinose, palatinose, nigerose, melibiose, melezitose, maltulose, maltotriose, maltotetraose, maltose, isomaltose, nigerobiose, trehalose, rhamnose and anothres (Fuente *et al*., 2011).

Sugars in honey are presented by monosaccharides, glucose and fructose, come after by disaccharides, sucrose, maltose, melezitose, maltotriose and tursnose. Disaccharides and trisaccharides such as sucrose and maltotriose and enzyme degradation monosaccharides. Sucrose contains of one molecule that it connected of fructose with glucose nevertheless α-1,4complex. It is decomposed by the invertase enzyme, yielding an equal blender of hexoses (Soldatkin *et al*., 2013).

1.2.4.2 Proteins

Apis mellifera honey included among 0.2% and 1.6% protein (Won *et al*., 2009). The protein composition of honey differ accordance with the types of the honeybees. Proteins and amino acids in honeys are give reasons for both animals and

vegetative origin, containing liquids and excretions of bee's salivary glands and pharynx of honeybees (Escuredo *et al*., 2013; Sak-Bosnar and Cabezuo, 2012), but the major content of protein is pollen.

Amino acids are subjected at 1% (w/w) of honey's compositions and their corresponding portions depending on the source of honey (nectar or honeydew). The mostly amino acid in honey and pollen is proline (Iglesias *et al*., 2006). As well proline, another amino acids represent in honey contain glutamic acid, alanine, asparagine, serine, lysine, ornithine, phenylalanine, tryptophan, leucine, isoleucines, cysteine, valine methionine, threonine, glycine, histidin, glutamine and aspartic acid (Keckes *et al*., 2013; Rebane and Herodes., 2010), with the most general of these being glutamic acid, isoleucine, leucine, tyrosine, phenylalanine and alanine (Girolamo *et al*., 2012)

Proline originated from the salivary excretions of honey bees (*Apis mellifera* L.) while the nectar convert to honey. In honey, proline presents of 50-85% amino acids (Iglesias *et al*., 2006; Truzzi *et al*., 2014). Proline benefits as a principle for estimation the maturation of honey, and in fewer studies, sugar contanination. At least 180mg/kg of proline is established as the limited amount of pure honey.

Diastase is a number of amylolytic enzymes that contain α- and β-amylase. A minor parts of the proteins represent in honey are enzymes as invertase, the α- and β-glucosidase, diastase, glucose oxidase, acid phosphatase and catalase (Sak-Bosnar and Sakac., 2012; Won *et al*., 2008). α-Amylase hydrolyzes starch chains into the α-D-(1,4) linkages, produced dextrin. β-amylase hydrolyzed starch chain at the end, produced to the structure of maltose (Sak-Bosnar and Sakac., 2012). Other enzymes represent in honey are glucose oxidase. It convertion to internal glucose within δ-gluconolactone, which is decomposed into gluconic acid. In addition δ-gluconolactone, glucose oxidase as well as prodution hydrogen peroxide, which has bactericidal processess (Moreira *et al*., 2007).

1.2.4.3 Organic acids

Follow by some authors; all honeys have a little acidic effect which is caused by almost 0.57% organic acids (Karabagias *et al*., 2014). These organic acids are comes from sugars by enzymes that are excreted by bees when changing nectar within honey or when get from nectar (Cherchi *et al*., 1994).

These acids are correlated to the color and flavor of honey and its chemical components as electrical conductivity, pH and acidity (Mato *et al*., 2006). Organic acids are also benefit to segregate honeys following by their geographical origin and/or botanical sources.

Any organic acids from distinctive areas of the world are represent in honey as acids; aspartic acid, butyric, citric, acetic, oxalic, tartaric, succinic, shikimic, quinic, pyruvic, propionic, 2-oxopentanoic, methylmalonic, malonic, malic, lactic, α-ketoglutaric, isocitric, α-hydroxyglutaric, 2-hydroxybutyric, glyoxylic, glutaric, glutamic, gluconic. formic, galacturonic, fumaric and formic (Cherchi *et al*., 1994; Mato *et al*., 2006; Nozal *et al*., 2003).

The dominant acid is gluconic acid. It's preresent in honey derived from glucose oxidase, which hoeybees add while maturing (Karabagias *et al*., 2014). Also gluconic acid, citric acid is further represent in honey, and the combination of these two element is benefit as positive criterion to separated floral honey from honeydew (Mato *et al*., 2006).

1.2.4.4 Phenolic and flavonoid compounds

Phenolic compounds are a chemical miscellaneous class, nearly 10,000 constituents, which are classes within distinc grouops accordance with their main chemical complex. These components have an aromatic ring with one or more hydroxyl groups in the complexs, which can devide from complex molecule that have high molecular weight (Pyrzynska and Biesaga., 2009). They may be separated within non-flavonoids (phenolic acid) and flavonoids (flavonols, flavones, flavonones, flavanols, anthocyanidin, isoflavones and chalcones) (Andersen and Markham, 2006) shown in figure 1.2.

Phenolic acids form is a great group of phenolic compounds with bioactive activity that are commonly found in vegetable and foods, and are secondary substances that are necessary for common activity of generally apperaring of plants. They are separate within 2 subclasses follow by their complex: the hydroxycinnamic and hydroxybenzoic acids (Challacombe *et al*., 2012). They are compositions that action as antioxidants, removing free radicals and preventing lipid oxidation.

Hydroxybenzoic acids have a common complex c1-c6 come from benzoic acid. Diversions in this complex can be found in hydroxylation and methylstion of the aromatic ring (Tsao, 2010). Acids which are come from hydroxybenzoic acids contain ρ-hydroxybenzoic, vanillic, syringic, salicylic (2-hydroxybenzoate), gallic and ellagic. These complexs may be represent in their soluble from in cells, connected with sugars or organic acids, or structure composed with cells connected with lignins (Rice-Evans and Packer, 2003).

Hydroxycinnamic acids have a common complex of c3-c6 and present variance in the arise ring components likes phenolic acids varies from ρ-coumaric, caffeic, ferulic and sinapic acids. These complexs appear commonly in their combined form as esters of hydroxyl acids likes tartaric acid shikamic including pure form (Rice-Evans and Packer, 2003).

Flavonoids have a c6-c3 nuclear complexs, including two benzene rings linked by a pyran ring. Replacement on the rings decisions in large groups of flavonoids. They present the major classes of plant phenolic compounds, which present besides 50% of all common phenolic compounds. They are generally present in the seeds, leaves, bark, and flowers of plants and trees. In plants, these complexs give keep opposite to herbivores, pathogens and ultra violet radiation. Plants have various derivatives of polyphenolics that great structures differences and elaboration, when honeybees gather nectar; these biological components may be changed from plants to honey (Silli *et al*., 2010).

Differ authors have studied the constituents of phenolic compounds in honey. They have found benzoic acid, hesperetin, gallic acid, rosmarinic acid, 4-hydroxybenzoic acid, chlorogenic acid, 3-hydroxybenzoic acid, galangin acid, ellagic acid, chrysin, pinocembrin, pinobanksin, myricetin, kaempferol, quercetin, ferulic

acid, β-coumaric acid, syringic acid, caffeic acid, vanillic acid and anothers (Alvarez-Suarez *et al*., 2012; Trautvetter *et al*., 2009).

Phenolic compounds represent in honey's benefit as a floral markers and have further raise the quantity of attraction candid to the study of antioxidant activities characteristics to these complexs because their capacity to eliminate or decrease the formation of free radicals (reactive oxygen species- ROS). Other studies present that flavonoids can prevent lipids from oxiadation of cell membrane (Sghaier *et al*., 2011). The major useful compounds of honey are flavonoids. They can provide significant antioxidant activity of honey, taking advantage results for human health (Alvarez-Suarez *et al*., 2012).

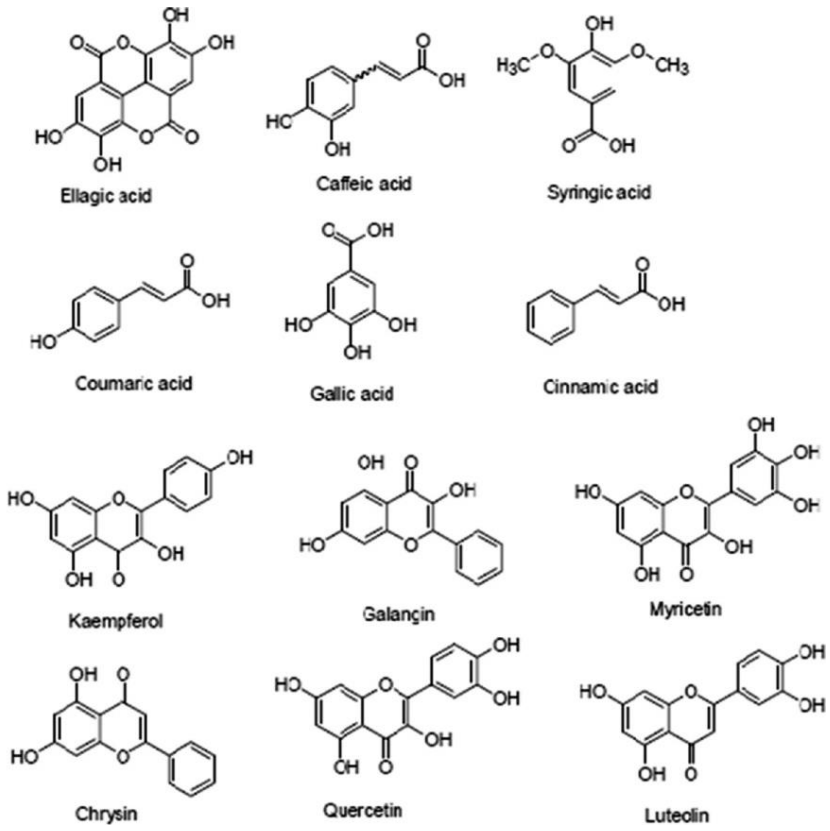

Figure 1.3 Chemical structures of some flavonoids and phenolic compound in honey
Source: Samarghandian *et al*., 2017

1.2.5 Honey produced in Thailand

In Thailand, there are five *Apis* species which are *A. andreniformis*, *A. florea*, *A. dorsata*, *A. cerana* and *A. mellifera* (Rattanawannee *et al*., 2007). The first four species are native to Thailand but *A. mellifera* has been introduced by man (anthropogenic) into the country for the apicultural industry (Wongsiri *et al*., 1996). To recognize these four native species, Rattanawannee *et al*. (2010) revealed that geometric morphometric analysis of the single wing alone could be used to identify four Asian honeybee species in Thailand and that the sex of the individual does not impede identification. The cavity nesting honeybee *A.cerana* and European hive honey bee *A. mellifera* are the mainly species purpose for beekeeping. *A. cerana* has been identified as native to eastern Asia containing Laos, Thailand, and Malaysia. Almost courses of *A. cerana* are little lower than *A. mellifera* and they have lower colonies. Dissimilar *A. mellifera*, *A. cerana* produces smaller products for beekeepers. *A. cerana* does not collect propolis and its colony has more ability to resist mites than that of *A. mellifera*. In common they made their nest in logs or in crater of abundant things, for example rock clefts, old wardrobes, or logs. Many local beekeepers construct beehives according to their folk wisdom. They driedout the center of tree trunks and wrap both ends with wooden caps. Three to five inches from the basement of the hive they build a hole for bee entry. Almost honeybee farms need moving-frame hives for *A. cerana*. Authrity of the colony is not difficult, but beekeepers have to always feed adequate food and prevent the colony from its enemies (ants, wasps) to small leave by colonies. Though *A. cerana* nests are found distributed throughout the country, a major beekeeping area of *A. cerana* is south of Thailand including Chumphon,Surat Thani, Nakhon Si Thammarat, Trang, Phattalung, Songkhla, Pattani, and Satun. *A. mellifera* is an introduced species and it is the predominant species for beekeeping since *A. mellifera* foragers have high ability to collect nectar. They use a specific chemical called “forage marking pheromone” to communicate to their nest mates. In addition, foragers will mark flowers with the pheromone after they have collected nectar. This will tell other foragers to avoid the flowers and go to other ones (Seanbualuang, 2012).

Bee products consist of honey, pollen, royal jelly, wax and broods with the total farm values of about 1,269 million THB (37.7 million USD) in 2013-2014 (Table 1.2). The major honey products are longan honey, lychee honey and white snakeroot honey. Among these three, longan honey has the highest volume with high quality and high price. The minor honey products have very small volumes with varying prices. Other bee products also have a small volume but the prices are quite high especially the royal jelly (Table 1.2). Honey was exported mainly to Germany, Taiwan and Saudi Arabia with the value of 577 million THB (17.1 million USD) in 2012 (Department of Agricultural Extension, 2014).

Table 1.2 Bee products in 2013-2014 with farm prices, volumes and values

	Products	Price/kg (THB)	Volume (Ton)	Value (THB)
Major honey	Longan	82.78	18,355.36	758,786,341.14
	Lychee	71.21	5,179.59	181,846,494.99
	Siam weed	67.46	3538.41	122,021,624.51
Minor honey	Sunflower	80.00	186.83	14,946,714.03
	Coffee	100.00	0.27	26.642.98
	Seasame	75.00	140.67	10,550,621.67
	Para rubber	60.00	125.89	7,553,285.97
	Rambutan	58.00	58.8	5,425,790.32
	American rope	110.00	11.99	1,318,827.71
	Romerillo	50.00	11.99	599,467.14

Source: Phankaew (2016)

Food sources of *Apis melifera* honeybees consist of 3 groups of crops. The first group is for nectar source only, the key crops are lychee, rambutan, para rubber and Siam weed. The second group is for pollen source only. The key crops are corn, giant mimosa and sensitive plant. The third group is for both nectar and pollen sources. The key crops are longan, sesame, sunflower and kapok (Table 1.3).

Table 1.3 Food sources with their distribution in different provinces

Key crops	Provinces
American rope	Chiang Mai, Chiang Rai
Coffee	Chiang Mai, Chiang Rai
Corn	Chiang Mai, Chiang Rai
Giant mimosa	Chiang Mai, Chiang Rai
Kapok	Chiang Mai, Chiang Rai
Longan	Tak, KhonKaen, Nakhon Ratchasima, Chiang Mai, Chiang Rai, Lumphun, Lampang, Phayao, Phrae, Nan
Lychee	Chiang Mai, Chiang Rai, Phayao, Samut Songkhram
Para rubber	Chiang Rai,Nakhon Sri Thammarat, Suratthani
Rambutan	Rayong, Chanthaburi, Suratthani
Romerillo	Chiang Rai
Sensitive plant	Chiang Mai, Chiang Rai
Sesame	Lopburi, Nakhon Sawan
Sunflower	Lopburi, Saraburi
Siam weed	Chiang Mai, Chiang Rai, Loei

Source: Phankaew (2016)

Apis mellifera honeybee is distributed mainly in the Northern provinces because the key food sources are in this area. There are two types of beekeepers, the first one keeps bee colonies at the same location all the time and the second one moves bee colonies to food sources. The key crops with their distribution in different provinces are shown in table 1.3. These crops are mainly distributed in the Northern provinces. The periods of the year which the key crops are available to honeybees are also important to apiculture industry. The first 3 months from January to March are the peak activity because the 2 key crops which provide high quality and big volumes of honey are available, also in December the activity is high because the availability

of the wild flower, white snakeroot, which is another main source of honey. The data in this regard is depicted in Table 1.4.

Table 1.4 Periods of the year with the availability of key crops

Month	Key crops
January	Lychee, Kapok
February	Longan, Rambutan, Coffee
March	Longan
April	Para rubber, American rope
May	Corn
June	Sesame
July	Corn
August	Corn
September	Romerillo
October	Giant mimosa, Sensitive plant
November	Sunflower
December	Siam weed

Source: Phankaew (2016)

Rambutan honey is one of natural medicine that often used in wound treatment. By means of smeared and drink, honey empirically is used to treat mouth burns, skin lesions and sores. In some health service, doctors use honey in patient's wound treatment. Indonesian community and government are trying to develop all the potential of the nature, including the proceeds of tropical fruit trees for example flower nectar and fruit. This effort is to support the repertoire of natural herbal remedy (Depkes, 2007). Rambutan honey is produced by *Apis mellifera*, a honey bee, from *Nephelium lappaceum* trees and collected from a beekeeping farm. Rambutan honey is known by Indonesian communities which empirically have been reported to heal wounds including oral mucosa wounds. With appropriate WHO quality standard, traditional medicine must meet several requirements including quality, safety and efficacy. The validity of safety/toxicity test is influenced by factors such as dosage test, preparation of dosage test, animals testing, doses, technique, national and international standardized testing procedures (Bpom, 2014).

Rambutan (N. *lappaceum Linn*.) is introduced in the Malayan archipelago, which contains Indonesia, Malaysia and South of Thailand (Indian Institute of Horticultural Research, 2012). The fruit shape in clusters; each fruit has a seed enclose by an aril which is wrapped by the pericarp and spinterns (1-1.5 cm long). The fruit is related affluent in sugar, vitamin and mineral compounds; a lower soluble solid composition of 16 °Brix may be analysis, and may influent the level as maximum as 17 to 21 °Brix which depended on cultivates (Kadar, 2013; Landrigan et al., 1998). Fullfillment of red color is the major harvest indicator. The postharvest element can reduce the attributes and flavor of the fruit with acutely maximum shelf life after 2-3 days at 40°C and 40 % RH. Fall of attributes is mainly because of spintern drying, even though color trouble can also provide to the fruit's deteriolation (O' Hare, 1992). As well as the result, the cost of rambutan can change based on the time taken during harvesting and selling to the consumers. Thus, the rambutan sugar granule improvement could so regared as an different to raise the fruit cost.

Northern Thailand is the leading longan-growing region with areas of more than 600,000 acres (ARDA, 2016). Longan honey is, in general, yellowish brown in color and has strong fruity scent and sweet taste (ATPC, 2016). During Thailand’s cold season, longan blossom becomes a good source of longan honey production.

Longan flower-honey is commercially produced in both Chiang Mai and Lumphun provinces Thailand. It is mainly used as a natural sweetener in various food products, and is composed of a concentrated aqueous solution of inverted sugars and various nutrients such saccharides, amino acids, phenolics, flavonoids, vitamins and minerals (Akhmazillh *et al*., 2013; Gheldof *et al*., 2002; Moniruzzaman *et al*., 2013).

1.2.6 Biological activities

Honey has numerous vital biological bioactive complexes including vitamins A (in form of Retinol), Vitamin B1 (Thiamine), Vitamin B2 (Riboflavin), Vitamin B6, Vitamin C (Ascorbic acid), Vitamin E (Tocopherol), Vitamin K

(AntiHaemorrhagic), Niacin, Panthothenic acid and fatty acids, phenolics, hydroxybenzoic acid, octadecanoic acid, cinnamic acid, flavonoids and ethyl ester (Bogdanov *et al*., 2008; Muhammad *et al*., 2015). Honey also contains apigenin, pinocembrin, acacetin and acids like abscisic and ferullic (Marghitas *et al*., 2010; Muhammad *et al*., 2014). Also, abundant amino acids of physiological significance are cysteine, arginine, proline, aspartic acid and glutamic acid (Qamer *et al*., 2007). It consist of any flavonoid, ascorbic acid, amino acid phenolic, protein and carotenoid composition according to their climate circumstances and topographical positions (Alvarez-Suarez *et al*., 2010). The present of active composition allow better sensitive of the promising biological character of the honey.

1.2.6.1 Antioxidant properties

The word “oxidative stress” explains the poor equity during free of charge radicals and antioxidant protective activity (Bogdanov *et al*., 2008). Antioxidant is a composition that can inhibit the oxidation of additional molecules. Oxidation is a biochemical respond that production free radicals for chain action that may decrese the cells and finally the physiological aims. Antioxidant like vitamin C (Ascorbic Acid) decline the chain actions to protect the body against free radicals. For perseverance the oxidative condition, human body assists involved system of excessiveing antioxidants. The food compose antioxidants have been assumed to prior the health. The literature advise that honey compose strong anti-oxidative agents. While an antioxidant capacity honey with phenolic compositions, has sufficient defensive activities in difference of various clinical conditions as aggravating disorders, cancer, coronary artery diseases, aging and neurological worsening (Kishore *et al*., 2011). Components similar polyphenols and phenolic acids provided in honey deviate according to the geographical position; for example, flavanol kaempferol compounds can be found in rosemary honey and quercetin composition in sunflower honey (Akan and Garip, 2011). Alvarez-Suarez *et al*. (2012) strong character of phenolics from monofloral honeys on humanoid Red Blood Cells (RBCs) membranes in conflict of oxidative deterioration. The result shows that honey build

RBCs oxidative deterioration most inference because its alteration within cell membrane and ability to entrance and appear at cytosol. Honey composes relevant antioxidants which are liable for biological reaction, increase of RBCs actions and defense. According to Bunting (2001) honey chelates and decommission free iron, which would alternatively catalyse the form of oxygen free radicals from hydrogen peroxide. However Alvarez-Suarez *et al*. (2010) found that all the Cuban honeys (85 monofloral honeys) studied produce the hydroxyl radical to changing ranges, upon extension of Fe^{2+} to the honey solution via Fenton-like reactions. A study by Henriques *et al*. (2006) established that singular the well-known manuka honey did not production few hydroxyl radicals via the Fenton action and that even the honeys that produced hydrogen peroxide and free radicals through the Fenton reaction like pasture honey also have an antioxidant capacity.

1.2.6.2 Wound healing

A report of honey's use in wound care by Molan (2006) has bring overpowering information that honey is a valid wound treatment alternative. With attention to wound treatment by honey function, the osmotic reaction of honey can produce dischange of lymph, which is capable to helped added oxygenation and contribute increase amount of nutrients on the wound external, including flushing away proteases that may prevent the restore processing (Molan, 2009). Furthermore, honey's osmotic reaction can generate a moist surrounds that is need for the fibroblasts to arrangement and pull the borders of the wound composed (Molan, 2001). The acidic pH of honey too addition the amount to aid wound healing because it can help to discharge the oxygen bring to haemoglobin (Molan, 2009). It has been signed that acidic of wounds can enhance the quickness of the healing processing (Kaufman, 1985; Leveen, 1973). A numerous of studies have thickly fortified that honey is an active medicinal healing for burns and infected wounds (Molan, 2001; Subrahmanyam, 1996) and it is many active like a dressing than many other present different (Vermeulen *et al*., 2005). Honey has been a device that has accepted a growing volume of concentration in wound care, specially in the responsibility of burn wounds. As ancient times, honey has been benefited by man for together food and medicine in individual perception (Jull *et al*., 2008). Conclusions the first

observation of honey in wound healing was in ancient Egypt during 2600 and 2200 BCE (Jull *et al*., 2008). Eventhough the accurate device for the useful conditions of honey in wound care is stable nameless, analysis has point to on the antibacterial activity of honey as a source. Honey is hygroscopic come from its large sugar composition, definition it has a dehydrating result that is inhibit for bacterial growth. Studies have show that the antibacterial activities of honey are more involved than just only large sugar composition. Ten Honeys concludes glucose oxidase, an enzyme that changes glucose to hydrogen peroxide, which may provide to few of its antibacterial activities (Jull *et al*., 2008; Simon *et al*., 2009; Langemo *et al*., 2009). Moreover, the antibacterial axtivities of honey present to diverge depend on the floral origin. Honey come from *Leptospermum* trees (manuka) or *Echium vulgare* bush (viper's bugloss) presented antibacterial activities liberated of hydrogen peroxide. It is accepted that other, yet undiscovered, composition of honey is important for the antibacterial activities. Diverse types of honey have been present to have antibacterial property against the following bacterial species *in vitro*: *Alcaligenes faecalis*, *Citrobacter freundii*, *Escherichia coli*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Mycobacterium phlei*, *Salmonella california*, *Salmonella enteritidis*, *Salmonella typhimurium*, *Shigella sonnei*, *Staphylococcus aureus*, and *Staphylococcus epidermidis*. Interesting, *Serratia marcescens* and the yeast *Candida albicans* were not prevented by honey (Lusby *et al*., 2005). Also, manuka honey has also been presented to have an inhibitory result on Pseudomonas aeruginosa, methicillinresistant *S. aureus* (MRSA), and vancomycin-resistant *enterococcus* species. In a current studied, ulmo honey presented greater anti-MRSA activity measured with manuka honey (Sherlock *et al*., 2010).Finally, honey shown combined with antifungal property.

1.2.6.3 Antimicrobial activity

Honey has an extensive past of benefit as an productive medicine because antique culture for a ample length of disease action (Molan, 2001). The physiological activity of honey has been characteristics to giving of hydrogen peroxide schemed by the enzyme glucose oxidase; antioxidant activities, below pH

value; osmotic reaction, and a difference of enzymes (Molan, 2009). One of the basics characteristic of honey is its antimicrobial activity, which admits honey to be collected for a long duration after come to destroyed (Al-Mamary *et al*., 2002). The antibacterial properties of honey are colleague with its huge osmolarity, acidity, production of hydrogen peroxide, and non-peroxide antibacterial components such as flavonoids, lysozyme, and the phenolic acids (Molan, 1992; Postmes *et al*., 1993; Sonwdon and Cliver, 1996; Wahdan, 1998; Willix *et al*., 1992). Hydrogen peroxide in honey is production by glucose oxidase excreted from the hypopharyngeal glands of bees (Molan, 2009). The leveled of hydrogen peroxide is capacity to relative leveled of glucose oxidase and catalase come from pollen (Weston, 2000). Nevertheless, not all honey has the alike therapeutic alike because huge difference in its antibacterial property (Molan, 2001). The changing antibacterial property between honeys depends on its floral origin (Allen *et al*., 1991). A study by Wahdan (1998) has displayed that with 21 forms of bacteria, containing *Escherichia coli*, *Klebsiella sp*, *Pseudomonas sp*, *Staphylococcus sp*, and two forms of fungi r*in vitro*, honey overcomed more pathogens than sugar command, and undiluted honey effectively prevent the growth of all 21 bacteria. The MIC (minimum inhibition concentration) of honey was found to be range from 1.8% to 10.8% (V/V) (Molan, 2001). Eventhough both of Gram-positive and -negative bacterial strains are conscious to honey, few Gram-negative bacteria (*Salmonella dublin* and *Shigella dysenteriae*) being more affected than gram-positive strains (*Bacillus cereus*, *Staphylococcus aureus*) (Bogdanov, 1984).

1.2.6.4 Anti-inflammatory activity

The anti-inflammation activities of honey have been known well (Molan, 2001). Honey has been found to have the difficulty of scavenging capacity of reactive oxygen species important for initation of inflammation (Greten *et al*., 2004). Though honey is used to wounds, it actively decreases the inflammation (Burlando, 1978; Subrahmanyam, 1998), including lowering oedema around wounds (Dumronglert, 1983; Efem, 1988; Efem, 1993) and sweat from wounds (Burlando, 1978; Efem, 1988; Efem, 1993; Hejase *et al*., 1996). Honey has also been examine to cure the pain that is a characteristics of inflammation (Burlando, 1978; Keast-Butler,

1980; Subrahmanyam, 1993). The studies of curing animal tissues have shown that the leucocyte numbers accompliled with inflammation have been fewer when the wounds have been delight with honey (Subrahmanyam, 1998). Like conclusions examined in animal study models have approved that the anti-inflammation reaction of honey cannot be because elimination of bacteria only (Oryan, 1998; Postmes, 1997). Moreover, honey has been display to reduce the stiffness of inflamed wrist combined of guinea pigs (Church, 1954). One of the anti-inflammatory results of honey can be featured to its antibacterial property because compositions of the bacterial cell wall are effective causes of the inflammatory reaction (Molan, 2009). The existence of slough in wounds too actions as an inflammatory stimulations, and slough elimination by honey function to wounds has display to relief reduce inflammation (Efem, 1988).

1.2.6.5 Antidiabetic activity

Diabetes mellitus is one of the highest diseases in current time, with over 285 million patients approximate in 2010 and around 438 million patients conclude for 2030 (Shaw *et al*., 2010). Diabetes predominance may be historical regulated or can be advances between life at some age. This disease has no detail of age for cases, but scientists studies explain that it is more ordinary indeveloping provinces than in the inactivity of the world (developed provinces and provinces in the third world) (Shaw *et al*., 2010). Eventhough diabetes mellitus is a chronic disease of endocrine diagnosis and still the main cause of death worldwide (Kokil *et al*., 2010; Roglic and Unwin., 2010). It’s not a death punishing. Present, the medical world is revolving increasingly swirling about the health benefits of natural products, medical herbs, and the honey with power of this disease, in the authority of this disease. Together with simple medical cure, using formulars of classic medicine, containing the benefit of apicultural products (such as honey), the diabetic patients can keep the blood insulin level and too their comprehensive health circumstances. The content of honey consist more than 200 compounds, with glucose, fructose, and water like major elements. Honey have benefit in folk medicine back in time at the starting of our stage, but their health is mutally beneficial, only on eye in vestigations, outsides

having some base for some scientific support. In the past decades, scientists have been concerned about explaning and testing the benefits of honey.

1.2.7 Oligosaccharides in the Gut Microbiota

The oligosaccharides are not digested by human intestinal hydrolases/hydrolyze enzymes (Manning and Gibson., 2004). The early section of the small intestine is the duodenum, which is acid (pH 4-5) and a comparatively huge amount of bacteria (10^2-10^4 CFU/ml) are found in this part (O'May *et al*., 2005) as displayed in figure 1.3. Lactobacilli, streptococci, veillonellae, staphylococci, actinobacilli, and yeasts are the major conspicuous structures in the jejunum and duodenum (Booijink *et al*., 2010). The GI microbiota variations significantly from the duodenum to the ileum because an addition in pH and decline of oxidation and reduction promisings, cause to an addition of the bacterial amount which can range to 10^6-10^8 CFU/ml. In the large intestine, the pH is impartial and insides transportation time additions, which establish the bacteria to change a lot (10^7-10^{12} CFU/ml) and are intense various (figure 1.3). At the same time, while the environment of large intestine is exacting anaerobic, require anaerobes, which come from the efficiency of fermentation, dominate in this section. More than 1200 bacterial groups have been recognized in the human's colon, each of which healthy distinctive pier at below 160 division groups (Qin *et al*., 2010). Nevertheless, most (>80%) of the GI microbiota cannot be cultured *in vitro*, constaining the benefit of molecular approach (Eckburg *et al*., 2005).

Current information shown that oligosaccharides can re-balance the equibilium of gut microbiota environment. Alternatively, they are digested, by gut microbiota, to production short-chain fatty acids (acetic, lactic, propionic and butyric), gases (CO_2, H_2, and CH_4) and other digestions (Delzenne, 2003; Manning and Gibson., 2004; Miller and Wolin., 1996; Grizard and Barthomeuf., 1999). Analysis indicated that oligosaccharides can restain growth and action of damage or pathogenic bacteria like Clostridium, Staphylococcus, Veillonella, Proteus, and Escherichia (Blaut, 2002; Gibson *et al*., 2004; Gibson and Roberfroid., 1995; Sabater-Molina et al., 2009). Determinant such as the group of sugar monomers, a description of

glycosidic bond during sugar moieties, and range of polymerization effect fermentation of oligosaccharides and another prebiotics in the gut microbiota (Swennen *et al*., 2006). The capacity of gut microbiota to discriminating digestible oligosaccharides is aspected to their intrinsically certained hydrolytic enzymes like xylanase, β-glucosidases, α-glucosidases, β-fructosidase, β-galactosidases and α-galactosidases (Manning and Gibson., 2004; Henrissat and Bairoch., 1993). Otherwise, oligosaccharides improve the growth and action of useful or non-pathogenic bacteria like bifidobacterium, lactobacillus, eubacterium, and few streptococcus, enterococcus, and lacteroides (Blaut, 2002; Gibson *et al*., 2004; Gibson and Roberfroid., 1995; Sabater-Molina et al., 2009).

A current study, in human flora-accompliced piglets, indicated that short-chain fructooligosaccharides (scFOS) excited the growth of useful bacteria, bifidobacteria and bacteroides, during FOS restained the growth of pathogenic Clostridium leptum subgroup (Shen *et al*., 2010). Human subjects who absorbed fructooligosaccharides were information to have gain fecal bifidobacterial amounts and beta-fructosidase action (Bouhnik *et al*., 2007). Other currently announced data showed that FOS supplementation in human subject's important growth fecal amounts and regular fecal gain of bifidobacteria (Yen *et al*., 2011). The study found that increase or growth of the piglets influenced the prebiotic result of scFOS on these microorganisms other than the bifidobacteria (Shen *et al*., 2010). The study additional indicated that the result of FOS on bifidobacteria growth was maintained after the break of FOS supplementation (Yen *et al*., 2011). Documentation besides showed that the short-chain fatty acids are fast consumed in the main intestine, and are afterwards metabolized in convinced tissues like liver and muscle (Sabater-Molina et al., 2009). The improved action or development of microbiota after oligosaccharide supplementation is attributed to the oligosaccharide come from metabolites specially the short-chain fatty acids (Swennen *et al*., 2006; Sabater-Molina *et al*., 2009). The short-chain fatty acids produced from hydrolysis of oligosaccharides role a valuable play in the useful and systemic results of oligosaccharides. Data also showed that FOS improves the intestinal digestion of mineral ions in rat (Wang *et al*., 2010).

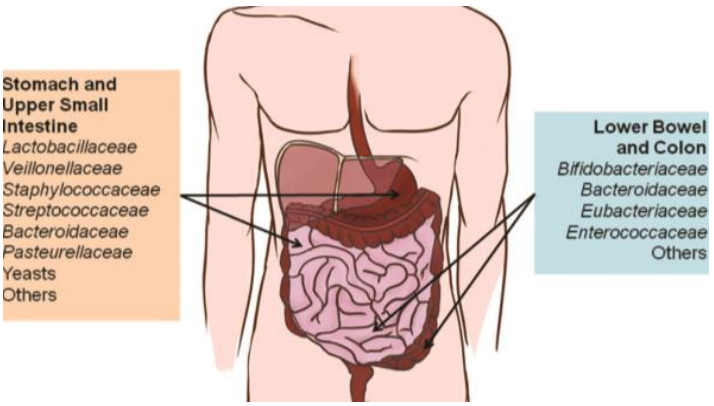

Figure 1.4 Outline of the human gut microbiota. The stomach and upper small intestine include yeasts and bacteria which both can enter up to 10^4 CFU/ml of gastric juice. In similarity, the lower bowel and colon include a wider differ of bacteria which can enter up to 10^6 CFU/ml in the lower bowel and as high as 10^{12} CFU/ml in the colon.

1.3 Objectives

1.3.1 To determine the physicocahemical properties of Thai originated honeys

1.3.2 To comparison of biological activity of Thai originated honeys

1.3.3 To evaluate the prebiotic potentail and growth of gut microbiota from oligosaccharides of Thai originated honey

1.4 References

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CHAPTER 2

PHYSICOCHEMICAL PROPERTIES OF THAI ORIGINATED HONEYS

2.1 Abstract

The aim of this chapter was to determined the physical and chemical properties of three honeys which were obtained from different sources of nectar. Three honeys including longan honey, rambutan honey and para rubber honey. The physical analysis includes pH, moisture content, electrical conductivity, viscosity and color. The range of pH in all honeys was 4.04 - 4.44, moisture content ranged 14.84% - 17.80%. The results showed electrical conductivity and viscosity in the range of 0.352-0.518 mS/cm and 2755.67-3220.33 cP, respectively. The Pfund color ranged 22-44. All obtained values are in the established codex standard values of honey. The total phenolic and flavonoid content were evaluated, and ranged from 217.80 to 428.59 mg gallic acid $100g^{-1}$ and 138.67 to 160.94 mg of quercetine $100g^{-1}$ of honey, respectively. The sensory analysis by 9 – points principal components showed higher value for overall acceptance in longan floral honey. Honey's sensory difference may due to the different in physical and chemical quality. From the obtained results, it was observed that longan honey exhibited the best of organoleptic attributes followed by the one rambutan honey, while the one from para rubber honey showed the least.

2.2 Introduction

Physical and chemical properties of honey is one character to judge the quality of honey. It is a natural food mainly composed of sugars and water together with minor constituent such as minerals, vitamins, amino acids, organic acids, flavonoids and other phenolic compounds and aromatic substances. The major constituents of honey (75%) are monosaccharides (fructose and glucose), with low quantities of disaccharides (sucrose) and polysaccharides. Honey contains different quantities of

minerals ranging from 0.02 g/ 100 g to 1.03 g/100 g, with potassium being the most abundant element comprising approximately one-third of the total mineral content (Chakir *et al*., 2011). The major physical properties include its color, viscosity, electrical conductivity, moisture contents. Both bio-chemical composition is particularly variable, depending on its source of collection, preservation and processing techniques and can considered as the important indicator for analyzing the honey quality.

The great valuable index is the origin of nectar and which is a foreign indexs. Extrinsic factors contain of region climatic indexs, kind of soil, distance and production prodecures of the beekeepers. The feature of honey is major accomplice with its botanical origin and chemical properties. Honeys that are of distinctive botanical and regional origin include varies compositions (Cinar, 2010). Chemically, honey is a highly concentrated solution of a complex mixture of sugars, predominantly fructose and glucose, with sucrose, maltose, and many other sugars at much lower concentrations. In addition to sugars, honey also contains small amounts of minerals, proteins, vitamins, and organic acids. The composition of phenolic compounds depends strongly on the plant species from which the nectar was collected (Iurlina *et al*., 2009). Furthermore, the resulting honey is also influenced by others factors, such as environmental conditions and climate (Iurlina *et al*., 2009; Pyrzynska and Biesaga, 2009; Montenegro *et al*., 2013).

In Australia, Ajlouni and Sujirapinyokul (2010) calculated the honeys of vary manufacturers in parts of HMF and amylase composition. Chemical properties of the honeys important different between the samples. pH, total acidity and moisture composition ranged between 4.02 - 4.69; 33.5 - 53.5 meq/kg; and 10.6% - 17.8%, respectively. The data indicated that heating processing affected on the contents of the honey, pH value and the botanical of honey all provided to thisdifference. Other country in which important studies concerning honey were achieves is Portuguese. Mendes *et al*. (1998) examined the characteristic factors of 25 honey samples and evaluated carbohydrate levels by HPLC analysis. Chemical criterions examined were HMF level, moisture and ash compositions, free acidity, diastase activity, and the content of insoluble materials. They also accomplish organoleptic evaluations.

Analysis of phenolic and flavonoid compounds has also been regarded as a very promising way of studying floral and geographical origins of honeys. For example, hesperetin has been used as a marker for citrus honey and keampferol for rosemary honey as well as quercetin from sunflower honey (Thomas-Barberan *et al*., 2001). Some phenolic acids (e.g., ellagic acid) (Antony *et al*., 2002) in honey and hydroxycinnamates (caffeic, *p*-coumaric and ferulic acids) in chestnut honey (Merken and Beecher, 2002) have also been used as floral markers honeys.

As known that physical and chemical properties are the characteristic of qualifised honeys. Due to those parameters of honeys originated in Thailand are still poorly explored, Therefore the aim of this work was to determined physical and chemical properties as well as sensory analysis to fulfill information and become beneficial data for apiculture and honey market from Thailand.

2.3 Materials and methods

Honey produced from different sources including longan (*Dimocarpus longan*), rambutan (*Nephelium lappaceum L.*) and para rubber (*Hevea brasiliensis*). Three species were obtained from beekeepers in different provinces of Thailand (Lumphun and Suratthani). All honeys were produced by *Apis mellifera*. The honey samples were stored at 4 ^{o}C and they were kept overnight at 25±2 ^{o}C before analyses.

2.3.1 Analysis of physical properties

2.3.1.1 pH

The honey solution was prepared by 5 g of honey in 20 ml of distilled water and was measured by a pH-meter (Metler Toledo, Model FiveEasy, Switzerland) according to AOAC method number 962.19 (AOAC, 1990).

2.3.1.2 Electrical conductivity (EC)

20 g of honeys were suspended in 75 mL of distilled water in volumetric flask and make the volume upto 100 mL. EC in mS/cm were measured by Orion 5 star multimeter (Mettler-Toledo, Switzerland).

2.3.1.3 Color

The color of honey was categorized by Pfund scale. Color grades were expressed in millimeter (mm) Pfund grades using glycerol as reference standard for comparison. HANNA colorimeter (C 221 Honey Color Analyzer, Hungary, Europe) was used to measure. Put the honey into the cuvette and read the data from colorimeter.

2.3.1.4 Viscosity

A Brookfield DVII fitted with needle number 64 (Brookfield Engineering Labs, MA) was used to determine viscosity at 25^{o}C. The honey samples were analysed in triplicate. Pour 500 ml of honey sample in to a beeker amount 600 ml before put the needle for determined.

2.3.1.5 Moisture content

Moisture contents in honey samples were analyzed using modified method of the Association of Official and Analytical Chemists (AOAC, 2000) by following steps: (1) heating the pre-weighed aluminum crucibles and lids at 105°C for 3 h and cooling them in a desiccator; (2) adding 5 g (W1) of honey samples to the crucible and heated in vacuum oven at 105°C for 24 h or until obtaining a constant weight; and (3) cooling in a desiccator and weigh the sample (W2). The moisture content of the sample can be calculated by below equation (1).

$$\text{Moisture (\%)} = \frac{(W1-W2)}{W1} \times 100$$

2.3.2 Analysis of chemical properties

2.3.2.1 Analysis of sugar profiling

Sugar and oligosaccharide of three honeys were analysed using by High-Performance Anion Exchange Chromatography coupled with Pulsed Amperometric Detection (HPAEC-PAD a Dionex-DX-300 equipment containing a gradient pump and an eluent degas module. Separation of carbohydrates was carried out on a CarboPac PA 10 anion-exchange column and 5 g of honey sample were dissolved in 50 mL DI water and filtered through a 0.45 µm filter. The 20 µL of honey samples was injected and flow rate was 0.7 mL/min and the sugar profile were detected by pulsed amperometric detection (PAD).

2.3.2.2 Determination of total phenolic content

The total phenolic content was analysis by the Folin-Ciocalteu method and the results were expressed as mg gallic acid/100g honey. First, Honey solutions were prepared at a concentration of 1g/mL. And aliquot of 1 mL of the stock solution was mixed with 0.3 mL of the Folin-ciocalteu reagent and 2 mL of 15 % sodium carbonate solution. Distilled water was added to a final volume of 5 mL. After that, incubated for 2 hrs, the absorbance of the reaction mixture was measured at 798 mm against a blank of distilled water. (Socha *et al*., 2007; Ferreira *et al*., 2009).

2.3.2.3 Determination of total flavonoid content

The total flavonoid content was determined using the method of Dowd as adapted by Arvouet-Grand *et al*., (1994) with some minor modifications. Briefly, 5 ml of 2 % aluminium trichloride ($AlCl_3$) in methanol was mixed with the same

volume of a honey solution (1g/ml). Absorption readings at 415 nm were measured in UV-visible spectrophotometer after 10 min against a blank sample consisting of a 5 ml honey solution with 5 ml methanol without $AlCl_3$. The total flavonoid content was determined using a standard curve with quercetin as a standard. The mean of three readings was used and expressed as mg of quercetin equivalents (QE) in methanol/100 g of honey.

2.3.2.4 Determination of proline content

Proline content in honey samples was measured using a 5 g honey in 100 mL distilled water. 0.5 mL of solution was put into test tube then 1 mL of formic acid, ninhydrin solution proline standard solution were added into tube. Then it was boiled in hot water for 15 min and incubated for 15 min at 70^{o}C in water bath. After the tube was cooled down for 45 min. Absorption was followed an UV/VIS spectrophotometer at 510 nm.

2.3.3 Sensory evaluation

The sensory evaluation was performed by untrained panelists using a nine point hedonic scale ranging from 1 (dislike extremely) to 9 (like extremely) for the parameters including Appearance, Color, Viscosity, Aroma, Flavor, Taste, Sweetness and Overall acceptance.

2.3.4 Statistical methods

The results were expressed in mean and standard deviation and correlation was obtained by using Microsoft Excel 2010. The significant difference were obtained by a one-way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT) ($p<0.05$) using SPSS software (version 16.0, IBM Inc., Chicago, IL, USA).

2.4 Results and Discussion

2.4.1 Physicochemical properties

Physical properties of honey (pH, electrical conductivity, moisture content, viscosity and color) shown in Table 2.1, The difference was statistically significant ($p<0.05$) in all parameter of all honeys. The moisture in the Thai honeys ranged from 14.84% to 17.80% and all of them were below the maximum limit of moisture content (≤20%) for honey recommended by the international quality regulations (Codex, 2001). The moisture is a very important parameter because it is a useful parameter to describe the humidity and viscosity of floral honey and it also depends on the several factor, for example climatic factors, harvest season, levels of moisture in bees and environmental factors. In addition, the moisture content of honey is also related to the botanical origin. Honey has a moisture content of less than 20% which is safety for fermentation. Moisture content is an important criterion in analyzing the ability of fresh and non-fermented.

As the moisture content present in Thai floral honey is important and contributes to its ability to resist fermentation and granulation during storage, low moisture content in the Thai honeys indicates its good storage ability and can lead to undesirable honey fermentation due to osmotolerant yeasts, which form ethyl alcohol and carbon dioxide (Bogdanov *et al*., 2004). To guarantee the quality of honey, the proper timing of honey extraction and degree of maturity of honey in the hives should be taken into account for each honey type. The electrical conductivity of honey samples is nearly relevant to the composition of mineral salts, organic acids and proteins; it is a factor that indicated high variability according to the floral origin and is recognized one of the high parameters for separated between blossom honeys and honeydews (Mateo and Bosch-Reig., 1998; Terrab *et al*., 2002).

The electrical conductivity (EC) is one parameter that correlated with pollen content in honey. Electrical conductivity of honey according to international quality reguations (Codex, 2001) was 0.8 mS/cm. The electrical conductivity of thai floral honey analyzed was in standardized of 0.352-0.518 mS/cm shown in Table 2.1.

The electrical conductivity was the highest in Para rubber honey and Longan honey respectively, the Rambutan honey is showed the lowest electrical conductivity. The EC values are almost nearly with the study from Thailand, 0.53 mS/cm to 0.71 mS/cm (Suntiparapop *et al*., 2012). The electrical conductivity expresses the ability of the aqueous solution to conduct an electric current. It is positively correlated with the soluble mineral content. The EC is an important criteria for determination of floral origin and also to regulate the concentration of organic acids, minerals, and proteins (Karabagias *et al*., 2014; Wanjai *et al*., 2012). Other factors, such as floral source, amount of organic acids and proteins, and storage time can also influence the electric conductivity of honey (Karabagias *et al*., 2014).

The pH is a measure of quality and contained in international standards is shown in Table 2.1. The results showed that Thai honeys has the properties of pH within the international standard (Bogdanov, 1999). All honey has a range of pH is 4.04 - 4.44. Longan honey has a higher pH values than other honeys. These values are similar to those reported for other honey samples from India, Brazil, Spain and Turkey, which would have a pH between 3.49 and 4.70, the variation in pH would be due to the buttered flora, the salivary secretion of the bee and the enzymatic and fermentative processes during the transformation of the raw material (Azeredo *et al*., 2003).

The three honeys showed that color ranging from 22 to 44 mmPfund scale (Table 2.1), which ranged from white to extra white amber. Honey color intensity depends on the floral origin. It is closely related to its chemical composition, mainly in the presence of colorants such as chlorophylls, carotenoids, flavonoids and derivatives of the tannins and polyphenols. Monica *et al*, (2007), reported that the nectar of honey color is dark and strong, resulting in the highest mineral content but also the presence of algae and green algae that part of the flora of the forest trees. The color of honeydew honey depends on the raw material composition of the food given to the bees.

The viscosity of a honey sample is a function of the composition of its sugars, water and colloid content. If the concentration of water is increased, honey

becomes less viscous. Proteins and other colloidal substances increases honey viscosity, but their amount in honey may be insignificant. The percentage of fructose content in honey has also been found to affect is viscosity and rheological properties. Honeys become less viscous with increase in fructose content. The viscosity of various honey samples from different sources around the globe had been reported (Karoui *et al*., 2007; Graciela *et al*., 2004; Hayette *et al*., 2007; Al-Khalifa and Al-Arify, 1999). Honey's viscosity may depends upon the amount of water and the type and amount of sugars it contains. If the concentration of water is increased, honey becomes less viscosity. Temperature also changes the viscosity of honey. The viscosity of honey samples ranged the viscosity of 2,755.67 to 3,220.33 Centipoise as shown in Table 2.1. but in this study not correlated between moisture and viscosity in longan honey due to not just only moisture content can correlated with viscosity but some substance such as protein and other affect to viscosity in honey, too.

Table 2.1 The various parameters tested for physical properties of Thai honeys

Thai honeys	pH	Electrical conductivity (mS/cm)	Color (Pfund scale)	Moisture content (%)	Viscosity (cP)
Para rubber	4.27 ± 0.04^{b}	0.518 ± 0.01^{a}	22 ± 1.53^{c}	14.85 ± 1.63^{b}	2755.67 ± 78.77^{b}
Rambutan	4.04 ± 0.02^{c}	0.352 ± 0.01^{b}	38 ± 1.00^{b}	14.84 ± 0.23^{b}	3090.33 ± 81.93^{a}
Longan	4.44 ± 0.01^{a}	0.353 ± 0.01^{b}	44 ± 0.58^{a}	17.80 ± 0.86^{a}	3220.33 ± 19.50^{a}

Mean±SD. Difference letter in a column show significant different ($P<0.05$). The difference letter $^{a, b, c}$ compared within same parameter among different honeys.

2.4.2 Proline content

The proline content of Thai originated honey samples is presented in Figure 2.1. The lowest proline content was determined in para rubber honey (116.25

mg/kg) and the highest content in Longan honey (785.75 mg/kg) and were following by rambutan honey (583.75 mg/kg). In the other honey types the proline content is higher than 500 mg/kg. In honeys the proline content changed on a wide range because in these honeys the nectar and pollen ratios are very different. As there is no limit value for proline content of honey. We used the international value, that is minimum 180 mg/kg, for measured is accepted in honey control laboratories (Codex, 2009). However, it should be taken into account that there is considerable proline variation, depends on honey origin. The honey proline content is a criterion of honey ripeness and in some of cases, also of sugar adulteration. The oxidative proline degradation pathway utilizes proline as a source of energy. Proline is rapidly metabolized and results in the production of multiple nicotinamide adenine dinucleotide phosphate (reduced form) equivalents and high levels of adenosine triphosphate (ATP). No other amino acid can be metabolized as rapidly as proline and release as much ATP without complete metabolism. (Carter *et al*, 2006). Another mechanism by which proline protects cells against stress has been suggested to involve the chelation of metals. High proline content in metal-tolerant plants is not unusual (Sharma and Dietz., 2006). One of the major toxicities of heavy metals is perturbation of cellular redox balance by ROS production. A potent oxidizing agent of biological macromolecules in the cell is the hydroxyl radical ($OH^{\bullet}$), which is formed by the reduction of H_2O_2 by transition metal ions such as Cu^{+} and Fe^{2+}. The function of proline as a metal chelator was suggested by Sharma *et al.*, who reported that proline can protect enzymes from zinc- and cadmium-induced inhibition by forming proline-metal complexes (Sharma *et al*., 1998). A copper–proline complex was also reported in the copper-tolerant.

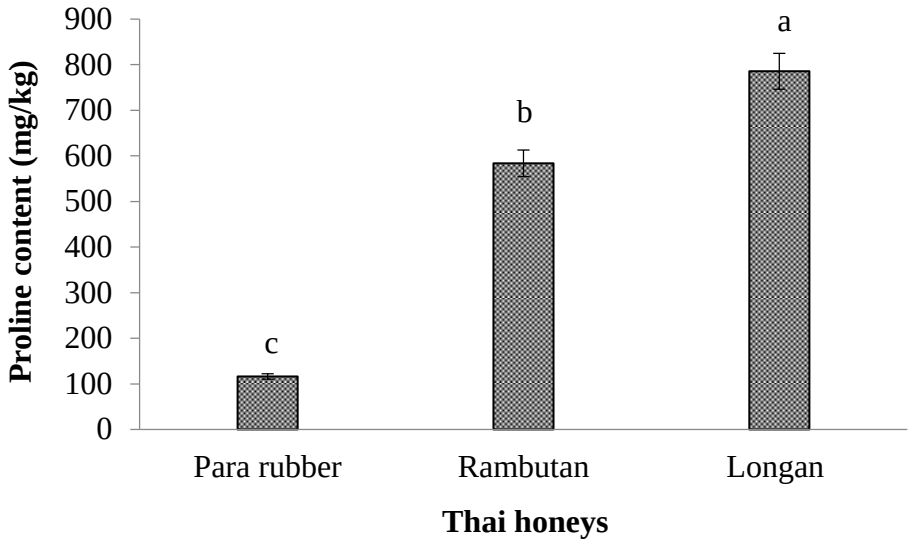

Figure 2.1. Proline content of honey samples. Values in the bar with different letters are significantly different ($P<0.05$). ^{a,b,c} compared among difference honeys.

2.4.3 Sugar and oligosaccharide contents

Reducing sugar was determined in rambutan honey (73.21%), followed by longan honey (71.38). The lowest % of reducing sugar (70.35%) was recorded in Para rubber honey (Table 2.2). Honey is a mixture of sugar and other compounds, honey is mainly composed with fructose (about 35.89-38.81%) and glucose (about 32.57-34.61%). Honey's remaining carbohydrates include maltose, sucrose, oligosaccharides and other complex carbohydrates. In this finding, monosaccharide content ranged from 70.35 – 73.21 g / 100 g of honeys and amount of fructose was abit higher than glucose as in the literature of honey sugar. For this study found fructose and glucose in all of honey samples as rambutan honey (38.60 and 34.61), longan honey (38.81 and 32.57) and para rubber honey (35.89 and 34.46), respectively. Almost 95% of the dry weight of honey contains of carbohydrates (White, 2005). The quantity of fructose and glucose, F/G ratios and onces the quantity of distinctive di- and oligosaccharide can be benefited in the characterization of unifloral honeys (Bogdanov *et al.*, 2004). In order to prevent customers against honey adulteration, the EU values for the total amount of F+G for nectar honey is raise to 60 g/100 g and for honeydew honey raised to 45 g/100 g (European Commission, 2002). Oligosaccharide in honey may have a distinctive act in its nutritional and health

benefits (Al-Qassemi and Robinson, 2003), but only the amount of maltose and raffinose in honeys has few value in the categorization of unifloral honeys. The crystallisation of honey is a very involved processing and it can be influenced by abundant factors, e.g. the quantity and equilibrium of fructose and glucose. The F/G ratios that are important in reconized the botanical source of honey can also be important in predicting the crystallization trend of honey. Provide that the F/G ratio more than 1.3 honeys does not crystallize. Otherwise, if the F/G ratio is below 1.3 the honey will crystallize very rapid (Manikis and Thrasivoulou, 2001). In the recent study, these common dietary fibers are mentioned to as regular dietary fiber. Almost prior studies have targeted on the results of oligosaccharides on provide gut microbes, with some studies analyzing the results of oligosaccharide and general dietary fiber on the gut flora as a entire (Dey, 2017). In this study, longan honey showed highest amount of oligosaccharides were raffinose. Therefore, longan honey was selected for further study on gut microbiota and promotion of prebiotic growth.

Table 2.2 Sugar and oligosaccharide content in Thai honeys

Sugar and oligosaccharide (g/100 g)	Longan	Rambutan	Para rubber
Monosacharides			
-Glucose	32.57	34.61	34.46
-Fructose	38.81	38.60	35.89
Total	71.38	73.21	70.35
Oligosaccharides			
-Maltose	0.88	0.70	1.26
-Raffinose	2.78	1.82	1.36

2.4.4 Total phenolic and flavonoid contents

Analysis of phenolic compounds used colorimetric method by spectrophotometer which a popular is folin-ciocalteu. The principle is to used folin-

ciocalteu, which reacts with the phenolic compounds in honey and the purple compound occur. Its absorbance was 765 nm and the absorbance was measured. Comparison of phenolic compounds from the standard graph used standard compound was gallic acid because it is a phenolic compound, the smallest unit of tannin found in plants. Therefore, the amount of phenolic compounds in honey shown the equivalent in milligrams of gallic acid per gram of honey.

Phenolic compounds are associated with antioxidant activities (Yangthong and Hutadilok-Towatana, 2014) because they have a structure that consists of an aromatic ring having at least one hydroxyl group its can give electrons to atoms or molecules with non-dual electrons, also known as radicals can helps inhibit or antagonize oxidation. Phenol compounds are polyphenol, the principle constituent of secondary metabolites in plants, which are naturally abundant in nature from simple molecules to complex molecules. This study showed that longan honey had the highest phenolic content (428.58 mg GAE/100g) followed by rambutan honey (262.52 mg GAE/100g), respectively. Para rubber honey showed a minimum amount of phenolic content (217.08 mg GAE / 100g) as shown in table 2.3. Limpawattana (2013) indicated the range and average values of total phenolic content observed for the Thai honey samples including (lychee, longan, korlan, sunflower, plaunoi, sesame and sabsue) are ranging from 587-1,652 mg GAE/kg. Venezuelan honey (3811,821 mg GAE/kg (Vit *et al*., 2009), Nigeria honey (362-1,028 mg GAE/kg) (Buba *et al*, 2013.) Czech (39-167.1 mg GAE/kg) (Lachman *et al*., 2010), Poland (71.7- 201 mg GAE/kg) (Kaskoniene *et al*., 2009), Malaysia (144-580 mg GAE/kg) (Moniruzzaman *et al*., 2013) and monofloral Cuban honey (213- 595 mg GAE/kg of honey) (Alvarez-suarez *et al*., 2010).

Flavonoids are an important group of polyphenols. Structural categories of flavonoids include flavones, for example, apigenin, luteolin, flavanones, for example, hesperetin, catechins, for example, epicatechin, epigallocatechin-3-gallate (EGCG), and anthocyanins, for example, cyaniding (Hendrich, 2006). The flavonoid compounds was achieved by the analysis of aluminum chloride and is common on the formation of a mixture during the aluminum ion, Al (III), and the carbonyl and hydroxyl groups of flavones and flavonols that provide a yellow color

(Popova *et al*., 2004). In this study, all three honeys flavonoids were high. Longan honey has the highest amount of flavonoids (160.94 mg QE/100 g) followed by rambutan honey (152.73mg QE/100 g) and para rubber honey (138.67 mg QE/100 g) (Table 2.3). The flavonoid contents in this study were more than the results gather for honey from the northeast of Brazil, which ranged from 2.5 to 83.8 mg of quercetin kg^{-1} of honey (Liberato *et al*., 2011). Previous studies have reported high flavonoid contents in different kinds of honey such as Portuguese, Burkina Fasan and Cuban honeys (Alvarez-Suarez *et al*., 2010). Flavonoids are recognized for their high pharmacological activities as radical scavengers. Recent interest in these substances has been stimulated by the potential health benefits arising from their antioxidant activities and free radical scavenging capacities in coronary heart disease and cancer (Yao *et al*., 2004)

Table 2.3 Total phenolic and flavonoid contents of Thai honeys

Thai honeys	Total phenolic contents (mg GAE/100 g honey)	Total flavonoid contents (mg QE/100 g honey)
Para rubber	217.08 ± 0.04^{c}	138.67 ± 0.03^{c}
Rambutan	262.52 ± 0.03^{b}	152.73 ± 0.04^{b}
Longan	428.59 ± 0.03^{a}	160.94 ± 0.01^{a}

Mean±SD. Difference letter in a column show significant different ($P<0.05$). The difference letter $^{a, b, c}$ compared within Thai honeys.

2.4.5 Sensory analysis

The sensory quality of honey shown in Table 2.4 by evaluated three honey samples including para ruber honey, rambutan honey and longan honey used 30 untrain-panellists. It was found that mean scores of color characteristic were 5.71, 7.52 and 7.35, sweetness were 6.32, 6.71 and 6.94, appearance were 6.10, 7.52 and 7.45, viscosity were 6.45, 6.90 and 7.42, aroma were 6.58, 5.84 and 7.61, flavor were 6.26, 6.65 and 7.13, taste were 6.26, 6.81 and 7.19 and overall acceptance were 6.45, 7.06 and 7.39, respectively. In term of viscosity, flavor and taste, the highest score

was obtained for longan honey compared with other two samples with approximate score of 7 (like moderately), para rubber honey has least approximate score of 6 (like slightly), and found that rambutan honey scores close to para rubber and longan honeys, which approximate score is ranged 6-7 (like slighty to like moderately). For aroma properties longan honey has the highest approximate scores of 7 (like moderately), which is significant different ($P < 0.05$) from para rubber honey and rambutan honey which was approximate score is 6 (like slightly). In contrast, appearance, color and overall acceptance found that para rubber honey obtained lowest score approximate score of 6 (like slightly) compared to rambutan and longan honey which significantly difference ($P < 0.05$). Rambutan honey and longan honey received similar approximate scores of 7 (like moderately) and sweetness found that three honey samples obtained no significant different score ($P < 0.05$) approximate scores of 6 (like slightly). Honey's sensory change may due to change in physical and chemical quality, and when sensory qualities are measured, the average acceptance score decreases. This research concludes that when considering sensory evaluation of honey, it was still acceptable from consumers.

Table 2.4 Sensory analysis of Thai honeys

Attributes	Thai floral honeys		
	Para rubber	Rambutan	Longan
Appearance	6.10 ± 1.78^{a}	7.52 ± 1.00^{b}	7.45 ± 1.63^{b}
Color	5.71 ± 1.60^{a}	7.52 ± 0.89^{b}	7.35 ± 1.14^{b}
Viscosity	6.45 ± 1.26^{a}	6.90 ± 1.10^{ab}	7.42 ± 1.39^{b}
Aroma	6.58 ± 1.26^{a}	5.84 ± 2.03^{a}	7.61 ± 1.20^{b}
Flavor	6.26 ± 1.44^{a}	6.65 ± 1.36^{ab}	7.13 ± 1.61^{b}
Taste	6.26 ± 1.67^{a}	6.81 ± 1.67^{ab}	7.19 ± 1.68^{b}
Sweetness	6.32 ± 1.64^{a}	6.71 ± 1.89^{a}	6.94 ± 1.46^{a}
Overall acceptance	6.45 ± 1.57^{a}	7.06 ± 1.00^{b}	7.39 ± 1.20^{b}

Mean±SD. Difference letter in a row show significant different ($P < 0.05$). The difference letter $^{a, b}$ and ^{c} compared within attributes.

2.5 Conclusions

The obtained results indicated that honey from the regions in Thailand have a low pH, normally value of electrical conductivity, low moisture and color, which indicated honey freshness and good conservation. The low moisture content helps to protect honey from microbiological activity and thus it can preserved for longer periods on time. Honey presents high fructose+glucose (more than 70%) and very low value of maltose (less than 2%); the remaining quality parameter agree in general, with national regulations. This study showed that the 3 samples of Thai originated honey contained phenolic compounds and flavonoids in variable quantities. The correlation between total phenolic acid and total flavonoid seems to depend on the composition of honey. The 9-points principal component analysis showed high intensity of sensory evaluation. Sensory quality parameters allow differentiation. Later research should be designed to know more on and biological activity in conjunction with physicochemical and properties and biochemical analysis to improves the assignation of botanical origin Thai honeys.

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CHAPTER 3

BIOLOGICAL ACTIVITIES OF THAI ORIGINATED HONEYS

3.1 Abstract

Honey is a natural product that has a sweet taste and has a high nutritional value and affects human health with anti-oxidant, anti-inflammatory, anti-inflammatory and antimicrobial effects including wound healing effects. The biological activities including, antioxidant activities, antidiabetic, enzymatic activities and prebiotic growth promotion by gut fermentation were evaluated in this chapter. The results of scavenging activity, DPPH (%) ranged from 30.51% to 40.53% and ferric reducing antioxidant power (FRAP) ranged 57.00 to 157.01 µM Fe II/kg. Activity of ferrous chelating assay showed range of % chelating from 23.11 - 49.89 and potential antioxidant activity based by ABTS ranged from 28.79% - 43.23%. The antidiabetic activity of honey samples was analyzed using the α-amylase and α-glucosidase enzyme inhibition assays. The result showed that all of honey samples can exhibit both enzyme activities. The para rubber honey showed %inhibition of 49.29% and 50.09%, rambutan honey were 53.94% and 57.02%, and longan honey were 61.64% and 66.67%, respectively, which, found highest percentage of inhibition against α- amylase and α-glucosidase enzymes. Analysis of enzyme activities presented in honey including diastase and invertase found that both enzymes are in the ranged of 6-12 Gothe Scale and 36.87-147.02 Unit/Kg honey. The oligosaccharide in longan honey was separated from honey by activated charcoal filtration. Prebiotic property of oligosaccharide was evaluated by *in vitro* batch fermentation. The oligosaccharides was separated by activated charcoal filtration. The result showed that oligosaccharide in longan honey stimulated fecal bacterial growth of *Bifidobacteria spp*. and *Lactobacillus spp.* were similar to that of fructooligosaccharides which significantly ($p < 0.05$) stimulated the growth of those species as well as production of SCFA such as butyric acid, acetic acid and propionic acid.

3.2 Introduction

Carbohydrates comprise between 95% and 97% (Alvarez-Suarez *et al*., 2010) in honey. Fructose and glucose are the main monosaccharides present in honey; it also contains disaccharides, trisaccharides and oligosaccharides (Saranraj *et al*., 2016). Among the organic acids, gluconic acid stands out, although the presence of formic acid, acetic acid, citric acid, lactic acid, malic acid, oxalic acid, pyroglutamic acid and succinic acid has also been identified (Ball, 2007; Alvarez-Suarez *et al*., 2010; Saranraj *et al*., 2016). The presence of all essential and non-essential amino acids is reported, except glutamine and asparagine; although proline, aspartate and glutamate predominate (Saranraj *et al*., 2016). The enzymes present in honey are: diastase, invertase, glucose oxidase and catalase derived from plants. It also contains all the vitamins soluble in water, with vitamin C predominating; and 31 minerals have been identified, among which predominate are: calcium, phosphorus, potassium, sulfur, sodium, chlorine and magnesium (Ball, 2007; Alvarez-Suarez *et al*., 2010; Saranraj *et al*., 2016 Sohaimy *et al*., 2015). These compounds contribute to the antioxidant (Al-Mamary *et al*., 2002; Alvarez-Suarez *et al*., 2013), antimicrobial (Al-Waili *et al*., 2011; Israili, 2014), anti-inflammatory (Vallianou *et al*., 2014), antiproliferative, anticancer and antimetastatic effects associated with honey (Saranraj *et al*., 2016; Afroz *et al*., 2016; Sipahi *et al*., 2017; Ahmed and Othman., 2013).

The relationship of the consumption of honey with type 2 diabetes mellitus type 2 (DM2) is an example that makes it possible to analyze the dynamics of the model of the natural history of the disease to incorporate scientific advances into medical practice in the prevention, diagnosis, treatment and control of DM2, moving from a mechanistic perspective towards understanding from a biopsychosocial and spiritual approach in the welfare state, sustaining a permanent process of adaptation to the environment to maintain the homeostatic balance of the internal environment in the patient with DM2 (Meo *et al*., 2017).

Honey is a saturated sweetener, especially fructose and glucose, and has many other sub-components (Viuda-Martos *et al*., 2008). Disaccharides such as sucrose and maltose and oligosaccharides, several units with 3-10 units of monosaccharide, with

values between 5 and 10% of honey, depending on the variety (Siddiqui and Furgala, 1967, 1968; Astwood *et al*., 1998; Weston and Brocklebank, 1999; Sanz *et al*., 2004; Bogdanov *et al*., 2008; Viuda-Martos *et al*., 2008). The nutritional composition of honey, which has a great influence on important physiological effects, depends on many considerations such as pollen sources, processing, storage and environment. In addition, oligosaccharides are less sweet than mono- and disaccharides. But most cannot be digested, which is needed for physiological prebiotics that may occur according to the production of metabolites. Honey has been used as food and medical products since the ancient time. It is used in many cultures for medicinal properties to treat burns, cataracts, ulcers and wound healing because it exerts a relaxing effect when used with open wounds. Depending on its origin, honey can be classified into different types, which monofloral honey is probably the most interesting and interesting as natural medicine. Honey has attracted a lot of researchers' attention for its biological properties, especially the antioxidant ability. Our study of chemical composition and variety of nutritional and health benefits of Thai honeys and other bioactive compounds. Then, the effects of Thai honey on biological activity, including antioxidant activity and other important biological effects.

3.3 Materials and methods

3.3.1 Materials and chemicals

All used chemicals and solvents were of analytical grade. DPPH (1,1-diphyenyl-2-picrylhydrazyl), TPTZ (2,4,6-tripyridyl-s-triazine), ABTS 2,2-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) diamonium salt, gallic acid, quercetin, HCl, $FeSO_4 \bullet 7H_2O$, $FeCl_3$, NaOH, $AlCl_3$, $K_2S_2O_8$, HNO_3 were purchased from (SAINT LOUIS MO, USA). Folin-Ciocalteu’s reagent was purchased from Carlo Erba (Milan, Italy).

3.3.2 Antioxidant analyses

3.3.2.1 DPPH free radical scavenging assay

The scavenging activity of Thai floral honeys was determined spectrophotometrically using the modified DPPH method (Yen and Chen., 1995). Briefly, floral honeys were dissolved in methanol and 1.5 ml of each sample or 1.5 ml of methanol (blank) was mixed with 3 ml of DPPH in methanol (0.02 mg/ml). The mixtures were left for 15 min at room temperature and then the absorbances was measured at 517 nm against reference mixtures that were prepared in the similar manner, by replacing the DPPH solution with methanol. The capability to scavenge the DPPH radicals, DPPH scavenging activity (SA), was calculated using the following equation:

$$SA_{DPPH^{\bullet}}(\%) = (A_0-A_x)/A_0 \times 100$$

Where A_0 is the absorbance of the blank and A_x is the absorbance of the sample.

3.3.2.2 Ferric reducing antioxidant power assay (FRAP)

The FRAP assay was performed according to a modified method described by Benzie and Strain (1996). Briefly, 200 µL of diluted honeys (1 g/5 mL) was mixed with FRAP reagent (1.5 mL). Then, the reaction mixture was incubated at 37^{o}C for 4 min and its absorbance was read at 593 nm against a blank that was prepared with distilled water. Fresh FRAP reagent was prepared by mixing of 300 mM acetate buffer (pH 3.6) with 10 mmol TPTZ (2,4,6-tripyridyl-s-triazine) solution in 40 Mm HCl containing 20 mM ferric chloride ($FeCl_3 \bullet 6H_2O$). The resulting mixture was then incubated at 37^{o}C. A calibration curve was prepared using an aqueous solution of ferrous sulfate ($FeSO_4 \bullet 7H_2O$). FRAP values were expressed as micromoles of ferrous equivalent (µM Fe [II]).

3.3.2.3 ABTS-radical cation decolorization assay

The TAC (total antioxidant capacity) was estimated by 2,2'-azinobis (3-ethylbenzthiazoline-6-sulphonic acid) radical cation decolorization assay ($ABTS^{\bullet}+$) for fast and slow antioxidant as described by Wnuk *et al*. (2010). A fresh solution of 2,2-azinobis (3-ethylbenzthiazoline-6-sulphonic acid) radical cation ($ABTS^{\bullet}+$) was prepared by dissolving 19.5 mg of $ABTS^{\bullet}+$ and 3.3 mg of dipotassium peroxodisulfate in 7 mL of 0.1 mol/L phosphate buffer, The solution was stored for 16 hours in the dark in order for the reaction to be complete. The $ABTS^{\bullet}+$ solution was then diluted in the 0.1 mol/L phosphate buffer to obtain an absorbance of about 1.0 at 414 nm. Aliquots (20 µL) of measured samples were added 980 µL of $ABTS^{\bullet}+$ solution and mixed thoroughly. The decrease in the absorbance of the mixture was measured spectrophotometrically at 414 nm exactly after 10 seconds (for fast antioxidants) and after 3 minutes (for slow antioxidants), after mixing the sample with the $ABTS^{\bullet}+$ solution.

3.3.2.4 Iron (II) chelating activity assay

The method described by Ramful *et al*. (2010). The reaction mixture consisted of 200 µL of samples at various concentrations and 50 µL of 0.5 mmol/L ferrous chloride tetrahydrate ($FeCl_2 \bullet 4H_2O$). The total volume of the reaction mixture was made up to 1 mL with water. The reacting solution was incubated for 5 min at room temperature, after which 50 µL of ferrozine (2.5 mmol/L) was added. The resulting purple coloration was read at 562 nm (Thermofisher scientific Genesys G10S). The control consisted of the reaction solution, without the samples or the positive control, ethylene diamine tetraacetic acid (EDTA). The chelating activity was calculated as followed:

$$\text{Chelating activity (\%)} = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} \times 100$$

3.3.3 Antidiabetic assay

3.3.3.1 α-Glucosidase inhibitory activity

The α-glucosidase inhibitory assay was determined according to Kim *et al*. (2004) with slight modification. 5 of 10 mL units/ mL α-glucosidase solution was pre-mixed with 10 mL of honey solution of 1g/ml. Then, 620 µl of 0.1 M potassium phosphate buffer (pH 6.9) was added, following incubation at 37.5^{o}C for 30 min, followed by adding 1 mL of Na_2CO_3 solution to terminate the reaction. The amount of occurring product (*p*-nitrophenol) was measured at 410 nm using a spectrophotometer. The acarbose were positive control.

3.3.3.2 α-Amylase inhibitory activity

The α-amylase inhibitory assay was determined according to the method previously described with slight modification of Kim *et al*. (2004). 20 µL of 40 U/mL α-amylase solution in 0.1 M potassium phosphate buffer (pH 6.9) was pre-mixed with 10 µL of honey solution of 1g/ml, following incubation for 15 min at 37.5^{o}C. Then, 300 µl 0f 1% starch solution in 50 mM sodium phosphate buffer (pH 6.9) was added to start the reaction. The reaction was carried out 37.5^{o}C for 5 min and terminated by adding 1.2 mL of DNS reagent (1% 3,5-dinitrosalicylic acid, 12% Na-K tartrate in 0.4 M NaOH). The reaction mixture was placed in a water bath at boiling temperature for 10 min then removed from water bath and cool downed to room temperature. The α-amylase activity was determined at 540 nm by spectrophotometer. The acarbose was used as a positive control and compared with honey.

Inhibitory activity of α-amylase and α-glucosidase were calculated by the following equation:

$$\text{Inhibitory activity (\%)} = (A-B)/A \times 100$$

Where A is the absorbance of reaction added buffer

Where B is the absorbance of reaction added honey floral

3.3.4 Enzymatic activities

3.3.4.1 Diastase analysis

Diastase number of Thai honey were analyzed by UV-Spectrophotometer (Rayleigh, VIS- 723G, England), following to the method advised by IHC (Bogdanov, 2009). Calibration of the starch solution to be used between the method was achieve with an absorbances value of 600 nm in the UV spectrophotometer and the solution of sodium chloride (NaCl) – acetate buffer solution to 10 g of sample. 10 ml honey solution with 5 ml starch solution in the test tubes were kept in distilled water for 15 min 40 ^{o}C. After that period, final solution was mixed again and transferred to the water bath. A 5 ml diluted iodine solution was then added to each 0 and 5 ml honey-starch solution with 11 ml distilled water; this solution was mixed with 5 minute intervals; and the absorbancy value was analyzed at the 5, 10, 15 and 20 min at 600 nm range from UV Spectrophotometer for detected of the diastase activity.

The results were expressed in diastase number (DN) as follows:

$$DN=300/t_x$$

where t_x is reaction time in minutes

3.3.4.2 Invertase activity

Place 5 mL of substrate solution in a test tube in the water bath at 40 ^{o}C for 5 minutes before adding the honey solution. Add 1 mL of honey solution (starting time). Mix the contents briefly in a mixer and incubated at 40 ^{o}C. After exactly 20 minutes add 0.5 mL of the reaction-terminating solution and mix again in a mixer (sample solution). For the blank, incubated 5 mL of substrate solution, stopper the tube, mix well and then add 0.5 mL of honey solution. Prepared a separated blank for each honey tested.

3.3.5 Prebiotic and gut microbiota evaluation

3.3.5.1 Preparation of honey oligosaccharides

Oligosaccharides in longan honey were excerpted by improved methods. In brief, 1 gram of honey were dissolved in 40 ml of deionized water and stirred with 6 grams of activated charcoal (Darco G-60, 100 mesh, Sigma Chemical Co., St. Louis MO, USA), in 500 ml of 10% ethanol in water for 30 minutes to eliminate monosaccharides and disaccharides. This mixture is filtered under vacuum and activated carbon was washed with 50 ml of 10% ethanol (v/v) in water. Oligosaccharides which adsorbed on activated charcoal were extracted by stirring for 30 minutes in 500 ml of 50% (v / v) ethanol. Activated charcaol was removed by filtering through paper and ethanol, evaporating under vacuum at a temperature of 30 degrees Celsius. The filtered sample was freeze-dried to gain honey oligosaccharide powder for fecal batch fermentation.

3.3.5.2 Fecal batch fermentation

The fermenters were containing the water-jackets with presterilized basal growth medium of 180 ml (0.025% (w/v) resazurin solution, bile salts 0.5 g/l, (pH 7.0), cysteine HCl 0.5 g/l, vitamin K 10 µL, haemin 0.02 g/l, Tween 80 2 ml, $NaHCO_3$ 2 g/l, $CaCl_2 \bullet 6H_2O$ 0.01 g/l, $MgSO_4 \bullet 7H_2O$ 0.01 g/l, KH_2PO_4 0.04 g/l, K_2HPO_4 0.04 g/l, NaCl 0.1 g/l and yeast extract 2g/l. Then, inoculated 20 ml of faecal slurry. Before adding faecal slurry mixed with homogenizing 10% (w / v), fresh voided material faecal, in 0.1 M phosphate buffer saline (PBS), pH 7.0, oligosaccharide was added for the final concentration 0f 1% (w/v). Each vessel was stirred by magnenically and the temperature set at 37 ^oC by circulating water bath. Automatically controlled the pH of vessel and maintained at 6.8. Anaerobic conditions were maintained by sparging the vessels with oxygen-free nitrogen gas at 15 ml/min. Samples (5 ml) were taken from each vessel at the beginning (T_0) and at intervals incubation period over a 48 hours.

3.3.5.3 Enumeration of fecal bacteria

Bacteria were counted used by fluorescent *in situ* hybridization (FISH), as described by Rycroft *et al*. (2001). Samples were diluted in 4% (w/v) paraformaldehyde and fixed overnight at 4 ^oC. Samples were washed twice with the filtered PBS and stored at -20^oC in PBS/ethanol (1:1, v/v) until analysis. Hybridization at the appropriate temperature was carried out using genus-specific 16S rRna-targeted oligonucleotide probes labeled with the fluorescent dye Cy3 for the various bacterial groups or with 4’6-diamidino-2-phenylindole for total cell. The probes using were Chis 150 specific for Clostridium (Franks *et al*., 1998), Lab 158 specific for *Lactobacillus* spp (Harmsen *et al*., 1999), Bac 303 specific for Bacteroides (Manz *et al*,, 1996), Bif 164 specific for Bifidobacterium (Langendijk et al., 1995) and Eub 338 specific for Eubacterium. Counted cells with a Nikon fluorescent microscope. Randomized at least 15 fields on each slide were counted.

3.3.5.4 Short chain fatty acid (SCFA) analysis

Samples were centrifuged at 13000 rpm for 15 minutes and injected 10 µl with automatic injector used by a High Performance Liquid Chromatography (HPLC) system (Aligent 1200 series) along with a UV detector. The column was an ion-exclusion Aminex HPX-87H (7.8 x 300 mm, Bio-Rad, USA) was maintained at 50^{o}C. Eluent used 0.005 mmol/L sulfuric acid in HPLC grade water and flow rate was 0.6 mL /min. Detection was done at 210 nm and received data using Chem Station software (Agilent Technologies, USA). Determination of the samples quantification was done using the calibration curves of butyric, propionic, acetic and lactic acids standard.

3.3.6 Statistical analysis

The results were expressed in mean and standard deviation and correlation was obtained by using Microsoft Excel 2010. The significant difference were obtained by a one-way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT) ($p<0.05$) using SPSS software (version 16.0, IBM Inc., Chicago, IL, USA).

3.4 Results and Discussion

3.4.1 Antioxidant activities

The scavenging activity of honey samples had been measured by using DPPH assay, and trolox was used as positive control. The unpaired electron of DPPH forms a pair with a hydrogen donated by free radical scavenging antioxidant from honey and thus converting the purple coloured odd electron DPPH to its reduced form in yellow. The degree of decolourization would be measured by UV-Visible spectrophotometer in order to determine the scavenging activity of honey. It was found that the EC_{50} of ascorbic acid was about a few thousands lower than all honey

samples. The lower the EC_{50} value the higher the scavenging capacity of honey, because it requires lesser amount of radical scavenger from the honey to reduce DPPH. The results of DPPH radical scavenging activity showed that darker honeys tended to be higher active in the reaction with DPPH●: in longan honey showed in Figure 3.1. The radical scavenging activity, calculated as percent of inhibition, reached 40.53%. The lowest radical scavenging activity, at a level of 30.51%, was reported for para rubber honey sample. The radical scavenging activity of honey varied from 23.81% to 100% in the DPPH reaction, as per the report of Wilczynska (2010), who recorded that the dark honeys were highly active in DPPH system. Moniruzzaman *et al*. (2014) showed strong correlation between the color intensities of the honey samples and their antioxidant parameters: phenolic acids, flavonoids, and reported the mean DPPH radical-scavenging activity of the Bangladeshi honey samples as 36.95 %; the highest DPPH radical-scavenging activity being 76.68 %.

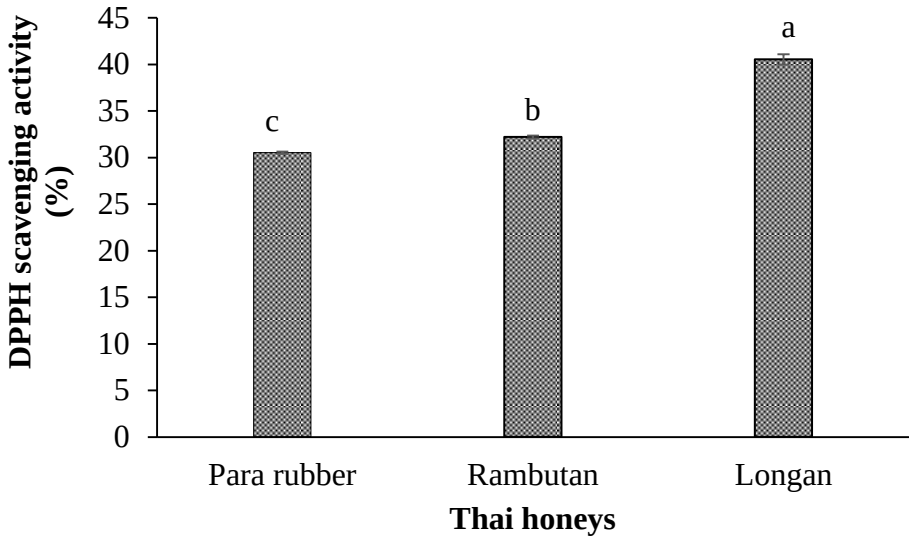

Figure 3.1 DPPH radical scavenging activity of honey samples**.** Values with different letters indicate significant different ($p < 0.05$). The difference letter ^{a,b} and^{c} compared between different honeys.

Total antioxidant potential of ferric reducing antioxidant power (FRAP) were varied from 57.00 (µM Fe (II)/kg) to 175.01 (µM Fe (II)/kg) in Thai originated honeys (Figure 3.2). The highest antioxidant potential of honeys were found in longan

honey in Lumphoon province at north of Thailand. While, the lowest antioxidant potential were observed in para rubber of south in Thailand. The results also showed the darker honeys had higher FRAP value or higher antioxidant potential. The longan honey with dark color had higher by FRAP value and rambutan and para rubber honeys with light color had lower FRAP value or antioxidant activity. FRAP method uses antioxidants as reductants in a redox-linked colorimetric analysis, applying an efficiency decreased oxidant system. Generally, aliquots of the honey solution are combined with a FRAP reagent (10 mM of TPTZ solution in 40 mM HCl, 20 mM $FeCl_3$ and 0.3 M acetate buffer at pH 3.6) according to spectrophotometer calculation of the absorbance of the action combined after incubation at 37°C for 10 min at 593 nm against the blank. The last effected can be expressed as the concentration of antioxidants having a ferric reducing capacity similst to that of 1 mM $FeSO_4$ used as the standard solution. Ammonium ferrous sulfate [Fe $(NH4)_2(SO4)_2 \cdot 6H_2O$] can also be used for the standard curve with the last effects expressed as micromoles of ammonium ferrous sulfate per 100 gram of honey. By the FRAP assay; few authors have also efforted to correspond the antioxidant capacity of honey with the quantity of ammonium ferrous sulfate (Ferreira *et al*., 2009; Gheldof *et al*., 2002; Kesic *et al*., 2009). FRAP assay nevertheless, has its own controls, specially for analysis lower non-physiological pH values i.e. at pH 3.6. Also, this assay is inadequated to distinguish slowly-acting polyphenolic compounds and thiols (Ou *et* al., 2002; Jerkovic and Marijanovic, 2010). Moreover, few components (even without antioxidant activities) with redox potential below that of the redox pair $Fe(3^+)$ / $Fe(2^+)$, can apparently decrease $Fe(3^+)$ to $Fe(2^+)$ providing to an raise in the FRAP value and so activating wrong positive results (Alvarez-Suarez *et al*., 2009). Otherwise, not all antioxidants decreased Fe (3^+) at a rate rapid plenty to admit its analysis into the consideration time (typically 4 min). Actully, abundant polyphenols reaction more slowly and need longer action times (30 min) for total measurement. Furthermore, any polyphenolic content like quercetin, caffeic, ferulic and tannic acids may have slower actions, need longer time (approximately 30 min) till the combine decline processing wasachieved. When used to analyzing the antioxidant activities of polyphenols in water and methanol (the solvent usually used for extraction of antioxidant from honey), the differ in absorbance reach after 4 min (Blasa *et al*.,

2006). So, the FRAP values for these contents cannot be properly analyzed in 4 min. Thus for this reason, the ideal action time should be more than 10 min.

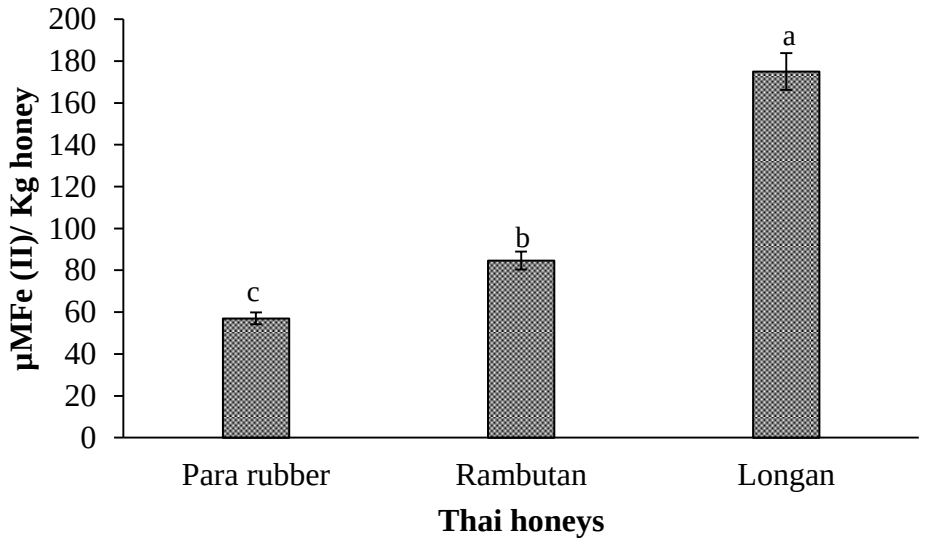

Figure 3.2 FRAP values in honeys. In each column different letters mean significant differences ($p < 0.05$). The difference letter ^{a,b} and ^{c} compared between different honeys.

ABTS is a measurement of antioxidant capacity in difference to antioxidant concentration which might contains a capacity of biologically-inactive antioxidants (Rice-Evans and Miller, 1995, Salah *et al*., 1995). ABTS authorized the measure of antioxidant capacity of combined of elements, so helped to analyzed during supplement and cooperative results (Miller *et al*., 1996, Rice-Evans *et a*l., 1995, Rice-Evans *et al*., 1996). The source method is based on the activation of metmyoglobin with hydrogen peroxide in the existence of ABTS to produce the radical cation either in the existence or not present of antioxidants. This has been disapproved on the based that the rapid acting antioxidants might also provide to the decline of the ferryl myoglobin radical (Re *et al.*, 1999). A more suitable method method is used a decolorization method due to the directly produced radical is relaible previous to reaction with the presumed antioxidants. This enhanced method for producing of ABTS affects the direct generation of the blue/green ABTS chromophore pass the action during ABTS and potassium persulfate (Re *et al.*, 1999)

which has absorption highest at wavelengths 645 nm, 734 nm and 815 nm (Miller, 1994; Miller, 1993; Re *et al*., 1999) with the many generally used highest absorbance reported to be at 415 nm (Huang *et al*., 2005, Re *et al*., 1999). The inclusion of antioxidants to the pre-formed radical cation decreases ABTS on a time-scale to a maintain amount, depended on the antioxidant capacity of the samples measurement, the concentration of the antioxidant and the extention of each action. So, the range of decolorization as percentage inhibition of the ABTS radical cation is analyzed as a capacity of these concentration and time and is evaluated related to the activity of Trolox standard below same conditions. A qualification of the assay used to measure the antioxidant activity of honey was also developed by (Piljac-Žegarac *et al*., 2009). For the calculation of antioxidant capacity, ABTS solution is diluted with ethanol (96%) to gained an absorbance of 0.700 (± 0.020) at 734 nm. 2 mL of ABTS solution are combined with 100 µL of honey solution in a cuvette and the decline in the absorbance is evaluated after 6 min, results indicated range of honey were 28.79% to 43.23% (Figure 3.3). Trolox was then used as the standard at different concentrations prepared in 96% ethanol and method below a same methods as that lead on the honey samples with the means of the three values expressed as mg (Trolox Equivalents) TE/100 g of honey.

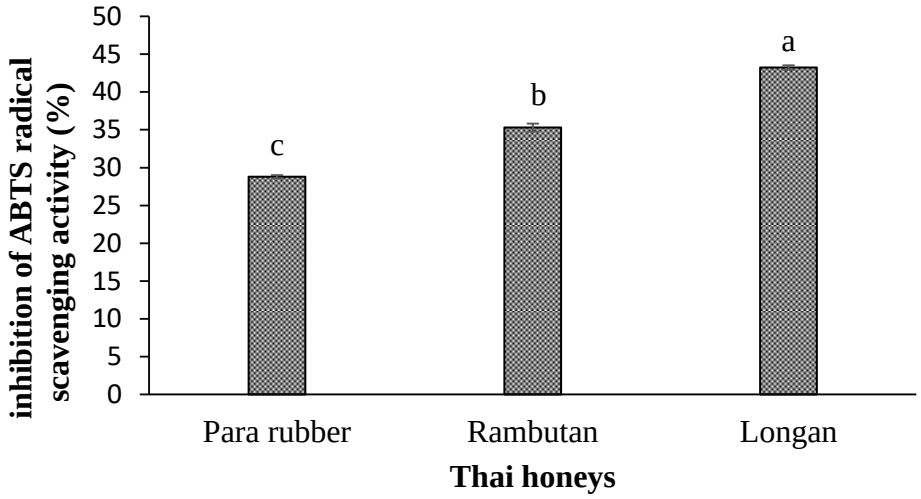

Figure 3.3 ABTS radical scavenging activity. Significant level of $p<0.05$. The difference letter ^{a,b} and ^{c} compared between different honeys.

Fe ++ ion chelating ability of honey samples shown in Figure 3.4. The honey samples showed 23.11 – 49.89 (µM Fe (II) Kg). Fe ++ ion chelating ability where as the standard EDTA showed at the same concentration. In this assay, honey samples and standard compounds interfered with the formation of ferrous and ferrozine complex which suggest that they have chelating activity and are capable of capturing ferrous ion before the formation of ferrozine. Iron is responsible to generate free radicals through the Fenton and Haber– Weiss reaction. But metal ion chelating activity of an antioxidant compound retard this oxyradical generation and the consequent oxidative damage. So, metal ion chelating capacity plays a significant role in antioxidant mechanism since it reduces the concentration of the catalysing transition metal.

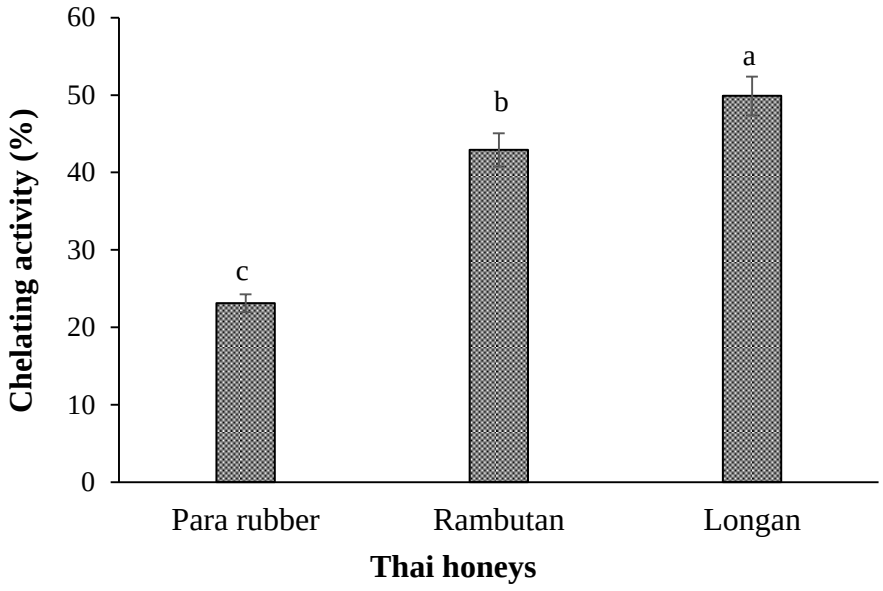

Figure 3.4 Ferrous ions chelating activity of honey samples. Values in the bar with different letters are significantly different (P<0.05). The different letter ^{a,b} and ^{c} compared between difference honeys.

3.4.2 Antidiabetic activities

3.4.2.1 α - amylase inhibition

The results of honey samples revealed that at all samples, longan honey achieved highest α - amylase (61.54%). All the other honeys exhibited different rate of inhibition as noticed among honey samples. Descending order of honey samples exhibiting alpha amylase inhibition were as follows: longan honey (61.54%) > rambutan honey (53.94%) > para rubber hoey (49.29%) showed in figure 3.5. The antidiabetic activity of honeys under this study was compared with a standard antidiabetic drug acarbose and it could be noted that the alpha amylase inhibition capacity for acarbose ranged from 24.02 % - 73.40 %. Even though the honeys under the study had moderate inhibition capacity, honeys proved to have comparatively good antidiabetic effect with regards to the standard acarbose. Evidence suggests that a number of oligosaccharides present in honey might play a role in the antidiabetic effect of honey (Erejuwa *et al*., 2012). The oligosaccharides in honey may contribute to the antidiabetic effect of honey either via modulation of gut microbiota or through the systemic effects of oligosaccharides (Erejuwa *et al*., 2012; Cani *et al*., 2006). The oligosaccharides analysis in honey was performed. Longan honey showed highest amount of oligosaccharides (raffinose). From previous reason, its showed that longan honey has the most effective against diabetic activity.

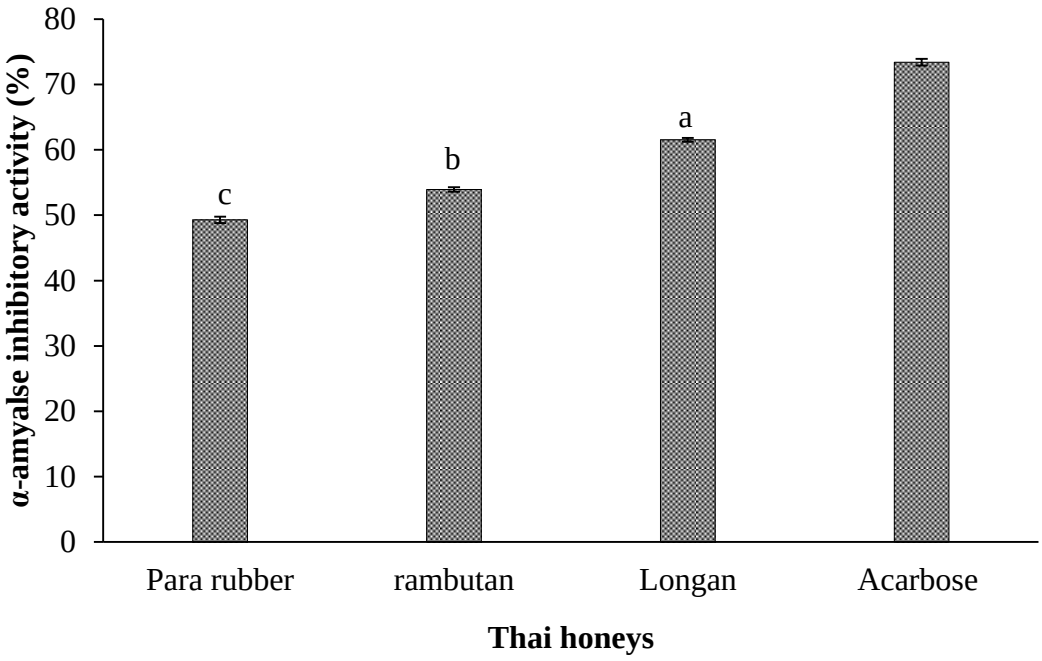

Figure 3.5 α-amylase inhibition capacity of honey samples. significant different among samples ($p < 0.05$). The difference letter ^{a,b} and ^{c} compared with acarbose standard.

3.4.2.2 α - glucosidase inhibition

The percentage inhibition of alpha glucosidase was analyzed in ten milliliter of each honey solution sample. The highest percentage inhibition was observed in longan honey (66.67%) followed by rambutan honey (57.02%). The least percentage of inhibition was noted in para rubber honey (50.09%) showed in figure 3.6. All the honeys analyzed were significant different. Along with enzyme inhibition capacity certain other established mechanisms responsible for the antidiabetic activity of honey are its effect on prolongation of gastric emptying, reduced rate of intestinal absorption and reduced food intake. As reported by Nasrolahi *et al*., (2012), antidiabetic drugs in combination with honey improved glycemic control, enhanced antioxidant defences and reduced oxidative damage thereby, reversed and degeneration of beta cells of pancreas and also enhancing the insulin production as well as reducing the insulin resistance towards the glucose moieties in circulation. Most polyphenols inhibit amylase and glycosidase activity

and, thus, inhibit glucose absorption in the intestine. In addition, polyphenols activate PPARγ and promote adiponectin production, thus subsequently improving insulin resistance. The antioxidative properties and antidiabetic efficacy of polyphenols are independent of each other and their interrelationship is still unclear. Nonetheless, in pre-symptomatic states, polyphenols contribute to the prevention of the type 2 diabetes through antioxidative activity. In the early phase of type 2 diabetes, polyphenols alleviate symptoms through PPARγ activation and inhibition of glucose absorption (Umeno *et al*., 2016). Due to previous study, longan honey contain the most polyphenol contents. It may be that longan honey can inhibit both type of enzymes (α-amylase and α-glucosidase) of antidiabetic better than rambutan and para rubber honeys.

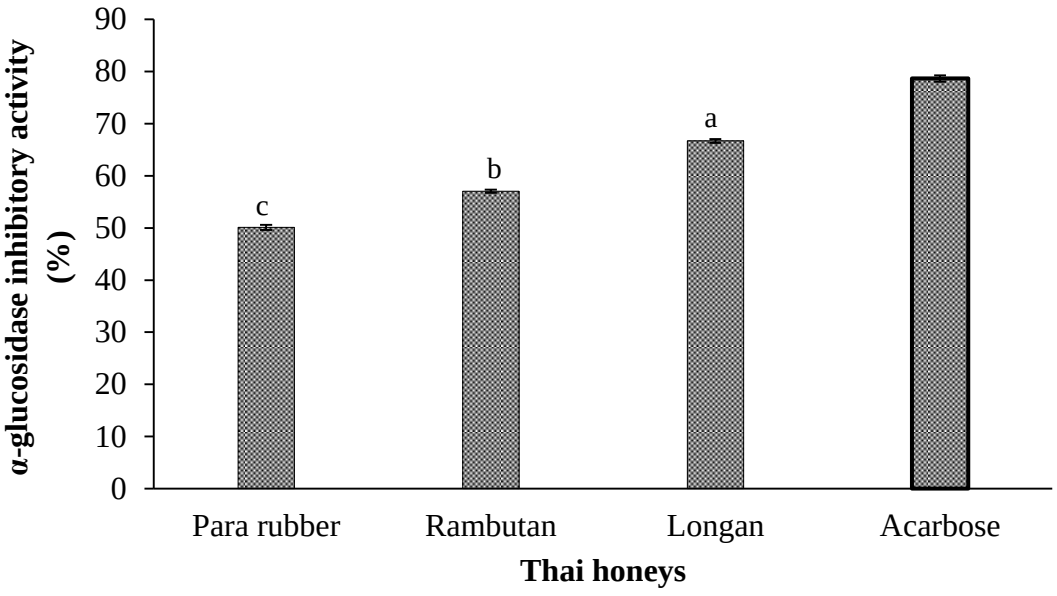

Figure 3.6 α-glucosidase inhibition capacity of honey samples. significant different among samples ($p>0.05$). The difference letter ^{a,b} and ^{c} compared with acarbose standard.

3.4.3 Activities of enzyme

Diastase is a common enzyme found in honey. The diastase activity in honey has been benefit as a freshness factor ancient the years. The action has decided a low level for diastase activity; it should not be less than 8 Diastase Number (DN)

units which 1 DN unit hydrolyses 1 ml of 1% starch used 1 g of honey for 1 h at 40^{o}C. The DN values were ranged between 6.0 and 12.0 Gothe units. Invertase is a general honey enzyme where is naturally using in Europe as a measurement of freshness. Its composition depending on the botanical and floral sources of the product, as well on its freshness. Table 3.1 demonstated that invertase activity values ranged from 36.9 to 147.0 unit/kg honey. The invertase is important for the chang of sucrose, maltose, melezitose, raffinose, melibiose and trehalose within glucose and fructose the dominant sugars in bee honey.

Table 3.1 Invertase and diastase activities of three honeys

Thai honeys	Invertase activity (Unit/kg)	Diastase activity (Gothe scale)
Para rubber	36.87±0.01^{c}	6.00±0.24^{c}
Rambutan	109.51±0.03^{b}	10.00±0.28^{b}
Longan	147.02±0.01^{a}	12.00±0.28^{a}

Different letters shown significant differences ($p<0.05$) for each samples. The difference letter ^{a,b,c} compared within the same enzymatic activity among different types.

3.4.4 Separation and characterization of honey oligosaccharide

Longan honey show above levels of oligosaccharide, primalily raffinose than rambutan and para rubber honey. Thus, longan honey was preferred to increasing the yield of oligosaccharide to study their result upon the growth of fecal bacteria. Figure 3.7 indicated the data of mono-, di-, and trisaccharides of longan honey before and after filtration by activated charcoal. Carbohydrate profile of the longan honey before filtration is displayed in Figure 3.7; 94.28% of total quantified corresponded to glucose and fructose, whereas 2.61% was maltose and only 3.11% was assigned as raffinose. Decreases of 20.20% and 20.10% were observed for glucose and fructose, respectively, whereas, maltose and

raffinose were increased 34.14% and 6.16%, respectively after charcoal filtration. The concentrations of glucose and fructose in the longan honey decrease meanwhile the concentrations of maltose and raffinose increased by activated charcoal filtration. So that, this treatment could increased the oligosaccharide content of honey. During activated charcoal filtration treatment, most of the glucose and fructose were removed, which, slightly decreased during this treatment. A high concentration of raffinose was detected with this method in longan honey.

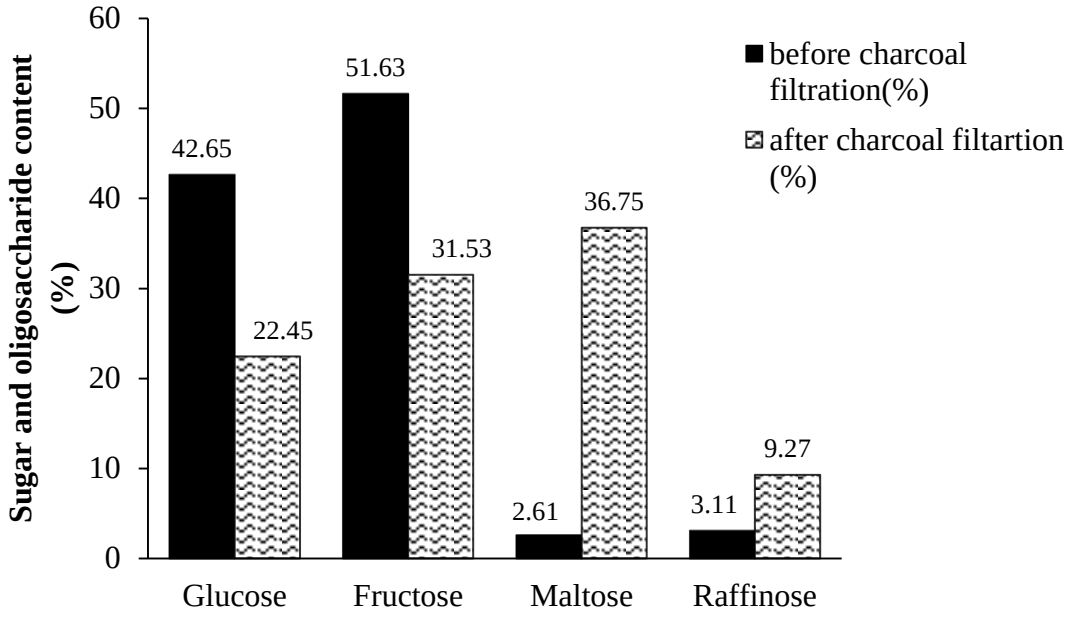

Figure 3.7 Sugar and oligosaccharide of longan honey content (%) before and after filtrated by activated charcoal.

3.4.5 Fecal bacteria enumeration

From this study, samples were collected from vessel of batch culture for fecal bacterial counts by fluorescene *in situ* hybridization (FISH) technique. The five bacteria groups that represented in the good and bad bacteria in the human gut were enumerated. It was found that at 0 h fecal fermentation. They are a number of *Bifidobacteria*, *Lactobacilli*, *Bacteroides*, *Clostridia*/*Enterobacter* and *Eubacteria* were 8.88, 7.52, 8.88, 9.22 and 9.89 log cell/ml, respectively (Figure 3.8

to 3.12). At 48 hours fecal fermentation of longan honey after filtration by activated charcoal, it was found that the number of bacteria in good group including bifidobacteria and lactobacilli increased significantly ($P < 0.05$) to 10.53 and 10.43 log cell/ml, respectively. However, it was also found that the number of bacteria in bad group clostridia/enterobacter decreased. There were no significant ($P \geq 0.05$) differences of eubacteria populations as shown in the Figure 3.12. So that longan honey before and after filtration by activated charcoal show similar effect on fecal bacterial fermentation by increased bifidobacteria, lactobacilli and bacteroides but not for clostridia/enterobacter. Moreover, longan honey showed similar effect comparable to a commercial prebiotic fructooligosaccharide (FOS) the number of good bacteria population increased and number of bad bacteria population decreased.

Gibson and Roberfroid (1995) found that increasing the number of bifidobacteria and lactobacilli populations in the colon helped to inhibit pathogens during fermentation because of production of free fatty acids, acetic acid and lactic acid resulted in a decrease pH, which inhibited the growth of pathogens and produced B vitamins which effect of inhibiting endemic and reducing blood lipids level as well.

The numbers of bacteria counts were calculated for the prebiotic index (PI). Sanz *et al*. (2005) showed honey oligosaccharides indicated potential prebiotic property (PI values between 3.38 and 4.24), increasing the populations of lactobacilli andbifidobacteria, while not to the levels of FOS (PI of 6.89). The PI of longan honey was 0.32. So it was found that longan honey contained oligosaccharide (raffinose) showed prebiotic effect because of value is positive. Based on previous research by Manderson *et al*. (2005), prebiotic properties of oligosaccharide from orange peels was compared with commercial fructooligosaccharides and it was found that PI of oligosaccharide from orange peel and fructooligosaccharide were 7.65 and 7.84, respectively. The value is positive and similar value. When comparing PI value with this study, honey gave lower PI value this may be due to variability of fecal bacterial composition and purity of oligosaccharide used. Honey contains small amount of oligosaccharide however is an

alternative source of prebiotics that has potential to promote good bacteria and do not support on growth of clostridia/enterobacteria.

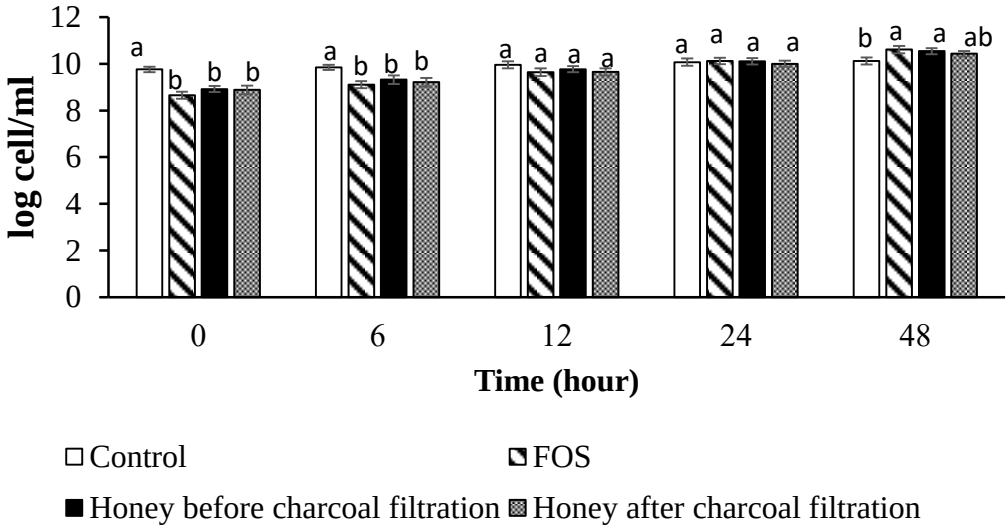

Figure 3.8 Changes in bifidobacteria populations (log cell/ml) during fecal fermentation of longan honey. Different letters indicate significant ($p < 0.05$) among samples within the same time.

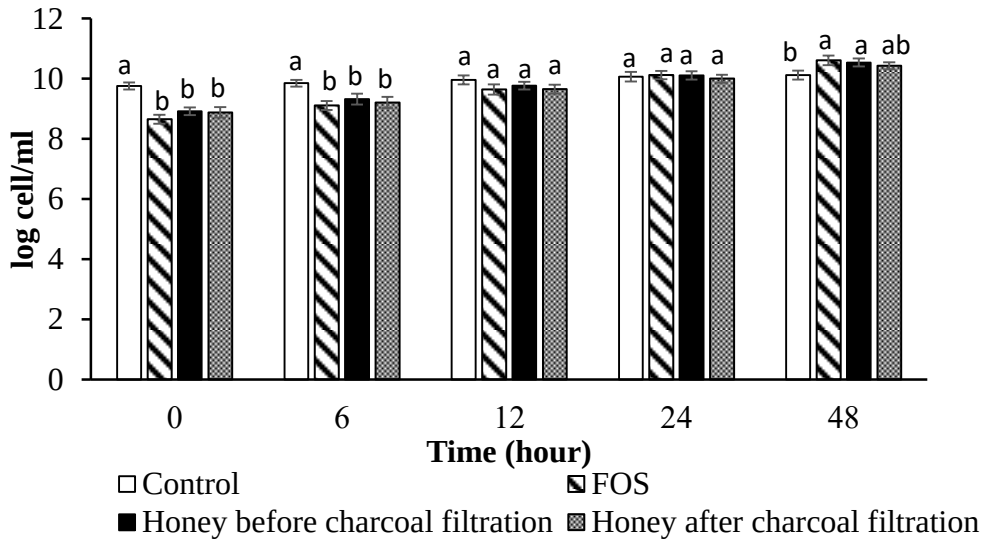

Figure 3.9 Changes in lactobacilli populations (log cell/ml) during fecal fermentation of longan honey. Different letters indicate significant ($p < 0.05$) among samples within the same time.

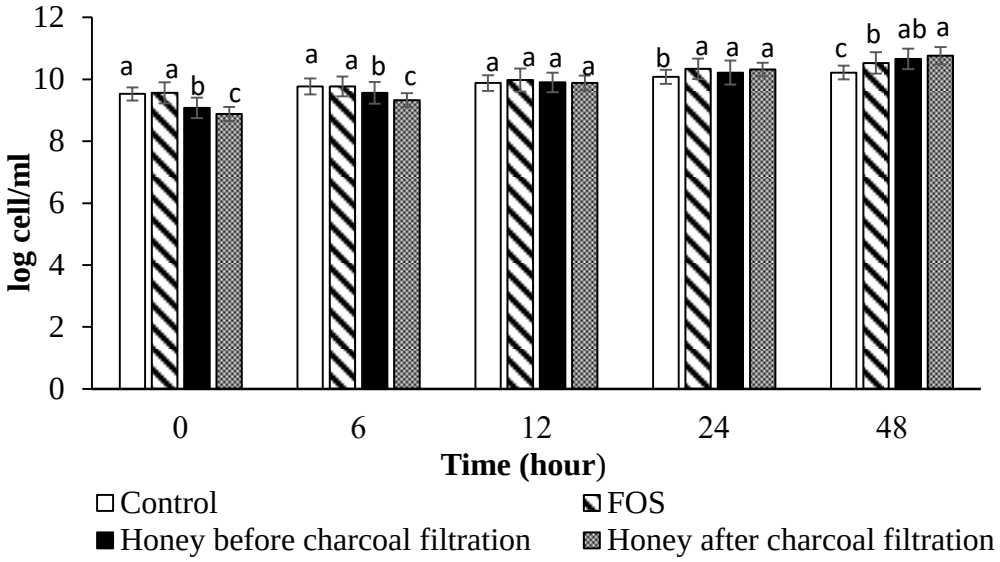

Figure 3.10 Changes in bacteroides populations (log cell/ml) during fecal fermentation of longan honey. Different letters indicate significant ($p < 0.05$) among samples within the same time

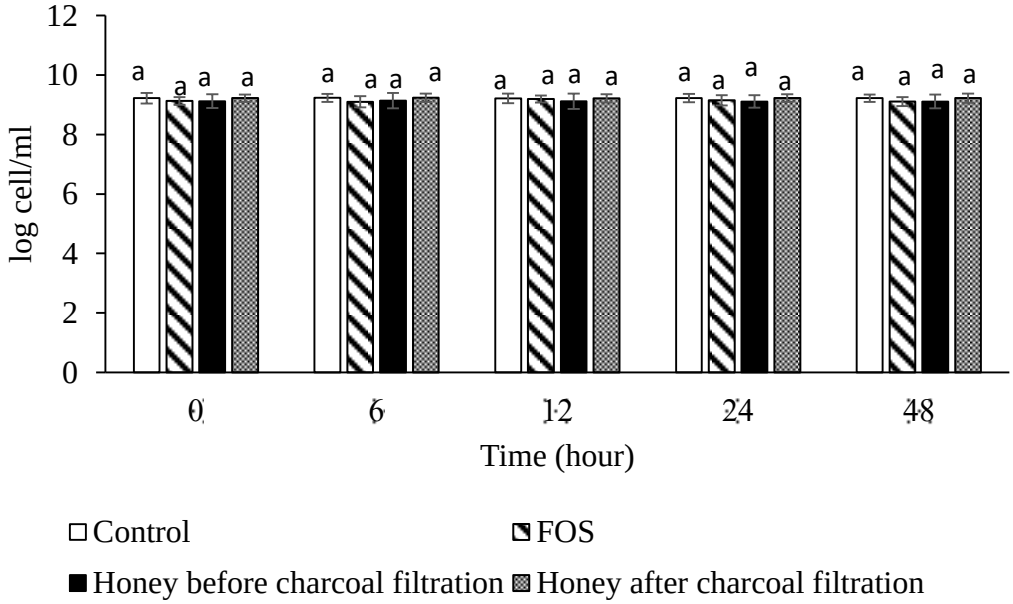

Figure 3.11 Changes in clostridia populations (log cell/ml) during fecal fermentation of longan honey. Different letters indicate significant ($p < 0.05$) among samples within the same time

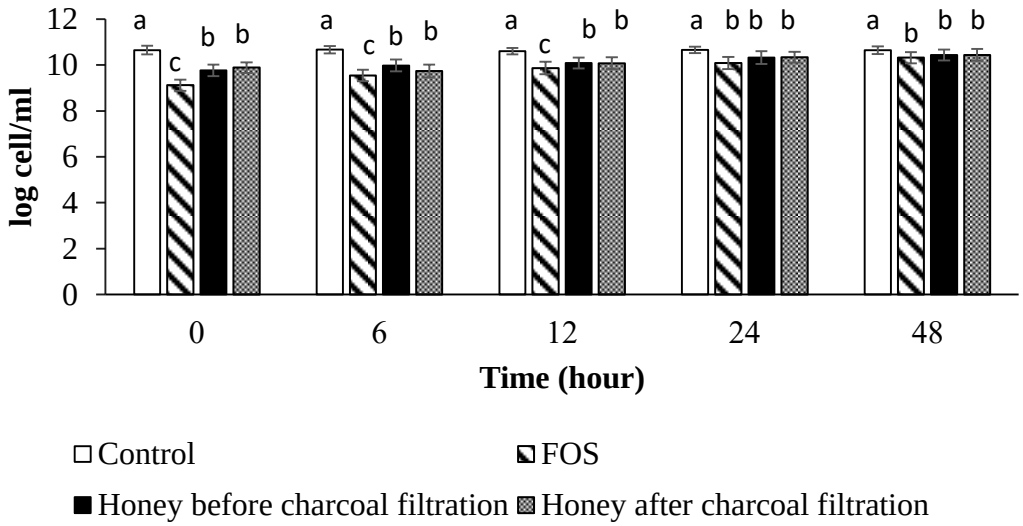

Figure 3.12 Changes in eubacteria populations (log cell/ml) during fecal fermentation of longan honey. Different letters indicate significant ($p < 0.05$) among samples within the same time

Concentration of short chain fatty acids (SCFA) produced by fecal fermentation of longan honey SCFA was determined. It was found that lactic, acetic and butyric acid were significantly ($P < 0.05$) increased from initial time of longan honey fermentation up to 48 hours (Table 3.2). The highest SCFA was acetic acid followed by propionic and butyric acid. These SCFA were derived from the fermentation of longan honey by either bifidobacteria, lactobacilli and other gut bacteria (Gibson and Roberfoid, 1995). Normally, short chain fatty acids derived from fermentation process within human colon have a ratio of acetate: propionate: butyrate was 60: 25: 10 mM per liter it depend on type and concentration of oligosaccharides, fecal composition and period time of fermentation (Basson *et al*., 1998).

Short chain fatty acid, both propionic and butyric are reported to have beneficial effects on health followed by Naidu *et al* (1999) indicated both of these SCFA support energy production in the large intestinal cells, while at the same time preventing the onset of pathogenic microorganisms in the intestinal mucosa and prevent tumors in mammalian cells. Cummings and Englyst reported that (1995) propionate sent to the liver for ATP synthesis and changes blood cholesterol levels and butyric used to break down colorectal cells, which that regulate cellular

proliferation and program cell death. Shamala *et al*. (2000) showed result significant increase in the amounts of lactic acid bacteria in the intestines of mice fed with honey, perhaps showed its character in revising the gut microbiota.

Table 3.2 Short chain fatty acid (SCFA) production by longan honey oligosaccharide fecal fermentation

Times (hour)	Samples	Acetic acid	Lactic acid	Propionic acid	Butyric acid
0	FOS	6.70±0.20^{c}	1.79±0.07^{b}	12.53±0.11^{a}	1.87±0.10^{a}
	Before filtration	7.76±0.12^{a}	1.64±0.20^{c}	3.57±0.10^{b}	1.79±0.12^{b}
	After activated charcoal filtration	6.91±0.06^{b}	4.62±0.33^{a}	12.59±0.17^{a}	1.75±0.10^{b}
6	FOS	13.66±0.21^{b}	3.80±0.05^{c}	15.81±0.22^{a}	2.72±0.07^{a}
	Before filtration	14.61±0.06^{a}	4.77±0.21^{b}	9.24±0.19^{b}	2.79±0.14^{a}
	After activated charcoal filtration	14.44±0.12^{a}	7.59±0.25^{a}	15.76±0.38^{a}	2.73±0.07^{a}
12	FOS	18.66±0.12^{c}	9.41±0.07^{b}	19.75±0.20^{a}	3.06±0.04^{c}
	Before filtration	19.21±0.19^{b}	8.72±0.23^{c}	13.62±0.18^{b}	3.57±0.18^{b}
	After activated charcoal filtration	20.78±0.11^{a}	9.80±0.17^{a}	19.73±0.18^{a}	3.73±0.09^{a}
24	FOS	23.15±0.08^{b}	12.87±0.05^{a}	20.80±0.11^{a}	4.63±0.17^{b}
	Before filtration	22.65±0.23^{c}	10.38±0.15^{c}	16.50±0.25^{b}	4.79±0.07^{a}
	After activated charcoal filtration	24.88±0.10^{a}	11.87±0.12^{b}	20.83±0.17^{a}	4.31±0.25^{c}
48	FOS	30.77±0.26^{a}	15.08±0.05^{c}	21.47±0.12^{c}	5.80±0.16^{b}
	Before filtration	28.67±0.14^{c}	13.81±0.12^{a}	21.73±0.22^{a}	5.62±0.28^{c}
	After activated charcoal filtration	29.47±0.17^{b}	13.66±0.13^{b}	21.61±0.25^{b}	9.76±0.29^{a}

Different letters indicate significant different ($p < 0.05$) for values in the same column and different sample.

3.5 Conclusion

In this study it had been established that three types of honey contain antioxidant property varied in different types of honey. Darker honey samples had highest antioxidant activity. The botanical origin of honey has the greatest influence on its antioxidant activity. The variation in the antioxidant; power among unifloral honeys with different geographical origin may be due to climate and environment factors such as humidity, temperature and soil composition. Honey can

not only act as a substitute for sugar among the diabetic, in fact it also contributes to curb the diabetic associated micro vascular complications and angiopathies as honey could revitalize the blood components along with its composition. On the basis data of this study obtained oligosaccharides from honey appeared to show potential prebiotic property, increasing the populations of bifidobacteria and lactobacilli. Some honey oligosaccharides have been demonstrated to be resistant to enzymes of digestive system. Also, its main compositions, Thai honey including a huge number of another components in trace and small numbers, able to untilized abundant biological and nutritional effects, similar to antioxiant and antidiabetic activities. The obtained results show that in gut microbiota, Thai honey offers advantages in controlling beneficial bacterial growth and in the treatment of several health problems, are valuable distinctive for the benefit of Thai honey.

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CHAPTER 4

SUMMARY AND SUGGESTIONS

4.1 Summary

1. Honeys has a physical properties including, color, conductivity, pH and viscosity complies with Codex Alimentarius Commission and Thai Agricultural Standard and chemical compositions include sugar, enzyme, proline and phenolic and flavonoid contents. Most of honeys consist of sugars which are fructose and glucose other solids.

2. Sensory evaluation of honeys has average of liking scores all of contributes has preference level in range of 6-7 (like slightly to like moderately) and still in acceptable level of consumers.

3. Analysis of phenolic and flavonoid compounds showed that longan honey had highest contents as well as antioxidant activities compared with the others two honeys. The obtained result seem that antioxidant activity in honeys influented by phenolic and flavonoid content.

4. Enzymes as honey components have been the object of much research over the years, the primary interest was as a possible means of distinguishing between natural and artificial honeys, but diastase and invertase are largely used as a measure of honey freshness, because their activity decreases in old or heated honeys.

5. Biological activities of honey samples including antioxidant, antidiabetic and potential prebiotic, longan honey found have highest all of activities more than rambutan and para rubber honey.

4.2 Suggestions

1. Active compounds in honeys or other biological activities could be studied further to obtain more information or data on health benefit.

2. The honey development for ingradient in food and neutraceutical applications which biological activities from this study should be studied.

3. Mechanism of each biological activity affecting to health benefit such as anti-aging, anti-inflammatory, and antioxidant should be further studied.

APPENDIX

APPENDIX A

Chemical preparation

APPENDIX A1.

0.05 M Sodium phosphate buffer (pH 6.8)

Reagent

A: 0.05 M dibasic sodium phosphate (7.80 g $Na_2HPO_4.2H_2O$ in 1000 mL of distill water)

B: 0.05 M monobasic sodium phosphate (8.90 g $NaH_2PO_4.2H_2O$ in 1000 mL of distill water)

Reagent A + Reagent B

pH	A (mL)	B (mL)
6.0	6.15	43.85
6.2	9.25	40.75
6.4	13.25	36.75
6.6	18.75	31.25
6.8	24.50	25.50
7.0	30.50	19.50
7.2	36.00	14.00
7.4	40.50	9.50
7.5	43.50	6.50
7.8	45.75	4.25
8.0	47.35	2.65

APPENDIX A2.

0.1 M Potassium phosphate buffer (pH 6.9)

Reagent

A: 1 M K_2HPO_4 (43.55 g in 250 mL of deionized (DI) water)

B: 1 M KH_2PO_4 (34.02 g in 250 mL of deionized (DI) water)

Reagent A + Reagent B

pH	A (mL)	B (mL)
5.8	2.13	22.88
6.0	3.30	21.70
6.2	4.80	20.20
6.4	6.95	18.05
6.6	9.53	15.48
6.8	12.43	12.58
7.0	15.38	9.63
7.2	17.93	7.08
7.4	20.05	4.95
7.5	21.65	3.35
7.8	22.70	2.30
8.0	23.50	1.50

APPENDIX A3.

300 mM Acetate buffer (pH 3.6)

Reagent

1. Glacial acetic acid
2. Sodium acetate trihydrate

Procedures

1. A 16 mL of glacial acetic acid was mixed with 3.1 g of sodium acetate trihydrate
2. The mixture solution was adjusted with DI water and made the final volume
3. Finally, the solution was measured the pH to 3.6 by pH meter with 1 N HCl

APPENDIX A4.

Dinitrosalicylic acid (DNS) solution

NaOH (g)	Distill water (mL)	Na2SO3 (g)	Sodium potassium tartrate (g)	3,5 Dinitrosalicylic acid (g)	Phenol (g)	Final volume (mL)
0.25	22.4	0.0125	5	0.25	0.05	25
0.5	45	0.025	10	0.5	0.1	50
1.0	90	0.05	20	1.0	0.2	100
2.5	225	0.125	50	2.5	0.5	250
5.0	450	0.25	100	5.0	1.0	500
10.0	900	0.5	200	10.0	2.0	1000

APPENDIX A5.

Phosphate Buffer Saline/Sodium Dodecyl Sulphate (PBS/SDS)

NaCl	8	g
KCl	0.2	g
K_2HPO_4	1.15	g
KH_2PO_4	0.2	g
HPLC distilled water	100	mL
10% SDS	100	µL

APPENDIX B

Standards

APPENDIX B1.

CODEX STANDARD FOR HONEY (CODEX STAN 12-19811)

MOISTURE CONTENT

(a) Honeys not listed below - not more than 20%

(b) Heather honey (Calluna) - not more than 23%

SUGARS CONTENT

Fructose and glucose content

(a) Honey not listed below - not less than 60 g/100g

(b) Honeydew honey, blends of honeydew honey with blossom honey - not less than 45 g/100g

Sucrose content

(a) Honey not listed below not more than 5 g/100g

(b) Alfalfa (Medicago sativa), Citrus spp., False Acacia (Robinia pseudoacacia), French Honeysuckle (Hedysarum), Menzies Banksia (Banksia menziesii),Red Gum (Eucalyptus camaldulensis), Leatherwood (Eucryphia lucida), Eucryphia milligani - not more than 10 g/100g (c) Lavender (Lavandula spp),Borage (Borago officinalis) - not more than 15 g/100g

WATER INSOLUBLE SOLIDS CONTENT

(a) Honeys other than pressed honey - not more than 0.1 g/100g

(b) Pressed honey - not more than 0.5 g/100g

APPENDIX B2.

Pfund scale of honey

VITAE

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Proceeding

Foomaneechot, O and Sirinupong, N. 2018. Physicochemical, Biochemical properties and Sensory evaluation of Thai originated honeys. In Proceedings of The International Conference on Food and Applied Bioscience. 1-2 February 2018, p. 1-6, Chiang Mai, Thailand.