

**THE EFFECTS OF STINGLESS BEE HONEY IN TRIS EXTENDER ON  
SEMEN QUALITY OF BOER GOAT**

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ON SEMEN QUALITY OF BOER GOAT

IJAZAH: SARJANA MUDA SAINS PERTANIAN DENGAN KEPUJIAN

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## ABSTRACT

The objective of this research was to determine the effects of stingless bee honey in Tris extender on semen quality of Boer goat. The research were conducted at Faculty of Sustainable Agriculture (FSA), Universiti Malaysia Sabah (UMS). Collection of Boer goat semen were done at the Goat Rearing Facility while semen evaluation were done at the Anatomy and Physiology Laboratory. Three (3) male Boer goat were randomly selected for this research and semen collection were done once a week using an Artificial Vagina (AV). Semen were collected 3 times and the experiment adopted the Completely Randomized Design (CRD). The fresh Boer goat semen were assessed in term of volume, colour, mass motility, and concentration. For this research, the collected Boer goat semen have a mean volume of  $1.83 \pm 0.17$  ml, concentration of  $2.21 \pm 0.13 \times 10^9$  sperm/ml, a creamy white appearance, and a mean mass motility of 5.00. Fresh semen with a minimum of 80% individual progressive motility were selected and proceed to further experimental treatment. Four (4) treatments is conducted on the semen, in which the first treatment (T1) serve as the control containing only Tris Buffer Solution (TBS) as the semen extender, while the second (T2), third (T3), and fourth (T4) treatment group contains TBS + 0.5%, 1.0%, and 1.5% volume per volume (v/v) of stingless bee honey respectively. Semen sample from each treatment were then assessed in term of sperm individual progressive motility, sperm viability and sperm abnormality through microscopic observation and eosin-nigrosin staining procedure. From this research, TBS + 0.5% stingless bee honey produced the best result together with the control treatment in term of sperm motility and viability. For sperm abnormality parameters, sperm from T1, T2, T3, and T4 showed no significant difference from each other. Therefore, it can be concluded that addition of 0.5% stingless bee honey in Tris extender are comparable to the control treatment, and can preserve sperm motility and viability up to 24 hours.

# KESAN MADU KELULUT DALAM PENCAIR TRIS TERHADAP KUALITI SEMEN KAMBING BOER

## ABSTRAK

Objektif kajian ini adalah untuk menentukan kesan madu kelulut dalam pencair Tris terhadap kualiti semen kambing Boer. Kajian ini dijalankan di Fakulti Pertanian Lestari (FPL), Universiti Malaysia Sabah. Pengumpulan semen dilakukan di Fasiliti Ternakan Kambing manakala penilaian semen dilakukan di Makmal Anatomi dan Fisiologi. Tiga (3) kambing Boer jantan dipilih secara rawak untuk kajian ini dan pengumpulan semen dijalankan seminggu sekali menggunakan faraj tiruan (artificial vagina). Semen dikumpul sebanyak tiga kali dan eksperimen ini menggunakan Rekabentuk Rawak Lengkap (Completely Randomized Design). Semen kambing Boer segar telah dinilai dari segi isipadu, warna, motiliti massa, dan kepekatan. Untuk kajian ini, semen kambing Boer yang diambil mempunyai purata isipadu  $1.83 \pm 0.17$  ml, kepekatan  $2.21 \pm 0.13 \times 10^9$  sperma/ml, berwarna putih krim, dan purata motiliti massa bernilai  $5.00 \pm 0.00$ . Semen segar dengan minimum 80% motiliti progresif individu telah dipilih untuk melalui rawatan eksperimen yang seterusnya. Empat (4) rawatan telah dilakukan terhadap semen tersebut, dimana rawatan pertama (T1) berfungsi sebagai kawalan yang mengandungi larutan penimbal Tris (TBS) sahaja sebagai pencair semen, manakala kumpulan rawatan kedua (T2), ketiga (T3), dan keempat (T4) masing-masing mengandungi TBS + 0.5%, 1.0%, dan 1.5% isipadu per isipadu (volume per volume, v/v) madu kelulut. Sampel semen dari setiap rawatan kemudian dinilai dari segi motiliti progresif individu sperma, kebolehidupan sperma, dan keabnormalan sperma melalui pemerhatian mikroskop dan prosedur perwarnaan eosin-nigrosin. Menerusi kajian ini, TBS + 0.5% madu kelulut menunjukkan keputusan terbaik bersama-sama dengan rawatan kawalan dari segi motility dan kebolehidupan sperma. Dari segi keabnormalan sperma, sperma dari T1, T2, T3, dan T4 tidak menunjukkan perbezaan yang signifikan antara satu sama lain. Oleh itu, boleh disimpulkan bahawa penambahan 0.5% madu kelulut dalam pencair Tris adalah sebanding dengan rawatan kawalan, dan mampu memelihara motiliti sperma dan kebolehidupan sperma sehingga jangka waktu 24 jam.

# TABLE OF CONTENTS

<b>Content</b>	<b>Page</b>
DECLARATION	ii
VERIFICATION	iii
ACKNOWLEDGEMENT	iv
ABSTRACT	v
ABSTRAK	vi
TABLE OF CONTENTS	vii
LIST OF TABLES	ix
LIST OF FIGURES	x
LIST OF SYMBOLS, UNITS AND ABBREVIATIONS	xi
LIST OF FORMULAE	xii
 <b>CHAPTER 1 INTRODUCTION</b>	 <b>1</b>
1.0 Introduction	1
1.2 Justification	2
1.3 Objective	3
1.4 Hypothesis	3
 <b>CHAPTER 2 LITERATURE REVIEW</b>	 <b>4</b>
2.1 Artificial Insemination in Goat	4
2.2 Semen Extender Properties	5
2.2.1 Glycerol	6
2.2.2 Energy Source	6
2.2.3 Milk Source	7
2.2.4 Egg Yolk	7
2.2.5 Buffer	8
2.2.6 Antimicrobials	8
2.3 Stingless Bee	9
2.3.1 Stingless Bee Honey	9
2.3.2 Chemical Constituents	10
2.3.3 Antimicrobial Activities	11
2.3.4 Antioxidant Activities	11
2.3.5 Effect on Fertility	12
 <b>CHAPTER 3 METHODOLOGY</b>	 <b>13</b>
3.1 Animals and Semen Collection	13
3.2 Reagent Preparation	15
3.2.1 Semen Extender	15
3.2.2 Eosin-Nigrosin Stain	16
3.3 Semen Quality Assessment	17
3.3.1 Semen Volume	17
3.3.2 Semen Colour	18
3.3.3 Semen Concentration	19
3.3.4 Subjective Motility	20
3.3.5 Live, Dead, and Abnormal Spermatozoa	21
3.4 Experimental Design	23
3.5 Statistical Analysis	23





<b>CHAPTER 4 RESULTS</b>	24
4.1 Fresh Semen Quality and Characteristics	24
4.2 Sperm Individual Progressive Motility	25
4.3 Sperm Viability	27
4.4 Sperm Mortality	29
4.5 Sperm Abnormality	31
<b>CHAPTER 5 DISCUSSION</b>	33
5.1 Sperm Individual Progressive Motility	33
5.2 Sperm Viability	35
5.3 Sperm Mortality	37
5.4 Sperm Abnormality	38
<b>CHAPTER 6 CONCLUSION</b>	39
6.1 Conclusion	39
6.2 Recommendation	39
<b>REFERENCES</b>	40
<b>APPENDICES</b>	47

## LIST OF TABLES

Table	Page
2.0 Physicochemical properties of stingless bee honey.	10
3.1 List of ingredients with quantity for preparing 1 L of TBS	15
3.2 Amount of TBS and pure stingless bee honey for preparing 50ml of treatment extenders	15
3.3 List of chemicals with quantity for preparing 100ml of eosin-nigrosin stain.	16
3.4 Scoring system for mass motility (MMOT) of sperm.	20
4.1 Mean volume (ml), concentration (sperm/ml), and mean mass motility (MMOT) of fresh Boer goat semen.	24
4.2 Mean percentage of sperm individual progressive motility with different concentration of stingless bee honey in Tris extender at 48 hour intervals.	26
4.3 Mean percentage of live sperm with different concentration of stingless bee honey in Tris extender at 48 hour intervals.	28
4.4 Mean percentage of dead sperm with different concentration of stingless bee honey in Tris extender at 48 hour intervals.	30
4.5 Mean percentage of abnormal sperm with different concentration of stingless bee honey in Tris extender at 48 hour intervals.	32

## LIST OF FIGURES

Figure	Page
3.1 (a) Male goat (buck) isolated from the female goat (doe); (b) Teaser doe restrained using a head crush before presented to the buck; (c) Example of artificial vagina and other apparatus used in semen collection; and (d) Warm water (45°C) poured into artificial vagina through the water inlet.	14
3.2 Eosin-nigrosin stain used for staining the sperm cell.	16
3.3 Graduated collection tube used in determining semen volume.	17
3.4 Creamy-white colour of Boer goat semen	18
3.5 Neubauer Haemocytometer used in determining semen concentration.	19
3.6 Example of a live spermatozoa (bottom) and a dead spermatozoa (top). Live sperm would appear white or translucent while dead sperm would appear pink or dark red.	21
3.7 Common abnormalities found in this research were (a) head detachment, (b) bent midpiece, and (c) coiled tail.	22

## LIST OF SYMBOLS, UNITS, AND ABBREVIATIONS

%	Percentage
°C	Degree Celsius
μl	Microlitre
μg	microgram
ANOVA	Analysis of Variance
AV	Artificial vagina
DMRT	Duncan multiple range test
<i>et al.</i>	<i>et alia</i> (Latin)
G	Gram
MMOT	Mass motility
mOsm	Milliosmole
mS	millisiemens
NAAB	National Association of Animal Breeders
OIE	World Organisation for Animal Health
Osm	Osmole
pH	Negative logarithm of the hydrogen ion concentration
SAS	Statistical Analysis Software
SEM	Standard error mean
Sp.	Species
TBS	Tris Buffer Solution
TMR	Total mixed ration
UMS	Universiti Malaysia Sabah
FPL	Faculty of Sustainable Agriculture
v/v	Volume per volume

## LIST OF FORMULAE

Formula	Page
3.1 Concentration (sperm/ml) = $\frac{n}{s} \times 25 \times \text{DF} \times 10^4$ n = total number of spermatozoa s = number of big square counted DF = dilution factor	19
3.2 Individual progressive motility (%) = $\frac{\text{Area 1} + \text{Area 2} + \dots + \text{Area 5}}{5} \times 100\%$	20
3.3 Viable spermatozoa (%) = $\frac{\text{Number of live sperm}}{\text{Total number of sperm assessed}} \times 100\%$	21
3.4 Dead spermatozoa (%) = $\frac{\text{Number of dead sperm}}{\text{Total number of sperm assessed}} \times 100\%$	21
3.5 Abnormal spermatozoa (%) = $\frac{\text{Number of abnormal sperm}}{\text{Total number of sperm assessed}} \times 100\%$	21

## **CHAPTER 1**

### **INTRODUCTION**

#### **1.1 Introduction**

Artificial Insemination (AI) is one of the method used to improve reproduction and genetics of farm animals. In this method, the semen from the male are deposited into the reproductive tract of the female by other than the natural means. Generally done in cattle, AI also proved to be successful in small ruminants such as goats. In Malaysia, goats are reared mainly for meat and milk production. Even though goat production in Malaysia are relatively small compared to other livestock, it still holds a large potential for improvement in the future.

One of the necessary component in AI are semen preservation. The collected semen from the male contains sperm cell which are sensitive to external and also internal condition on which it resides. To overcome this problem, semen are usually mixed with an extender, in which the sperm cell are provided with optimum condition to thrive. Extender contains buffer solution to reduce the pH fluctuation due to the sperm cell's metabolism. Yolk are also added to provide nutrient for the sperm cell, while giving protection from damage during cryopreservation. To some extent, antimicrobial agents are also needed to prevent foreign organism from contaminating the semen



Stingless bee, or known locally as Lebah Kelulut in Malaysia has recently received commercial attention. Stingless bee honey has been sold at premium price due to the difficulties in gathering it. However, due to limited quantity and lack of marketing strategies, stingless bee honey's sale are still very low compared to regular honey produced by honey bees. By extending the usage of stingless bee honey, beekeepers income in Malaysia can be increased. Moreover, the properties of the stingless bee honey itself, such as the antimicrobial activity, high sugar content and its effect on fertility should be emphasized. Research on stingless bee in Malaysia are still pretty scarce, and alternative usage of the honey may be of significant value. Therefore, the objective of this research is to determine the effect of different concentration of stingless bee honey in Tris extender on semen survivability of Boer goat.

## **1.2 Justification**

Honey is easily obtained in Malaysia. There are several types of honey found in Malaysia, each consisting of different chemical properties. By extending their usage other than for human consumption, the commercial value of honey will increase and therefore increase the revenue of the beekeepers. Compared to other type of honey, stingless bee honey are still less known by the public, and research towards its properties are still lacking. Finding an alternative use for this type of honey, can shade some light towards its potential while also increases public interest.

Other factor includes finding an alternative to the conventional tris-citrate-yolk semen extender. Several studies has shown that egg yolk can cause negative effect to spermatozoa survivability by affecting the plasma membrane, acrosomal integrity and reducing motility. Thereby, a substitute should be found in order to increase the success rate of semen preservation. By studying the effect of stingless bee honey as goat semen extender, the semen quality may be prolonged in vitro compared to other conventional semen extender.

### **1.3 Objective**

Objective of this research was to determine the effects of stingless bee honey in Tris extender on semen quality of Boer goat.

### **1.4 Hypothesis**

The hypothesis for this research are;

H<sub>0</sub>: Stingless bee honey in Tris extender will not improve the semen quality of Boer goat.

H<sub>A</sub>: Stingless bee honey in Tris extender will improve the semen quality of Boer goat.



## CHAPTER 2

### Literature Review

#### 2.1 Artificial Insemination in Goat

Artificial Insemination or AI is a type of assisted reproductive technology. In this procedure, the semen collected from the male animal will be deposited into the reproductive tract of the female animal by artificial mean, in which human interference is involved. Generally, semen will be collected from the male either by the use of Artificial Vagina (AV) or an electroejaculator. The collected semen will then be evaluated in term of its concentration, sperm motility, and viability. Semen that meets the required standard will be extended, in which it is mixed with an extender. The function of extender are mainly to dilute the semen or increase its volume, so that it can be used for more than one female. It also serves to nourish the sperm cell and increase its viability by providing optimum of pH, energy source and also cryoprotectant. After extension, the semen is packed, usually in straws, and can be stored indefinitely. However it depends on the preservation method that are being used. Fresh semen can only be used for a few hours, chilled semen can last for a few days, while semen that are frozen using liquid nitrogen can be used even after several years. The standard procedure for inseminating female goats or does involves raising the rear quarters of the doe, with the forequarters on the ground. After that, the use of speculum and light source is needed to locate, and also depositing the semen on the right position on the female reproductive tract (Tsuma *et al.*, 2015).



The two basic technique for AI in goat are laparoscopic insemination and trans cervical insemination (Cseh *et al.*, 2012). Inception rate are generally higher when laparoscopic insemination is being used. However, this technique require skills and knowledge. The use of anaesthesia and minor surgical procedure also makes supervision of veterinarians a necessity in laparoscopic insemination. Foote (1999), favoured trans cervical insemination in which a small volume of concentrated semen are passed through the cervix and into the uterus, helped by elevating the hind quarters and using a lighted speculum to view the cervix. Otherwise fertility is found to be lower with frozen semen and a greater number of fresh sperm are required. Another advantage of trans cervical insemination is that the techniques can be learned and performed by the producer himself. Application of anaesthesia and surgical entry are also not necessary (Farin, 2013).

## **2.2 Semen Extender Properties**

Semen extender are used to increase the volume of the semen. One ejaculation contains a concentrated amount of spermatozoa, and therefore capable to inseminate more than one female. Increasing the volume can therefore save time and cost needed to prepare the breeding doses. However, the most important function of the extender are to increase the semen survivability. The sperm cell are found to be deteriorating in quality after it is exposed to the environment outside of the donor's body, thus chilling and freezing processes are done to preserve its integrity. However, chilling and freezing also pose its own detrimental effect to the sperm, as found by many previous study. Nowadays, semen extenders that are used will share the same basic components, such as glycerol, energy source (sugar), milk, egg yolk, buffer, and antimicrobial agents.

### 2.2.1 Glycerol

Sperm cell may be damaged following cryopreservation, therefore different cryoprotective agents have been used. According to Holt (2000), Curry (2000) and Evans and Maxwell (1987), glycerol can be classified as penetrating cryoprotectant and has been used extensively as semen diluters. First discovered by Polge *et al.* (1949), discovery of glycerol has provided cryoprotection for sperm cell while increasing its post-thaw quality. According to Foote and Bratton (1949) addition of glycerol to egg-yolk based extender will increase post-thaw quality of semen by 15%.

Glycerol provides cryoprotection, or protection from damage due to freezing, by inhibiting formation of intracellular crystalline. This property makes it a necessary component found in conventional extender. However, effect on fertilizing ability of spermatozoa have been found. An increase in the transient osmotic pressure prior to equilibration around the cell membrane will ultimately increase osmotic pressure and affecting the semen quality parameters (Pommer *et al.*, 2002). Hammerstedt *et al.* (1992) also proposed that thermal changes will disturb the lipid to lipid and lipid to protein interaction within the cell membrane, resulting in loss of function.

### 2.2.2 Energy Source

In sperm cell, mitochondria can be found in the midpiece. Production of energy will help in the movement of sperm tail or flagellum, which is responsible for the movement of sperm cell (Bahr and Engler, 1970). According to Lamirande and Gagnon (1992), process of oxidative phosphorylation inside the mitochondria are the main source of Adenosine Triphosphate (ATP) molecule. Naturally, semen contains fructose as source of energy to facilitate sperm movement. Glucose found in egg yolk extender are also found to be utilized by the sperm cell (Salisbury *et al.*, 1978). Potential of different kinds of sugars such as glucose, trehalose, ribose, raffinose, saccharose and galactose as energy source for sperm cell are still actively research. In ruminant however, fructose sugar based extenders are proven most effective, with less negative effect as compared to others (Kasimanickam *et al.*, 2011).



### 2.2.3 Milk Source

Lactose molecule cannot diffuse readily across the cell plasma membrane. This property will aid in creating osmotic pressure around the cell and prevents intracellular crystallization (Barbas and Mascarenhas, 2009). Casein in milk not only reduce seminal proteins binding, but also reduce cell membrane lipid damage, improve motility and viability of sperm (Bergeron *et al.*, 2007). Previous studies done in the mid-20<sup>th</sup> century has bring many advancement towards cryopreservation of semen, especially on extenders containing both milk and glycerol (Michajilov, 1950; Thacker and Almquist, 1953). This can therefore provide an alternative other than egg yolk to be used as semen extender. Acrosomal integrity of goat spermatozoa are found to be compromised when being used with egg yolk based extenders (Aboagla and Terada, 2003). Steroidal compound such as pregnelenone and progesterone found in egg yolk may cause acrosomal reaction in the sperm cells which increases microbial contamination and hormonally active substance in semen extended with egg yolk (Muller-Schlosser *et al.*, 1995).

### 2.2.4 Egg Yolk

Egg yolk is still used as primary non penetrating cryoprotectant in semen extenders. Nowadays, 20% egg yolk in semen extender has been used as a standard in most underdeveloped countries as it is cheap and readily available (Almquist and Wickersham, 1962). Philips (1939) was the first to report egg yolk use in the diluents, with a ratio of 1:1 to phosphate buffered solution (v/v) and become more popular. It was reported that 4 % (v/v) addition of egg yolk produced satisfactory results for semen quality parameters (Salisbury *et al.*, 1941). In egg yolk, Low Density Lipids (LDL) abundance is considered a main cryoprotective agent as it adheres to the cell membrane and protects the cell from cryoshocks (Smith *et al.*, 1979). The LDLs are composed of 17-60 nm spherical molecules, with lipid and triglycerides in the core surrounded by protein and phospholipid thin layers. The LDL contains 11-17% proteins and 83-89% lipids (Graham and Foote, 1987). The addition of egg yolk increased post thaw motility by solubilizing the cell membrane lipids and binds to the sperm (Moussa *et al.*, 2002).



### 2.2.5 Buffer

Buffer is added to semen to preserve the optimum pH for the sperm cell to thrive. Following normal cellular metabolism, sperm cell may increase the pH of the semen and eventually die due to unsuitable pH. Among many type of buffers, hydroxymethyle aminomethane (Tris) and citric acid are commonly used in semen diluents, especially for semen. Developed in 1963, Tris-egg yolk-glycerol extender was most popular for both fresh and frozen semen (Foote, 1998). Use of phosphate buffer as extender was limited due to poor visibility when examined under a microscope (Philips, 1939). According to Rigau *et al.* (1996), increased cellular metabolic activities during semen processing will lead to production of lactic acid. Together with other acids, the extracellular environment become more acidic in nature, therefore having reduced pH. Low pH will reduce cellular activates within spermatozoa and also affect storage life of semen. Bicarbonates and sodium citrate are among the simplest buffer that can be used, however Tris are more preferred due to it stability at high temperature and other environmental conditions (Gadea, 2003).

### 2.2.6 Antimicrobials

Antimicrobial agents are added to extender to keep microbial contamination low in the extended semen. However, presence of sugar (fructose) in semen extender and room temperature (20°C) during semen processing provide a suitable media for bacterial growth. This is especially true in bull semen collection using artificial vagina. Among the commonly found contaminant in extended semen are Gram positive bacteria, and also harmful organism such as *E. coli* and *Salmonella* spp (Gadea, 2003). Michajilov (1950) found that contagious bacteria such as *Brucella abortus*, *Vibrio fetus*, and *Mycobacterium* sp. posed a risk in being transferred through cryopreserved semen. Competition for nutrient source between the sperm cells and contaminating bacteria may affect the motility and viability of spermatozoa, leading to loss of fertilizing ability. Research also shown that contamination of bacteria will affect semen pH, acrosomal integrity and sperm motility (Althouse *et al.*, 2000). World Health Organization (WHO) and World Organisation for Animal Health (OIE) recommended that semen extender components from animal source should be free from all kinds of microorganisms (Marco-Jime Nez *et*

*al.*, 2004). According to Foote and Bratton (1949), Cornel extender was the first standard diluent which incorporate Penicillin G, Streptomycin, and Polymixim-B as antimicrobial agent and gain approval by the National Association of Animal Breeders (NAAB) for several years. Since the first recipe of Cornel extender were introduced in 1950, penicillin and streptomycin are still used at the rate of one gram per litre.

## 2.3 Stingless Bee

Stingless bees are from the tribe of Meliponini have more than one type of genera, i.e. Melipona, Scaptotrigona, and Trigona. The stingless bees are native to tropical and subtropical parts of the world such as Central and South America, Africa, Asia, and northern Australia (Boorn *et al.*, 2010). The *Trigona* sp., known as the 'Kelulut' is the stingless bees species found in Malaysia. These bees produce stingless bee honey, a multifloral honey which is stored in clusters of small resin pots near the extremities of their nests while honeys from *Apis* sp. are stored in hexagonal-shaped honey combs. The stingless bee honey has been reported to have qualitatively excellent antibacterial potency (Zainol *et al.*, 2013), which is useful medically and therapeutically. Biluca *et al.* (2014) stated that honey from stingless bees has a distinct taste and aroma, more fluid in texture, and undergoes slow crystallisation. The distribution of stingless bee honeys in the world market however is limited as compared to honeys from the *Apis mellifera* owing to a limited supply, a lower shelf life, and also lack of an institutional quality standard due to less knowledge available on the product (Guerrini *et al.*, 2009).

### 2.3.1 Stingless Bee Honey

Stingless bee produce their own honey, and are found to be different from the one produced by the bees of genus *Apis* (regular honey bee). Several research has shown that there is a difference between the colour, taste and viscosity between honey from a stingless bee and honey from the honey bee (Almeida-Muradian *et al.*, 2014; Guerrini *et al.*, 2009). The stingless bee honey product are traditionally consumed or are used in medical practices. The honey is harvested either directly from the forest or from the meliponary (bee farm) (Souza *et al.*, 2006).



### 2.3.2 Chemical Constituents

Approximately 200 compounds can be found in honey such as vitamins, enzymes, amino acids, and minerals. Major content of honey are water and sugar, with sugar comprises 95-99% of the total honey's dry matter. Fructose is the most prevalent sugar in honey, contributing 32-38% out of the total sugar. Moreover, honey also contain glucose and several other disaccharides and oligosaccharides. Vitamin C and Vitamin B constituent can also be found in honey (Ciulu *et al.*, 2011). Even though acting as minor constituent, enzymes and protein are present in honey, exhibiting antimicrobial activity and facilitating calcium absorption (Ariefdjohan *et al.*, 2008). Several research have reported (Can *et al.*, 2015; Escriche *et al.*, 2014; Flores *et al.*, 2015; Habib *et al.*, 2014) that phenolic compounds and flavonoids present in honey plays an important role in ameliorating oxidative stress or acting as antioxidants.

Table 2.0 Physicochemical properties of stingless bee honey.

Physicochemical properties	Stingless bee honey
Appearance	Amber brown
Moisture content	25.02 g/100g
pH	3.05-4.55
Total reducing sugars	55.00-86.00%
Glucose	8.20-30.98 g/100g
Fructose	31.11-40.20 g/100g
Sucrose	0.31-1.26% g/100g
Electrical conductivity	0.49-8.77 mS/cm
Ash content	0.01-0.12 g/100g

Source: Souza *et al.*, 2006.



### 2.3.3 Antimicrobial Activities

In a research done by Miorin *et al.* (2003), the biological activity of honey from stingless bee *Teagonsca angustula* was found to possess good antimicrobial activity against *S. aureus* bacteria. Another study also revealed that *T. angustula* honey has significant antimicrobial activity against several different bacterial strains, including *Bacillus cereus* and *Pseudomonas aeruginosa* as well as against yeasts such as *Candida albicans* and *Saccharomyces cerevisiae* (DeMera and Angert, 2004). Honey from *Trigona laeviceps*, a stingless bee found in Thailand, also exhibit antimicrobial activity against several types of bacteria (*E. coli* and *S. aureus*), fungal strain and yeast (Chanchao, 2009). Various research also supported the antibacterial properties of honey bee honey against various type of bacteria, fungi, and viruses (Aggad and Guemour, 2014; Cooper *et al.*, 1999; Nasir *et al.*, 2010). Manuka honey is one of the most potent and well-investigated honeys for its antimicrobial and wound healing activities (Al Somal *et al.*, 1994; Willix *et al.*, 1992). Tualang honey from Malaysia was also reported to have significant antimicrobial and wound healing activities (Bergman *et al.*, 1983).

### 2.3.4 Antioxidant Activities

Antioxidant activities of honey have spark various research, including towards stingless bee honey. Vattuone *et al.* (2007) have conducted research on microbial activity of honey from stingless bee *T. angustula* and *Plebeia wittmanni*, whereas Da Silva *et al.* (2013) studied antioxidant potential of stingless bee honey from *Melipona* (Michmelia) *seminigra merrillae*. Both research reported that the honey exhibit good antioxidant activity. Honey bees from different geographical region may exhibit different antioxidant activity. Kishore *et al.* (2011) reported that scavenging activity of Tualang honey bees is high, while its honey showed antioxidant activity superior to other honey type. Flavonoids and polyphenols found in honey also reported to have antioxidant activity (Pérez-Pérez *et al.*, 2013).





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