EFFECTS OF HONEY CONSUMPTION ON BONE METABOLISM IN FEMALE ATHLETES

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ABSTRACT

Reproductive hormone disturbances, diet restriction and minimal involvement in weight bearing exercises are some of various risk factors for low bone mass density (BMD) in female athletes. Honey has been scientifically proven to prevent bone loss, improve estrogen secretion and reduce oxidative stress but limited to studies conducted on rats. It is inconclusive whether honey supplementation would have the same benefit in humans, especially female athletes. First study aimed to assess the association between BMD and energy availability, body composition, estrogen level and bone loading status in female athletes and to what extend those factors predict BMD. Body composition, BMD, energy intake, energy expenditure, Bone Physical Activity Questionnaire (BPAQ) score and estrogen level were determined from 85 female athletes aged 18–29 years. Results showed that 53% of female athletes had low BMD and the mean (SD) of energy intake was 1291 (33) kcal/day. BMD was positively associated ($p<0.05$) with BPAQ score and body weight but negatively associated ($p<0.05$) with energy expenditure. There was no association between estrogen, energy intake and energy availability with BMD. Energy expenditure and BPAQ score were the main factors that contributed to low BMD in female athletes. Second study was to investigate the acute effects of high and low Tualang honey (TH) dosages on antioxidant activity and oxidative stress. 20 female athletes were assigned equally into low honey (LH) and high honey (HH) dosage group. Blood was collected before (0 hours) and after 0.5, 1, 2 and 3 hours honey ingestion to determine Total phenolic content (TPC), Ferric reducing antioxidant power (FRAP), Malondialdehyde (MDA) and Reactive oxidative species (ROS). Both LH and HH groups increased antioxidant activity and reduced oxidative stress and no difference found between groups. The third study was to determine whether BMD, bone metabolism, estrogen levels, and oxidative stress are influenced by the consumption of TH or a combination of TH and jumping

exercise for 8 weeks in female athletes. Forty-five female athletes with low BMD were equally assigned into three groups: honey (H), honey combined with jumping (HJ) and jumping (C). BMD was measured, and blood was analyzed for estrogen level, bone biomarkers [Bone Alkaline Phosphatase (BAP), C-terminal telopeptides type 1 collagen (ICTP)] and oxidative stress biomarkers (MDA and ROS) pre- and post- intervention. After 8 weeks, H and HJ showed an improvement in BMD score, estrogen levels and bone biomarkers but no significant difference were observed between the three groups. MDA and ROS values were significantly reduced (p<0.05) in H and HJ but were significantly increased in C. In comparison to C, H and HJ were significantly lower (p<0.05) for ROS but not MDA. In conclusion, low BMD in female athletes is associated with high energy expenditure and poor mechanical bone loading. To counter these conditions, TH intake for 8 weeks or combined with jumping exercise were found to be an alternative treatment for female athletes with low BMD as it appeared to improve estrogen levels and have preventive effects towards bone loss and oxidative stress.

ABSTRAK

Gangguan hormon pembiakan, sekatan diet dan penglibatan yang minimum dalam latihan menggunakan berat badan merupakan faktor risiko menyebabkan ketumpatan mineral tulang (KMT) yang rendah di kalangan atlet wanita. Madu telah terbukti secara saintifik dapat mengelakkan tulang rapuh, meningkatkan rembesan estrogen dan mengurangkan tekanan oksidatif, namun kajian hanya terhad ditemui dijalankan ke atas tikus. Tidak diketahui sama ada suplemen madu akan mempunyai faedah yang sama kepada manusia, khususnya, atlet wanita. Kajian pertama bertujuan untuk mengenalpasti kaitan antara KMT dan ketersediaan tenaga, komposisi badan, tahap estrogen dan status bebanan tulang di kalangan atlet wanita dan dan bagaimana faktor ini meramal KMT. Komposisi badan, KMT, pengambilan tenaga, penggunaan tenaga, skor soalselidik fizikal aktiviti tulang (SFAT) dan tahap estrogen didapati daripada 85 atlet wanita terlatih berumur 18-29 tahun . Keputusan menunjukkan 53% daripada atlet wanita mempunyai KMT yang rendah dan min (SD) pengambilan tenaga adalah 1291 (33) kcal/hari. KMT adalah berkait ($p \leq 0.05$) secara positif dengan skor SFAT dan berat badan tetapi berkait ($p \leq 0.05$) secara negatif dengan penggunaan tenaga. Tiada perkaitan ditemui antara estrogen, pengambilan tenaga dan ketersediaan tenaga dengan KMT. Penggunaan tenaga dan skor SFAT adalah faktor utama penyumbang kepada KMT yang rendah di kalangan atlet wanita. Kajian kedua adalah untuk menyiasat kesan akut terhadap dos madu Tualang (MT) yang tinggi dan rendah ke atas aktiviti antioksidan dan tekanan oksidatif. 20 atlet wanita telah dibahagikan samarata kepada kumpulan dos madu rendah (DR) dan madu tinggi (DT). Darah dikumpulkan sebelum (0 jam) dan 0.5, 1, 2 dan 3 jam selepas pengambilan madu untuk mengenalpasti kandungan jumlah fenolik, (TPC), ferik mengurangkan kuasa antioksidan (FRAP), malondialdehide (MDA) dan reaktif oksidatif spesies (ROS). Kedua-dua kumpulan DR dan DT telah meningkatkan aktiviti antioksidan dan mengurangkan tekanan oksidatif dan tiada

perbezaan antara kumpulan ditemui. Kajian ketiga bertujuan untuk menentukan sama ada KMT, metabolisma tulang, tahap estrogen, dan tekanan oksidatif dipengaruhi oleh pengmbilan MT atau gabungan MT dan senaman melompat selama 8 minggu di kalangan atlet wanita. Empat puluh lima atlet wanita dengan KMT yang rendah telah dibahagikan kepada tiga kumpulan: madu (M), madu digabungkan dengan aktiviti melompat (ML) dan melompat (K). KMT diukur, dan darah dianalisis untuk tahap estrogen, biomarker tulang [Alkali Phosphatase Tulang (BAP) dan C-terminal telopeptida jenis 1 kolagen (ICTP)] dan biomarker tekanan oksidatif (MDA dan ROS) sebelum dan selepas intervansi. Selepas 8 minggu, M dan ML menunjukkan peningkatan pada skor KMT, tahap estrogen dan biomarker tulang tetapi tiada perbezaan yang ketara diperhatikan antara tiga kumpulan. Nilai MDA dan ROS telah dikurangkan ($p < 0.05$) dalam kumpulan M dan ML tetapi telah meningkat dengan ketara dalam kumpulan K. Berbanding dengan kumpulan K, M dan ML menghasilkan ROS yang rendah ($p < 0.05$) tetapi tidak untuk MDA. Kesimpulannya, KMT yang rendah dikalangan atlet wanita dikaitkan dengan penggunaan tenaga yang tinggi dan kekurangan mekanikal bebanan tulang. Untuk mengatasi keadaan ini, pengambilan MT selama 8 minggu atau digabungkan dengan senaman melompat telah ditemui sebagai rawatan alternatif untuk atlet wanita yang mempunyai KMT rendah kerana mampu meningkatkan tahap estrogen dan mempunyai kesan pencegahan terhadap tulang rapuh dan tekanan oksidatif.

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LIST OF SYMBOLS AND ABBREVIATIONS

BAP	:	Bone Alkaline Phosphatase
BMD	:	Bone Mineral Density
BMI	:	Body Mass Index
BMR	:	Basal Metabolic Rate
BPAQ	:	Bone Physical Activity Questionnaire
BW	:	Body Weight
C	:	Control
CHO	:	Carbohydrate
DEXA	:	Dual Energy X-ray Absorptiometry
DPPH	:	2,2-diphenyl-1-picrylhydrazyl
DR	:	Dos Madu Rendah
DT	:	Dos Madu Tinggi
EAT-20	:	Eating Attitude Test-20
EM	:	Emission Wavelength
EX	:	Excitation Wavelength
FAMA	:	Federal Agriculture Marketing Authority
FAT	:	Female Athletes Triad
FFM	:	Fat Free Mass
FRAP	:	Ferric Reducing Activity
FSH	:	Follicle Stimulating Hormone
GI	:	Glycemic Index
GnRH	:	Gonadotropin Hormone
GPx	:	Glutathione Peroxide
GRF	:	Ground Reaction Force

H	:	Honey
H_2O_2	:	Hydrogen Peroxide
HH	:	High Honey
HJ	:	Honey Jumping
HRT	:	Hormone Replacement Therapy
ICTP	:	C-Terminal Telopeptidase Type-1 Collagen
K	:	Melompat
KMT	:	Ketumpatan Mineral Tulang
LH	:	Low Honey
M	:	Madu
ML	:	Madu dan Melompat
MT	:	Madu Tualang
MDA	:	Malondialdehyde
NTx	:	Amino-terminal Cross-linking Telopeptidase of Type-1 Collagen
OC	:	Osteocalcin
OPG	:	Osteoprotegerin
ORAC	:	Oxygen Radical Absorbance Capacity
PAL	:	Physical Activity Level
QUS	:	Quantitative Ultrasound
RANK	:	Receptor Activator of Nuclear Factor Kappa B
RANKL	:	Receptor Activator of Nuclear Factor Kappa B Ligand
RDA	:	Recommended Dietary Allowance
RMR	:	Resting Metabolic Rate
RNI	:	Recommended Nutrient Intake
ROS	:	Reactive Oxygen Species
SFAT	:	Soalselidik Fizikal Aktiviti Tulang

SD	:	Standard Deviation
SEM	:	Standard Error
SOD	:	Superoxide Dimutase
SOS	:	Speed of Sound
TBARS	:	Thiobarbituric Acid Reactive Substances
TEA	:	Thermic Effect of Activity
TEE	:	Total Energy Expenditure
TEF	:	Thermic Effect of Food
TH	:	Tualang Honey
TIA	:	Time in the Air
TPC	:	Total Phenolic Content

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CHAPTER 1: GENERAL INTRODUCTION

In general, the major factor for nutrient deficiency is low energy intake (less than 2000 kcal/day). Low energy intake together with high energy expenditure is associated with a condition called low energy availability (Burke et al., 2003; McLean et al., 2001). Chronic low energy availability can alter normal body weight and reduce body fat as well as suppress reproductive hormones, in which the imbalance of these hormones may cause menstrual irregularities (da Costa et al., 2013; Quah, Poh, Ng, & Noor, 2009) and failure to gain bone density (Rodriguez-Pacheco et al., 2009). It has been proven that deficiency of reproductive hormones level will lead to increase osteoclast activities which then will cause increases in bone resorption (Kassem, 1997) and reduce bone mineral density (BMD) (MacKelvie, McKay, Petit, Moran, & Khan, 2012). Therefore, based on previous evidence, it was established that energy balance, anthropometric measures and menstrual disturbance are among the potential factors associated to low BMD in female athletes.

In Malaysia, study showed that the majority of young female athletes (age 15-21 years old) particularly athletes that were in leanness sports (aesthetic, endurance and weight category sport) had low BMD (-2.0 SD < z-score< -1.0 SD) than the athletes from the non-leanness sports group (Quah, Poh, Ng & Noor, 2009). To date, the published data on the factors associated with bone quality which was limited to body composition, eating disorder, menstrual irregularity and calcium intake had been focusing on elite female athletes (Quah et al., 2009). Furthermore, data on energy intake and energy expenditure among the female athletes was last published in 1997 (Ismail, Wan Nudri, and Zawiah, 1997). Since the number of women competing in sports at various levels had increased for the past years, therefore, there is a need to look at their

bone status and possible associated factors such as energy availability, estrogen level and osteogenic effect that may impact their bone health.

It is important to increase awareness about bone health among female athletes because physiologically the bone continually remodeled throughout life where bone resorption activity by osteoclasts is always followed by bone formation by osteoblasts (Christenson, 1997). The imbalance between osteoblast and osteoclast activity could also influenced by oxidative stress which leads to bone loss (Schramm et al., 2003). Oxidative stress can results from excessive free radicals production and low antioxidant properties activity during intense physical activity. The natural antioxidant in the body that could reduce bone stress is estrogen, which is important to increase the expression of glutathione peroxidase (GPx) in osteoclasts (Badeau, Adlercreutz, Kaihovaara, & Tikkanen, 2005). However, if estrogen secretion is reduced then an alternative ergogenic aid is needed to improve bone formation and bone resorption among the female athletes.

Previously, the alternative treatment used for bone loss was calcium supplementation (Mackerras & Lumley, 1997) and antioxidant food such as fruits, vegetable, and herbs. Antioxidants in fruits and vegetables (onion, broccoli, and cauliflower) have demonstrated beneficial effects on bone metabolism in rats because it reduced oxidative stress (Mühlbauer, Lozano, & Reinli, 2002). Honey contains phytochemical compounds such as phenolic and flavonoids which has been shown to have high antioxidant properties that could enhance intestinal calcium absorption and prevent bone loss (Omotayo et al., 2010; Zaid, Sulaiman, Sirajudeen, & Othman, 2010; Erejuwa, Sulaiman, & Ab Wahab, 2012) in human (Griffin, Davila, & Abrams, 2002) and rats (Mühlbauer et al., 2002) studies. Honey consumption also resulted in prolong

survival of osteoblasts and reduction of osteoclastogenic activity, and consequently, reduction in bone loss (Mohd Effendy et al., 2012).Antioxidant activity of honey generally are known to exert significantly *in vitro* (Gheldof & Engeseth, 2002), however there is limited data on the *in vivo* antioxidants activity. The past acute and chronic honey supplementation studies had used various honey dosage from as low as 20 mg/day up to as high as 1.5 g/kg body weight which observed an increased in antioxidant activity and reduced oxidative stress. However, these studies were conducted on postmenopausal women (Shafin, Othman, Zakaria & Nik Hussain, 2014) and healthy male subjects (Tartibian & Maleki, 2012; Schramm et al., 2003), either with fix amount or based on g/BW. However, to date there is no study available on female athletes. The exact dose of honey that is optimal for protection against oxidative damage also is still debatable and therefore its acute effects will be determined in the present study.

Tualang honey (TH) had the highest free radical scavenging and antioxidant activity compared to nine different Malaysian honeys from different origins (using Manuka honey as a gold standard) (Kishore et al., 2011). A study conducted on female rats showed that TH supplementation combined with jumping exercise (Mosavat, Ooi, & Mohamed, 2014a; Tavafzadeh, Ooi, Oleksandr, & Sulaiman, 2011) for the duration of 8 weeks observed beneficial effects on bone metabolism and reproductive hormone. In human study, TH consumption for the duration of 16 weeks showed an improvement in BMD on postmenopausal women (Shafin et al., 2014). It is inconclusive whether honey supplementation would have the same benefit on human as in rats, especially female athletes as an alternative treatment for low BMD. Thus, in the present study, it is expected that these positive effects of TH with appropriate dosage may improve bone

metabolism (osteoclast and osteoblast activity), reproductive hormone i.e. estrogen and reduce oxidative stress especially in females athletes with low BMD.

1.1 Problem Statement

1) Studies on low BMD among female athletes in Malaysia had been focusing on energy intake, menstrual irregularities and eating disorder but limited to elite athletes. To date, no study looking at other possible factors such as energy availability, body composition, estrogen level and bone mechanical loading among non-elite female athletes.
2) Honey has been shown to have antioxidant properties that protect against oxidative stress in sedentary individuals. However the recommended dosage of TH for optimal effects in female athletes is not known.
3) The consumption of TH for 8 weeks either with or without high impact activity have shown positive effects on bone metabolism and reproductive hormones in rats, to date no study conducted on human especially in female athletes.

1.2 Hypotheses of the Study

Hypothesis 1: Energy intake, energy availability, body composition, mechanical bone loading and estrogen are positively associated with BMD in female athletes

Hypothesis 2: TH with high dosage (1.5 g/kg body weight) shows a higher antioxidant activity and lower oxidative stress compared to TH with low dosage (0.75g/kg body weight) in female athletes.

Hypothesis 3: TH consumption combined with jumping exercise for 8 weeks induced better effects on bone metabolism, estrogen and oxidative stress than TH consumption alone in female athletes.

1.3 Research Objective

1) To determine the factors associated with BMD such as energy availability by measuring energy intake and energy expenditure, body composition, bone mechanical loading and estrogen level, and to identify to what extent do these factors predict BMD in female athletes.
2) To examine the postprandial responses of antioxidant activity and oxidative stress biomarkers after acute consumption of TH with high and low dosages.
3) To investigate the impact of the consumption of TH alone or TH combined with jumping exercise for the duration of 8 weeks on bone metabolism, oxidative stress and reproductive hormone among female athletes.

CHAPTER 2: LITERATURE REVIEW

2.1 Energy Intake and Energy Requirement of Female Athletes

Adequate nutrition intake is a crucial part for athletes to achieve optimal performance. It is essential for the athletes to practice a healthy diet to meet the demands of exercise and/or competition. An appropriate energy intake is important for health maintenance as energy require supporting optimal body function. An athlete‘s energy intake from food, fluids and supplements can be measured from weighed food records (typically 3 to 7 days), a multi-pass 24-hour recall or from food frequency questionnaires (Deakin, 2000). Low energy intake can be caused by inadequate intake of macronutrients (carbohydrates, essential amino acids and fatty acids) and/or micronutrients (minerals and vitamins), which are necessary for health (McLean, Barr, & Prior, 2001).

The energy requirement of Malaysian adults aged 19-29 years for moderate physical activity level (PAL: 1.75) was estimated 2065 kcal/day (NCCFN, 2005). An athlete‘s energy requirements depend on the training volume and intensity training and competition cycle, and will vary from day to day throughout the yearly training plan. Energy requirement also increase due to high stress, exposure to cold or heat, high altitude, some physical injuries, specific drugs or medication (e.g. steroid, nicotine), increases in fat-free mass (FFM) and during the luteal phase of menstrual cycle (Manore & Thompson, 2006).

Thus, to measure energy balance, energy intake should be equal to total energy expenditure (TEE), which calculated based on the total of basal metabolic rate (BMR),

the Thermic Effect of Food (TEF) and the Thermic Effect of Activity (TEA) (Equation 1) (Zanker & Swaine, 1998).

TEE = BMR + TEF + TEA (Equation 1)

TEA = Planned Exercise Expenditure

+ Spontaneous Physical Activity

+ Non-exercise Activity Thermogenesis

Techniques used to measure or estimate components of TEE in sedentary and moderately active populations can also be applied to athletes, but there are some limitations to this approach, particularly in highly competitive athletes. Since the measurement of BMR requires subjects to remain exclusively at rest, it is more practical to measure resting metabolic rate (RMR). The estimated BMR can be acquired by using either the Cunningham (Cunningham, 1980) (Equation 2), the Harris-Benedict (Harris & Benedict, 1919) (Equation 3) or the equation for Malaysian adult athletes (Equation 4) with an appropriate activity factor being applied to estimate TEE which are encouraged to be used for specific population (e.g. athletes). TEA measurement consists of planned exercise expenditure, spontaneous physical activity (e.g. make a small movement (kicking), and non-exercise activity thermogenesis.

BMR in women (MJ/day) = 500 + 22(FFM in kg) (Equation 2)

BMR in women (kcal/day) = 655.1 + 9.563(BW) + 1.850(Height) – 4.676(Age) (Equation 3)

BMR in women (kcal/day) = 669 + 13 (BW in kg) + 192 (gender: women 0) (Equation 4)

TEE also can be estimated through factorial calculations based on PAL. PAL is calculated using a physical activity questionnaire and 24-hour physical activity recall. PAL is calculated as the ratio of TEE to BMR. BMR can be calculated from Ismail, Ng, Chee, Roslee, and Zawiah (1998) and Poh et al., (2010) predictive equations or using indirect calorimetry. Thus, the TEE for women aged 18-30 years old can be calculated using equations below (Equation 5 & 6):

Calculate Women BMR = 0.0535 (W) + 1.994 (Ismail et al., 1998) (Equation 5)

Calculate TDEE= BMR x Activity Factor (PAL) (Poh et al., 2010) (Equation 6)

2.1.1 Nutrient intake recommendations

In general, the body needs carbohydrate (CHO), fat, protein, vitamin and mineral for maintaining normal physiological activity of the body. There are guidelines for female athletes to estimate the daily nutrient needs. To determine the amount of CHO needed, the athlete‘s BW, energy requirement, type of sport and intensity of training or game schedule. CHO are the main source of energy to fuel the muscles and brain activities. CHO rich foods including fruits, vegetables, breads and grains should cover up 55-65% of daily caloric intake (Burke, Cox, Cummings, & Desbrow, 2001). CHO intake for endurance athletes are suggested to be higher than athletes who undergo general training needs ranges between 7 to 10 g/kg/day and 5 to 7 g/kg/day respectively (Burke et al., 2001). However, the amount has been proposed for extreme and prolonged activities as high as to 12 g/kg BW/day. Thus, recommended CHO intake should be individualized depends on body weight and cannot be the same for all the athletes. Besides that, the periodized training schedule over the week, training cycle of seasonal calendar should be reviewed according to changes in exercise frequency and session. Due to restriction of total energy intake, the athlete less likely to achieve CHO

intake goals. It happens in female athletes especially for endurance athletes who aim to achieve or maintain low levels of body fat (Burke et al., 2001). Professional consultation by nutritionist may help female athletes to find a balance between BW control issues and CHO intake goals.

Meanwhile, the needs for protein are 15 - 20% of total daily energy intake. Protein is very important to repair body cell, growth of muscle and maintenance of body health especially after strenuous activity. A proper balance of plant (e.g. cereal, nuts, beans and soy products) and animal (e.g. egg, milk, meat and fish) proteins will also help the physiological activity such as enzymes and hormone secretion which necessary during intense exercise. However, it is important to maintain a positive nitrogen balance, a protein product especially to athletes who are trying to build muscle or injured. Based on RNI women aged 19 to 29 years old need 55g/day of protein. For team sport athletes such as football, netball and handball, protein intake between 1.2 to 1.7 g/kg BW will result in positive nitrogen balance (Tarnopolsky, 2004). A sufficient dietary protein requirement for endurance activity (low to moderate intensity) is 1.0g/kg BW/day (Tarnopolsky, 2004). The only situation where dietary protein requirement exceed those for relatively sedentary individuals is in elite athletes where the maximal requirement is approximately ~1.6g/kg/d (Tarnopolsky, 2004).

Fat intake typically ranges from 25% to 35% of the total calorie intake. Consuming less than 20% of energy intake from fat, does not benefit body and performance goal. Intake of fat by athletes should be based on individualized and also depends on training level and body composition goals (Rosenbloom, 2012). Type of fat such as plasma free fatty acids, triglycerides and adipose tissues contributes a fuel substrate to increase fat availability that most important during endurance training.

Vitamins also are very important for body activity. There are categorized as micronutrients and are necessary for the sedentary individual and active person (Woolf & Manore, 2006). Vitamins can be divided into two groups which are water soluble vitamin and fat soluble vitamins. Water soluble vitamins consist of vitamin B complex and vitamin C. Meanwhile, fat soluble vitamins are vitamin A, D, E and K. To ensure individual get an adequate amount of vitamin, they should be eating a variety of food especially, fruits and vegetables (Lappe et al., 2008). Other than vitamins, minerals, inorganic nutrients also are very important to maintain a normal body function. The examples of minerals are calcium, magnesium, zinc, and iron which are needed in daily diet (Beshgetoor & Nichols, 2003; Lukaski, 1995). In Malaysia, the recommended nutrient intake is referred to RNI and is categorized according to gender and age. An example of the RNI for Malaysian women aged 19 to 29 years old are shown in **Table 2.1**.

Table 2.1: Recommended Nutrient Intake (RNI) for women (age: 19-29 years old) in Malaysia (adopted from NCCFN,2005).

	Unit	Amount
Energy	Kcal	2000
Protein	G	55
Calcium	Mg	800
Iron	Mg	29
Zinc	Mg	4.9
Selenium	Mg	25
Thiamin (B_1)	Mg	1.1
Riboflavin (B_2)	Mg	1.1
Niacin (B_3)	Mg	14
Folate	Mg	400
Vitamin C	Mg	70
Vitamin A	Mg	500
Vitamin D	Mg	5
Vitamin E	Mg	7.5

2.2 Energy Availability of Female Athletes

It is important to consume adequate nutrition to achieve athletic performances goals and also for maintaining good health and nutritional status among female athletes. The recommended energy requirement of moderately active adults aged 19 to 29 years (PAL 1.75) is 2065 kcal/day (WHO/FAO, 2003). However, it was found that female college athlete‘s reported low energy intake of 1939kcal/day (Shriver et al., 2013). Lower energy intake of 1988kcal/day and 1491kcal/day were also reported among elite cross-country skiers (Papadopoulou et al., 2012) and elite skaters (Dwyer et al., 2012) respectively. Energy intake of less than 2000kcal/day cannot support strenuous activity

and nutritional demands of female athletes (McLean et al., 2001). Poor energy intake among active women was causing from poor food selection which subsequently lead to insufficient or poor intake of protein, carbohydrate and essential fatty acid intake (Manore, 2002). A few studies reported that poor dietary pattern increases dietary restraint (Burrows, Nevill, Bird, & Simpson, 2003; Viner, Harris, Berning, & Meyer, 2015).

Recent studies also showed that nutrient intake for female athlete mostly lower than recommended in nutritional standard especially in CHO, protein and fat (Arieli & Constantini, 2012; Shriver et al., 2013). For example, one study reported that female college athletes‘ (18 or older), carbohydrate intake was significantly lower than the minimum recommended intake of 5g/kg/day, whereas protein intake did not differ significantly from the minimum protein recommendations of 1.2g/kg/day and the mean proportion of total energy coming from fat is less than 20% (Shriver et al., 2013).The most common micronutrients intake that is below recommendation are calcium, vitamin B, iron, and zinc. Iron and zinc intakes are typically low among vegetarian female athletes. Female athletes ingest low magnesium, zinc (Burrows et al., 2003), vitamin B, calcium (Burrows et al., 2003; Manore, 2002), vitamin K (Booth et al., 2003), vitamin D (Tenforde, Sayres, Sainani, & Fredericson, 2010), vitamin C and selenium (Rivas, 2012) intake.

The daily total energy expenditure for a different type of sports had been reported to be significantly higher than energy intake in female athletes (Shriver et al., 2013). The time athletes spent during intense exercise (17% of the day) was reported to be similar to those soldiers in base camp (Ismail, Isa, and Janudin., 1996). For instance, the energy expenditure was found to be 2099 kcal/day in athletics, 2731 kcal in shot-put,

3098 kcal in basketball, 2522 kcal in pencak silat and 2295 kcal in swimming among female athlete aged between 16 to 25 years old at who were based at National Sport Institute (Ismail et al., 1997). Based on the data, it was shown that most of the athletes had low energy intake and high energy expenditure. Low energy intake coupled with high energy expenditure could lead to negative energy balance or known as low energy availability.

Energy availability, defined as dietary intake minus exercise energy expenditure normalized to FFM, is the amount of energy available to the body to perform all other functions after the cost of exercise is subtracted. Low energy availability may occur from insufficient energy intake, high TEE or a combination of the two (Loucks, 2013). The concept was first studied in females, where an energy availability of 45 kcal/kg FFM/day was found to be associated with energy balance and optimal health. Meanwhile, a chronic reduction in energy availability, (particularly below 30 kcal/kg FFM/d) was associated with impairments of a variety of body functions (Loucks, 2013). An example of estimation of energy availability in a 60kg female is demonstrated below:

Body weight (BW) = 60 kg

Body composition, % Body fat (BF) = 20 %

Fat free mass (FFM) = 80 %

FFM in kg = % FFM x BW

= 0.8 x 60

= 48 kg

EI = 2400 kcal/d,

Energy expenditure from exercise (EEE) = 500 kcal/d

EA = (EI – EEE) / FFM = (2400-500) kcal·d / 48.0 kg = 39.6 kcal/kg FFM/day

Energy availability is pronouncing more in athletes compared to inactive peers. (Muia, Wright, Onywera, & Kuria, 2015). It seems that low energy availability in athletes, regardless males or females may disturb the athletic performance in the long-term period. Long term of low energy availability may reduce secretion of hormones such as estrogen, cortisol, and leptin as well as may impair bone formation (Aurelia Nattiv, Loucks, Sanborn, & Warren, 2007). Unbalance of energy availability, poor reproductive hormone secretion and bone status are known as female athletes triad (FAT) (De Souza et al., 2014). A proper treatment guideline and early health screening have been established for controlling of low energy availability including assessment of dietary intake using the Eating Attitude Test (EAT-20) (De Souza et al., 2014; Mountjoy et al., 2014).

2.2.1 Factors associated with low energy availability in female athletes

2.2.1.1 Manipulating body weight and body fat in sports

The incidence of low energy availability is greater among physically active females compared to inactive individuals (Reinking & Alexander, 2005). A possible reason could be because female athletes are more concerned with their body weight (Quah et al., 2009). Specifically, in a research comparing female athletes and sedentary, female athletes had significantly lower scores in body satisfaction, and ideal body weight (shape or body size) (Reinking & Alexander, 2005). There are a few types of sports that place particular importance to body size. For instance, ballet dancers are very concerned

about their body shape, appearance, and terrified of gaining body weight which cause to poor eating attitude (Hidayah & Bariah, 2011) and increased prevalence of FAT (Doyle-Lucas, Akers, & Davy, 2010). Marathon female runners also involved in dietary restraint practices, whereby runners avoid ingesting low calorie intake and restricted intake of carbohydrate, fat, protein and calcium which could subsequently lead to low body weight (Barrack, Rauh, Barkai, & Nichols, 2008; Hoch, Stavrakos, & Schimke, 2007). Meanwhile, 70% of cyclists were identified as restrained eaters who consciously restrict energy intake for weight control (Viner et al., 2015). Sometime, due to high desire to achieve the ideal training body weight, most female athletes trapped in low energy availability condition (Nichols, Sanborn, & Essery, 2007). Overall, leanness-dependent (e.g. gymnastic) and weight category (e.g. karate) sports are the groups that most concern on body size, body weight and body fat compared to other sports (Sundgot-Borgen & Torstveit, 2004).

Most of the scientific data found that female athletes had low fat percentage (Brito et al., 2012). It was reported that the mean fat percentage in moderately trained female athletes was 18.4±3.29% among volleyball players and 15.7±5.05% among basketballers, and was similar to the reported values in elite adult players in team sports (Tsunawake et al., 2003). Pollock et al. (2010) mentioned that female athletes also undergoing a high training frequency (Lim, Omar-Fauzee, & Rosli, 2011) intensity (Manore, 2002) and long duration endurance exercise (Hind, Truscott, & Evans, 2006) exercise that reduced body fat, body mass index (BMI) (Hind et al., 2006),and lean fat mass (Dolan et al., 2012).

2.2.1.2 Low fat percentage that lead to hormonal disturbance

It is undeniable that low energy availability is one factors that could contributes to low fat percentage that might affect reproductive hormone secretion (Loucks, 2003;

Sundgot-Borgen & Torstveit, 2007). As adipose tissues are responsible for leptin hormone production, low adiposity could reduce leptin, insulin, thyroid hormone (Misra, 2008; Thong, McLean, & Graham, 2000) and insulin-like growth factor I secretions (Christo et al., 2008). This condition were higher among oligo/amenorrheic than eumenorrheic runners (Cobb et al., 2003). Imbalance of those hormones could suppress the secretion of gonadotropin-releasing hormone (GnRH) which signals the hypothalamus in brain to reduce the luteinizing hormone pulsatility in the blood. Low level of luteinizing hormone could then reduce the secretion of estrogen hormone by ovaries.

2.2.1.3 Eating disorder in female athletes

Eating disorder experienced in anorexic and bulimic patients due to serious disturbance in eating attitude. The occurrence of eating disorders inducing low BMD is greater in athletes compared to sedentary individuals, also higher among female than male athletes (Sundgot-Borgen & Torstveit, 2004). Further, this condition is more common among lean sports athletes like swimming and cross-country compared to non-lean sports such as soccer, field hockey, and softball (Reinking & Alexander, 2005). Besides, athletes that involved in leanness-dependent and weight-dependent sports had similar risk of eating disorder (Gibbs, Williams, & De Souza, 2013; Reinking & Alexander, 2005; Sundgot-Borgen & Torstveit, 2004). Pressure to maintain body weight has been found to lead to low energy intake and cause some female athletes to develop low energy availability and eating disorder (Nichols et al., 2007). It was proven in a previous study that athletes engaging in dietary restriction practices were (14.5%), high frequency of training (4.6 ±5.3 h/wk), have eating disorder history (13.7%), actively trying to lose weight (80.3%), report irregular menstrual cycles (34.7%), resulting low confident and higher perceived stress (McLean & Barr, 2003).

2.2.2 Low energy availability and bone loss

The incidence of low BMD is higher in America (Kanis, Johnell, Oden, Sernbo, et al., 2000), Sweden (Kanis, Johnell, Oden, Jonsson, et al., 2000) and Europe (National Osteoporosis Foundation, 2002) and were rising in Malaysia. Meanwhile, it was found that Caucasians and Asians have higher risk of developing lower BMD than African-American (Kumar & Clark, 2002; Nattiv, 2000).

Low energy availability is more extreme when athletes consume a low energy intake (Loucks, 2007). It was suggested that an exposure to low energy availability among athlete could be caused by poor dieting habit (Wan Nudri et al., 2003). Such condition occur particularly among vegetarian athletes who practice low calorie and fat intake (McLean & Barr, 2003) which increases the risk of low bone quality. Adequate intake of vitamin B (B1, B2, B4 and B6) is necessary to produce energy and repair or build a new muscle tissue (Manore, 2002). In addition, vitamin B, magnesium, zinc, as well as calcium and vitamin D (Tenforde et al., 2010) have been categorized as bone building nutrients (Manore, 2002, Burrows et al., 2003). A significantly ($p=0.036$) lower incidence of bone loss was found in women who took calcium supplementation regularly as opposed to those who did not, (18.7% vs. 29.3%) (Lim et al., 2005). Additionally, study by Booth et al. (2003) showed that low dietary vitamin K intake was associated with an increased risk of hip fracture. Selenium and vitamin C that acted as antioxidants were also found to be low among the athletes (Rivas, 2012). Besides, it was found that female athletes who are treated under drug therapy for a long period such as corticosteroids, heparin, cytotoxins and certain anticonvulsants put the person at risk of low BMD (Ferguson, 2004). Such risks were also seen among athletes who consumed diet pills and laxatives (Hidayah & Bariah, 2011).

Low energy availability, low body fat percentage and high energy expenditure could negatively affect the reproductive hormone regulation in female athletes (Thong & Graham, 1999). Furthermore, low body fat and its effect on estrogen secretion are important in understanding low BMD among female athletes. Low estrogen level was found to be reducing if hormones like insulin, thyroid, and leptin are reduced (Thong et al., 2000). Additionally, leptin are secretion by adipose tissues in fat cell. Thus, a number of fat stores are important to help the secretion of leptin by adipose fat cells. There have been several studies suggested that leptin may be a metabolic signal that provides a link between adipose tissue, energy availability, and the reproductive hormones (Thong et al., 2000). However, De Souza et al., (2008) reported leptin as significant marker to predict a bone formation, but not markers of bone resorption (De Souza et al., 2008).

2.3 Menstrual Cycle

In reproductive system, the menstrual cycle is a regular natural change in female (Silverthorn, 2013). At the age of twelve until fifteen years old, the first period usually come and known as age of menarche (Zareen et al., 2016). The gap duration between first day of period and last day of period ranged between 5 to 12 days and typically length for the next period is 21 to 35 days in female adults (an average of 28 days) (Diaz, Laufer, & Breech, 2006). Each cycle can be divided into three phases based on events in the ovary (ovarian cycle) or in the uterus (uterine cycle) (Silverthorn, 2013). The ovarian cycle consists of follicular phase, ovulation phase and luteal phase whereas the uterine cycle is divided into menstruation, proliferation phase, and secondary phase.

The first part of ovarian cycle is the follicular phase. In follicular phase, egg is release in ovum after the ovarian follicles mature (Silverthorn, 2013). Then a few ovarian follicles are stimulated during the rising of follicle stimulating hormone (FSH)

(Fanchin et al., 2003). In a second phase, known as ovulation, a mature egg is released into the oviduct. After that, estrogen hormone will suppress the production of luteinizing hormone from anterior pituitary gland. Ovulation process may start around day 12 and may last for up to 48 hours of average cycle (Dunson, Colombo, & Baird, 2002). After ovulation, FSH and luteinizing hormone turn the remaining part of dominant follicle into corpus luteum in luteal phases (Weschler, 2003). This process are continued and yield significant amounts of progesterone and estrogen (De Souza, 2003). Progesterone are important to receive fertilised egg for implantation, whereas estrogen may produce one or two days of fertile cervical mucus after ovulation (Weschler, 2003). Menstrual bleeding or menstruation usually last around 2 to 7 days (Diaz, Laufer, & Breech, 2006). Menstruation ceases after menopause which usually occurs around 45 and 55 years of age (Meguid & Wise, 2001)

2.3.1 Reproductive hormone level in female athletes

Márquez and Molinero (2013) concluded that if young or adults female are under nutrition and have low body weight with less fat percentage, then estrogen secretion reduced and affect menstrual cycle. This condition also results from the suppression of the pulsatile secretion of a hypothalamic GnRH which leads to a reduced secretion of luteinizing hormone and FSH, thus preventing ovarian stimulation (Zaid et al., 2010). Additionally, previous study also found that low energy availability of less than 30kcal/kgFFM/day will reduce the secretion of luteinizing hormone (Loucks & Thuma, 2003). In Malaysia, it was found that 47.6% of 30 elite athletes from leanness sports (gymnastic, karate, taekwondo, pencak silat), significantly experienced irregular menstrual cycle compared to athletes from non-leanness sport. More concerning is that 26.8% of the athletes fit the criteria for primary amenorrhea, 5.3% for secondary amenorrhea and 17.9% for oligomenorrhea (Quah et al., 2009). Thus, nutritional intake

among the athletes are the crucial element that need to give extra attention because it could contribute to menstrual irregularities and bone loss.

2.3.2 Female athletes, estrogen and bone loss

Amenorrhea is a common problem among female athletes. It is a condition of abnormal absence of menstruation. Mostly athletes with amenorrhea had a lower BMI and BMD than eumenorrheic athletes (Christo et al., 2008; Misra, 2008). In athletes, most menstrual disorders result from a disturbance of the GnRH pulse generator in the hypothalamus of the brain leading to disruption of the pulsatile release of luteinizing hormone in the blood, on which ovarian function critically depends (Loucks, 2003). Low luteinizing hormone leads to reduced estrogen release which negatively affect bone formation and resorption, and finally contribute to net bone loss (reduced osteoblast, increased osteoclast) (De Souza et al., 2008; Loucks, 2003).

Estrogen also can be considered as an endogenous antioxidant. Abnormal estrogen secretion then will trigger the disruption of bone formation and bone resorption which could lead to bone loss. In female, bone loss progresses much more rapidly due to estrogen deficiency (MacKelvie et l., 2002). Low estrogen levels will reduce the quantity of calcium in the bone, as the effect from removal of calcium from bone (Quah et al., 2009). Declining serum estradiol levels can increase the lifespan of osteoclast, leading to bone resorption and bone loss in the female. It is because, estrogen is essential to help in development of epiphyseal growth plates in bone (Kassem, 1997).

Estrogen also was found to exhibit antioxidant protection of lipoproteins in the aqueous system and was shown to increase the expression of GPx in osteoclasts (Badeau et al., 2005). When the body is subjected to high level of oxidative stress

following estrogen reduction, lipid accumulation will occur. This will promote osteoblast apoptosis and simultaneously upregulate reactive oxygen species (ROS) production**,** particularly hydrogen peroxide (H_2O_2) and superoxide anion (Almeida, Han, Martin-Millan, Plotkin, et al., 2007). ROS may increase bone resorption through activation of NF-κB which plays an important role in osteoclastogenesis. ROS can also promote osteoclast resorption activity directly or by mimicking receptor activator of NF-kB (RANK) signaling which results in osteoclast differentiation (Wauquier et al., 2009). Oxidative stress decreases the level of bone formation by modulating the differentiation and survival of osteoblasts (Bai et al., 2004).

Artistic gymnasts and rhythmic gymnast show high incidence of amenorrhea and oligomenorrhea due to low caloric intake. Low caloric intake coupled with strenuous training exercise, result in unbalance of reproductive hormone secretion (estrogen), low body weight (Manore, 2002) and subsequently low BMD (Helge & Kanstrup, 2002). Oligo/amenorrheic runners reported a low BMD at different site such as spine, hip, and whole body for 5,6 and 3% respectively than eumenorrheic runners (Cobb et al., 2003).

2.4 Bone Formation and Resorption Activity during Exercise

The study by Clarke (2008) discussed on the anatomy and physiology of the bone. It is found that bone undergoes modelling, and remodeling throughout life. Modelling and remodeling in bone occur when the bone received mechanical force during exercise. In response to biochemical force on bone, the activity of osteoblast and osteoclast may influence the bone formation and bone resorption process (Wauquier et al., 2009). Bone formation is very important to maintain the bone strength and mineral content. Meanwhile, continuous removal of old bone and replace with new bone involved mainly in bone resorption process (Burr, 2002). It is important to prevent accumulation

of bone micro damage (Altindag, Erel, Soran, Celik, & Selek, 2008). Both process may develop at different site randomly but mainly are targeted to areas that require repair (Parfitt, 2002).

Unbalance of bone formation and bone resorption leads to the activation of osteoclasts to enhance bone resorption, and subsequently affect the bone loss (Banfi, Iorio, & Corsi, 2008). In osteoclast precursors, RANKL-induced activation of RANK to stimulates ROS production, which also known as osteoclastogenesis (Almeida, Han, Martin-Millan, O'Brien, & Manolagas, 2007). Then, ROS enhances the osteoclast numbers by stimulation of RANKL expression through activation of NF-kB (Manolagas, 2010). RANKL will binds to the receptor of RANK and subsequently differentiate into mature osteoclast. Nevertheless, OPG could work as a decoy receptor to prevent the binding between RANKL with RANK.

Other than that, ROS production also can be controlled by production of gluthathione peroxidase which is associated with bone resorption (Fuller, Lean, Bayley, Wani, & Chambers, 2000). Bone resorption process occurs within 2 to 4 weeks to maintain bone health (Boyle, Simonet, & Lacey, 2003). However, 4 to 6 months were needed for bone formation to complete, thus the combination of organic matrix and mineral will increase concentration of calcium and phosphate (Anderson, 2003). At the end of bone formation process, approximately 50 to 70% of osteoblasts cell will be converted into bone lining and osteocytes. Bone lining formation is essential to regulate the movement of mineral such as calcium, phosphate, vitamin D into bone. Bone-lining cells may regulate influx and efflux of mineral ions into and out of bone (Dobnig & Turner, 1995). Alkaline phosphatase plays a role in mineralization of bone during bone formation also could works as a biomarker (Whyte, 1994).

2.4.1 Measurement of bone density, bone formation and bone resorption

There are many different equipment used to measure bone density. Dual energy X-ray absorptiometry (DEXA) was widely used in clinical setting. The DEXA method is costly, non-portable and involved complicated procedure to operate the DEXA machine. Instead a more economical and simple method of assessment using quantitative ultrasound (QUS) could be recommended in a study by Van den Bergh, Smals, Schweitzer, and Hermus (2001). A QUS measurement of the calcaneus was compared to broadband ultrasound attenuation (in dB/MHz) and speed of sound (SOS, in m/s). It was showed that the mechanical properties of trabecular bone (stiffness and strength) can be better predicted with QUS than with DEXA (Alexandersen et al., 2005). In a systematic review Prins, Jorgensen, Jorgensen, and Hassager (1997) mentioned that unlike to the more established method of bone densitometry (e.g. using DEXA), QUS does not involve exposure of individuals to ionizing radiation. It is cheaper, takes up less space and is simpler to use. Moreover, high significant correlation (*r*-values are between 0.6 and 0.9) was found between QUS and DEXA machine (Trimpou et al., 2010), thus QUS is believed to reflect mainly BMD.

It is also important to correlate a findings of the ultrasound BMD assessment with measurement of bone serum biomarkers. The bone formation biomarkers commonly used are osteocalcin (OC) and BAP while bone resorption biomarkers are tartrateresistant acid phosphatase (TRAP) and C-terminal telopeptides of type I collagen (ICTP). Most bone biomarkers (bone formation and bone resorption) can be measured using commercial kits available in the markets. There are advantages for using bone biomarkers as the markers because it is less dangerous, more sensitive to changes and more easily to analyse compared to radiological determination (Banfi, Lombardi, Colombini, & Lippi, 2010). Bone biomarkers mostly used to analyze serum

concentration of bone metabolism after exercise intervention, however, it is shown that less improvement occur after exercise less than 3 weeks. Bone serum markers were used in some studies to measure BMD after endurance training consisting of walking, running, aerobic dance and stair climbing in both males and females athletes (Adami et al., 2008; Eliakim, Raisz, Brasel, & Cooper, 1997). In pre-menopausal women following 4 weeks of exercise, an increase in bone formation was found, however no improvement in bone resorption markers was found (Adami et al., 2008). Another study reported a rise of bone formation markers and a decrease in bone resorption markers in adolescent males in 6 weeks exercise (Eliakim et al., 1997). Following 8 weeks exercise, bone formation increased significantly accompanied by an increase in resorption in male and female army recruits (Evans et al., 2008). After 16 weeks of training (marching, running, jumping), no additional increases in bone formation were reported and bone resorption returned to baseline levels. Thus, the studies speculated that exercise more than 8 weeks may enhance bone resorption rather than bone formation which may contribute to bone loss. In contrast, after involved in 12 weeks of resistance training (Lester et al., 2009) and 8 weeks jump (Erickson & Vukovich, 2010) protocol, bone biomarkers showed an increased bone formation markers with no changes in bone resorption which determine the effectiveness of exercise prescription and an 8 week jump protocol. Thus, it show that bone formation markers are more sensitive than bone resorption markers, and stimulation of osteoblast and/or osteoclast functions is exercise dependent but the response is usually not seen immediately.

During training, ICTP, a bone resorption marker, was shown to be less sensitive than amino-terminal cross-linking telopeptide of type I collagen (NTx) and urinary pyridinolines, which were sensitive to anaerobic exercise (Evans et al., 2008). Whereas, the bone formation markers, BAP and OC changed after 1 month and 2 months of an

exercise programme, respectively (Lester et al., 2009). After 2 months, while BAP normalized, it was found to be sensitive to aerobic exercise and OC was found to be sensitive to anaerobic exercise. Thus, choosing suitable bone biomarkers are so important to get the adequate results.

2.4.2 Impact of exercise on bone mineral density

It was proven that specific exercise seems to have a great effect on bone health apart from nutritional strategies. Current strategies for bone loss prevention should be started from childhood during bone growth and development to avoid bone loss later in life. It was found that low load physical activity enhance bone loss because low load avoid calcium removal in the bone during physical activity (Lester et al., 2009). Another study by Hagihara et al. (2009) discovered that the appropriate exercise volume to enhance BMD of long bone was 4 to 5 days per week of 30 minutes in each session.

In term of type of exercise, the best exercises for bone loading are high impact exercise, muscle-strengthening exercise, stair-step machines, jumping or walking (National Osteoporosis Foundation, 2002). Matthews et al. (2006) agreed and suggested that at least three mechanisms are involved when doing weight bearing physical activity. First, exercise generates mechanical forces that stimulate bone-building osteoblasts. Second, the chronic force of muscles pulling against bones also promotes osteoblast activity. Third, physical activity increases secretion of growth hormone and estrogen as well as other hormones that encourage bone and muscle growth.

Additionally, high impact exercise such us jumping, skipping and running produces peak forces and bone load seems to be effective in producing great osteogenic effects could elicit beneficial effects on bone health. (MacKelvie, Khan, Petit, Janssen, &

McKay, 2003; Matthews et al., 2006). Jumping at an appropriate height (depends on landing force result using force plate form) increased BMD of the metaphyseal trabecular of tibia and decreased serum TRAP levels (bone resorption marker) but did not alter BAP levels (bone formation marker) (Mosavat et al., 2014a; Ooi, Tavafzadeh, Hung, Hung, & He, 2014; Tavafzadeh et al., 2011; Tavafzadeh, Ooi, Chen, Sulaiman, & Hung, 2015). This observation suggested that, jumping may have more effect on trabecular bones than cortical bones because trabecular bones contain more osteoclast. In the present study, jumping exercise is suggested as an alternative to weight-bearing exercise other than strength training and aerobic dance exercise.

A large and growing body of literature has showed the association between bone mechanical loading with BMD. Scofield and Hecht (2012) mentioned that high impact exercise or also called as weight-bearing exercise is helpful on trabecular microarchitecture (Modlesky, 2008) of bone. This is because weight-bearing exercises involve bone mechanical loading that produces an osteogenic effect on bone. This occurs when mechanical stimuli are present to stimulate bone formation, on the other hand, degradation will occur if such stimuli are absent (Nichols et al., 2007). High impact sports such as gymnastics, rugby, or volleyball could perform high osteogenic response compared to low impact sports such as cycling, rowing and swimming (Nichols et al., 2007). The similar finding was also found in high impact sports associated with boxing and figure skater (Dolan et al., 2012; Prelack, Dwyer, Ziegler & Kehayias, 2012). Type of sport that produces high risk of developing low BMD are long distance running (Nieves et al., 2010), dancing (Hidayah & Bariah, 2011) cycling (Viner et al., 2015) swimming, diving (Mudd, Fornetti, & Pivarnik, 2007) and figure skating (Prelack et al., 2012) compared to inactive peers (Scofield & Hecht, 2012). Antigravitation sports such as swimming and diving also have low average leg BMD

(Mudd et al., 2007; Scofield & Hecht, 2012; Sundgot-Borgen & Torstveit, 2004) because of low bone loading.

2.4.3 Exercise, oxidative stress and bone metabolism

As prevention for bone loss, it was found that physical activity was correlated with bone health (Bonaiuti et al., 2002). However, chronic exercise represents a form of oxidative stress to the organism and therefore could alter the balance between pro-oxidants and antioxidants (Finaud, Lac, & Filaire, 2006). Oxidative stress resulted from a disturbance in the balance between free radicals production and antioxidant protective activity during exercise (Wang, Wen, Huang, Chen, & Ku, 2006). In athletes, oxidative stress can easily develop owing to their active physical activity, physical injury or emotional stress (Bloomer, Goldfarb, Wideman, McKenzie, & Consitt, 2005). The relationship between physical activity and oxidative stress depends on mode, intensity, and duration of exercise. Involvement in strenuous and intense training leads to high oxidative stress production that overwhelm the endogenous antioxidants level in the body.

Several pathways have been suggested by which exercise could increase ROS production. The primary pathway involves electron leakage from mitochondria during aerobic exercise due to increase in oxygen flux, resulting in an increase in free radical production (Deaton & Marlin, 2003). Furthermore, auto-oxidation of catecholamines, which are released from the adrenal glands in greater quantities during exercise, induces an increase in free radical production. Similarly, haem proteins such as oxymyoglobin can undergo auto-oxidation as a result of exercise, thus leading to free radical formation, especially that of superoxide.

Generally, exercise can have positive or negative effects on oxidative stress depending on training load, training specificity and the basal level of training. When discussing the different types of exercise, aerobic exercise is accompanied by an increased oxygen consumption which may increase ROS activity. However, this phenomenon does not occur with low exercise intensity (under 60% VO_{2max}). Regular moderate exercise (70% VO_{2max}) increases antioxidant defences of the skeletal muscle through up-regulation of superoxide dismutase (SOD) and GPx genes expression, thereby adapting stronger oxidative stresses. This adaptation may occur very quickly (within 5 minutes) after ROS production and seems to be specific to oxidative muscular fibres which are the main ROS production location during exercise (Finaud et al., 2006).

On the other hand, anaerobic exercises are a type of exercise including a large variety of sport activities (e.g. sprints, jumps or resistance exercise [eccentric or concentric]) which also could increase body oxidative stress (Fisher-Wellman & Bloomer, 2009). ROS production during anaerobic exercise arises from inflammation and cellular damage, which often happen after traumatizing exercise such as impact sports and eccentric exercise and cause bone loss. **Figure 2.1** shows the pathway on how ROS reacted on bone cell. Strenuous and intense training could cause overproduction of ROS and the inability of endogenous antioxidants to remove ROS could lead to oxidative stress. As a result of high oxidative stress, osteoblast is reduced, whereas osteoclast activities are increased due to imbalance of bone coupling (Wauquier et al., 2009)

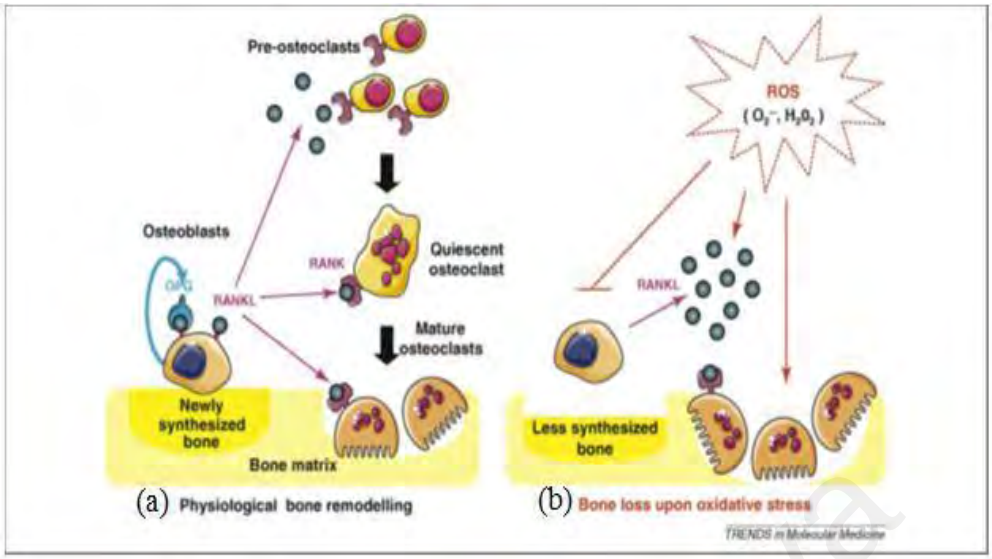

Figure 2.1: Physiological of **(a)** bone remodeling in normal condition without oxidative stress activity and **(b)** bone loss upon ROS activity which cause bone formation by osteoblasts is reduced, whereas osteoclast activities is increased (adopted from Wauquier et., 2009)

In the presence of the RANKL, expressed by osteoblasts and haematopoietic precursors differentiate into multinucleated osteoclasts. RANKL binding to the receptor RANK on the surface of pre-osteoclasts stimulates cell fusion, activates resorption capabilities and enhances cell survival. OPG, a decoy receptor for RANKL, prevents osteoclast differentiation (Wauquier, Leotoing, Coxam, Guicheux, & Wittrant, 2009). By degrading bone, osteoclasts create lacunae that are filled with newly synthesised matrices by osteoblasts. As a result of oxidative stress (increase ROS production) this bone coupling is unbalanced, and bone formation by osteoblasts is reduced, whereas osteoclast differentiation and activities and subsequent bone resorption are enhanced directly or indirectly through an increased RANKL production (Wauquier et al., 2009). Abnormalities of the RANK-RANKL-OPG system with an unbalanced increase in RANKL activity have been implicated in the pathogenesis of various skeletal diseases, including various forms of osteoporosis and bone disease secondary inflammation (Mazziotti, Bilezikian, Canalis, Cocchi, & Giustina, 2012).

2.4.3.1 Measurement of oxidative stress in human studies

Malondialdehyde (MDA), have been frequently used as markers of oxidative stress in response to exercise. The most common method used to assess changes in MDA with exercise is the thiobarbituric acid (TBARS) assay (Halliwell & Chirico, 1993; Sen, Packer, & Hänninen, 2000). Resting plasma MDA was found to be higher in sprint trained athletes and marathon runners compared with control subjects (Marzatico, Pansarasa, Bertorelli, Somenzini, & Della Valle, 1997). Santos-Silva et al. (2001) also found elevated resting MDA levels in trained adolescent swimmers compared with control subjects. In contrast, Niess, Hartmann, Grünert-Fuchs, Poch, and Speit (1996) reported higher plasma MDA in untrained subjects compared with trained subjects, while Miyazaki et al. (2001) observed no change in erythrocyte MDA after a 12-week training program. Several studies reported single bouts of exercise increase blood levels of MDA (Hartmann, Nieβ, Grünert-Fuchs, Poch, & Speit, 1995; Koska et al., 2000; Miyazaki et al., 2001). Marzatico et al. (1997) found plasma MDA increased over 48h post-sprint type exercise in sprinters and immediately post-endurance exercise among marathon runners. Kanter, Lesmes, Kaminsky, La Ham-Saeger, and Nequin (1988) reported increases in plasma MDA (70%) following an endurance event in elite athletes. Similarly, Child, Wilkinson, and Fallowfield (2000) found an increase in MDA level of about 40% immediately after a half marathon.

2.4.3.2 Antioxidant and bone health

While hormone replacement therapy (HRT) use dominates to treat bone loss, it appeared that diet may give a similar effect with no side effect on bone. Million Study Collaborator (2003) found that the side effects of HRT are not suitable for long-term treatment patients. Hence, there is a need to find a comparable alternative treatment to reduce low BMD risk. A recent study by Mielgo-Ayuso et al. (2015) have suggested

that an accurate assessment of nutritional status by a nutritionist is essential to improve bone health in athletes. During exercise, it appeared that moderate training on regular basis was beneficial for oxidative stress and health. Alternately, acute exercise was detrimental for health as oxidative stress would be increased, although this exercise stimulus was necessary to allow an up-regulation of endogenous antioxidant defences. Supporting endogenous defences with additional oral antioxidant supplementation may represent a suitable non-invasive tool for preventing or reducing oxidative stress during training (Karacabey, 2005). Whole foods, rather than capsules, contain antioxidants in natural ratios and proportions, which may act in synergy to optimize the antioxidant effect (Chun et al., 2005)

Recently, more and more athletes are taking supplements of antioxidants even though there is inconclusive evidence about the benefits. However, it is believed that athletes can achieve a balance between antioxidants and oxidants through adequate dietary/supplemental antioxidant intake. Dietary rich antioxidants food has been found to significantly strengthen the endogenous antioxidant defense system, hence, decreasing the adverse effects of ROS on normal physiological functions in human (Aruoma, 1994). ROS is an oxidative stress marker produced when an imbalance occur between free radical production and antioxidant defense system. Fruits and vegetables contain numerous antioxidant phytochemical (i.e phenolic acid and flavonoid) that was shown to play an important role in bone metabolism (Prior & Cao, 2000). It was suggested that the intake of antioxidants may influence BMD by acting as free radical scavengers, preventing oxidation-induced damage to bone cells (Rivas, 2012). Normal diet that includes fruits like orange, prune, and citrus significantly inhibited bone resorption (Chiba et al., 2003; Mühlbauer, Lozano, Palacio, Reinli, & Felix, 2003). Grape seed and pomegranate extract also contain high antioxidant compound such as

Vitamin C which can improve BMD (Chidambara Murthy, Jayaprakasha, & Singh, 2002). The authors speculated an increase in absorption of antioxidant compounds with these dietary practices. Powers, Nelson, and Larson-Meyer (2011) proposed that antioxidant supplements is advisable to be taken by endurance athletes because of its potential to remove free radicals that contribute to muscle fatigue during prolonged exercise and also act as bone-building (Manore, 2002). Thus it is highly encouraged to include fruits and vegetables in daily diet.

There are also some dietary recommendations for women to increase bone density, most of which are related to high vitamins and minerals content. Vitamin C supplement appears to have a beneficial effect especially among postmenopausal women using concurrent oestrogen therapy (Morton, Barrett-Connor, & Schneider, 2001) and premenopausal women because it could increase the femoral neck BMD (Macdonald et al., 2004). Other than that, vitamin C procollagen, enhance collagen synthesis, and stimulate BAP, a marker for osteoblast formation (Morton et al., 2001). A previous study by Hadzir et al. (2014) also found 30-90 g/mL of ascorbic acid was the optimal dose that is able induce osteoblast differentiation. The authors also suggested that ascorbic acid can be used as a nutritional supplement for cellular therapy of bone-related disease (Hadzir et al., 2014).

Apart from vitamin C, there is an increase in interest towards vitamin D supplementation as a result of studies discovering vitamin D deficiency in the athletic populations (Powers et al., 2011). One study has shown that Vitamin D supplements are able to reduce the incidence of stress fractures (Tenforde et al., 2010). However, results of meta-analysis showed that vitamin D supplement present a small benefit at the femoral neck with weighted mean difference only 0·8%. Meanwhile, no effect at any

other site was reported, including the total hip (Reid, Bolland, & Grey, 2014). Omotayo et al. (2010) found that vitamins have the ability to enhance intestinal calcium absorption. Higher intakes of calcium also correlated with improvement in femoral Neck BMD (Macdonald et al., 2004). Similar with Merrilees et al. (2000) finding, a significant increase in BMD of the trochanter femoral neck and lumbar spine was observed among women supplemented with calcium intake of 1160 mg/day.

Athletes involved in strenuous exercise may increase the production of oxidative stress in the body, thus the intake of antioxidant food or supplements were found to give a beneficial effect after exercise. However the timing of antioxidant intake are crucial either before or after training, thus the individualization requirement will be the important part to consider to make sure the athletes received the exact amount and timing (Puel et al., 2006). Most studies concerning the area of recovery from exercise in athletes focus on the use of antioxidant supplements rather than from natural source (e.g food) (Vassalle, Pingitore, & De Giuseppe, 2015). The study on natural food is limited due to inconsistent of antioxidant contents in every type of food. It is because whole food contain antioxidants in natural ratios and proportions, which may act in synergy to optimize the antioxidant effect compared to capsule. In this context, a diet rich in antioxidants may really be a nonpharmacologic and natural opportunity to maintain a physiological antioxidant status.

More recently, several studies have been focusing on honey as an alternative treatment for bone loss. Honey has high levels of flavonoids and phenols (Schramm et al., 2003). Honey could exert a hypoglycemic effect and reduced oxidative stress in kidneys (Omotayo et al., 2010). Honey also elicited beneficial effects to reduce the

cortisol and increase progesterone levels (Mosavat, Ooi, & Mohamed, 2014b). The nutrient content in honey such as calcium, antioxidant and mineral are found to increase bone density and reduce body weight (Zaid et al., 2010). The study by Gheldof and Engeseth (2002) showed that ORAC activity of seven types of honey (3-17μmol TE/g) were similar to ORAC values of many fruits and vegetables (0.5-19μmol TE/g fresh weight) (Cao, Sofic, & Prior, 1996; Chun et al., 2005). These results indicate that honey is comparable to fruits and vegetables in antioxidant capacity on fresh weight basis. Although honey is consume in small amount and not consumed in quantities equivalent to that most fruits and vegetable, it can be used as a healthy alternative to sugar and thereby serve as a supplementary source of dietary antioxidants. Furthermore, Shriver et al. (2013) suggested that nutritional planning with high calcium, antioxidant and carbohydrate are recommended especially for female athletes.

2.4.3.3 Antioxidant measurement

The estimation of total antioxidant activity was described by Benzie and Strain (1999) using the FRAP assay. At low pH, reduction of ferric tripyridyltriazine measures the change in absorption at 593 nm and can monitor complex formation to an intense blue coloured ferrous form. The change in absorbance is therefore directly related to the combined or total reducing power of electron donating antioxidants present in the reaction mixture, for example, of human serum compared to the standard with ascorbic acid. The FRAP assay was also used by Kappus et al. (2011) for comparing supplementation of multi-flavanoids with placebo in athletes before, immediately and 30 min after an acute bout of aerobic exercise. The FRAP assay showed a significant increase after supplementation compared to the placebo group.

Other than that, the total phenolic content (TPC) of heparin-treated plasma was determined with Folin-Denis reagent. This method estimates phenolics by absorbance at 760 nm relative to a standard curve for gallic acid.

2.5 Honey

Honey is a sweet food made by bees using nectar from flowers. In Malaysia there are three well known type of honey which are Tualang (*Koompassia excelsa*), Gelam (*Melaleuca cajuputi*) and Acacia. Honey would have a beneficial effect on human health including increased bone health (Kishore, Halim, Syazana, & Sirajudeen, 2011; Ooi, 2011; Zaid et al., 2010; Zaid et al., 2012). TH is a Malaysian multi floral jungle, honey. It is dark in colour, with a very sweet taste, and nectar are collected from the Tualang tree, a very tall tree found in a tropical rainforest (Ahmed & Othman, 2013). The TH is produced by the rock bee (*Apis dorsata*) (**Figure 2. 2**), which builds hives on branches of tall Tualang trees. The physical characteristic and large variation of nutrient content of honey depends on various factors including geographical regions, floral source, climate, temperature, handling, and storage. In addition, Alvarez-Suarez, Tulipani, Romandini, Bertoli, and Battino (2010) also demonstrated a significant correlation between color and nutritional content (i.e antioxidant capacity) of honey. Generally, darker colored honey has a higher level of antioxidant.

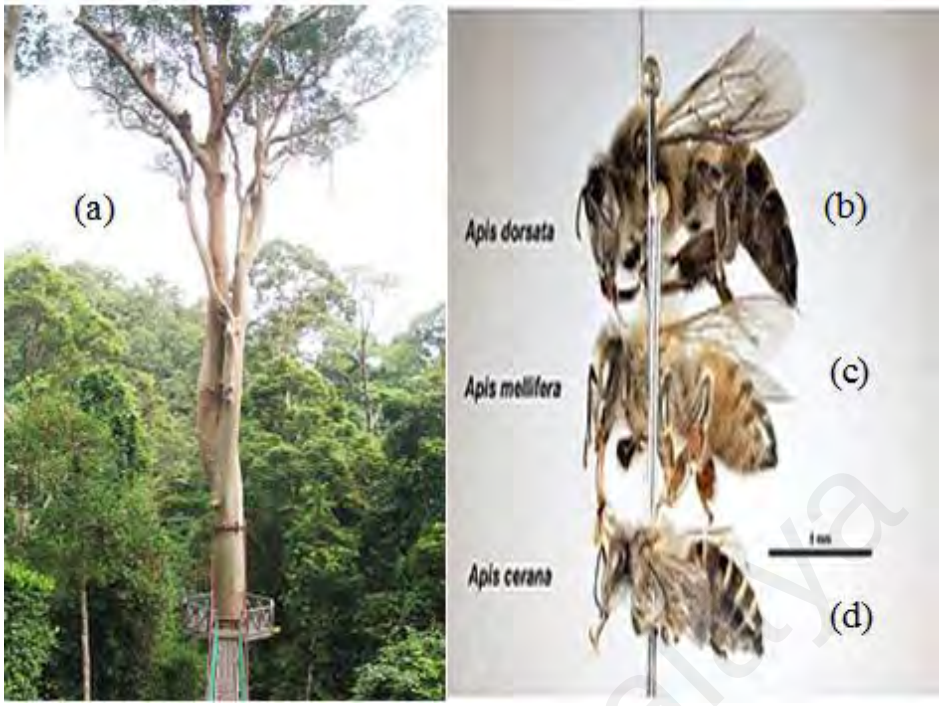

Figure 2.2: The illustration of **(a)** Tualang tree in a tropical rainforest (adopted from Laura, 2015) and three type of rock bee **(b)** *Apis dorsata*, **(c)** *Apis mellifera* and **(d)** *Apis cerana* (adopted from Walker, 2005)

Meanwhile, the glycemic index (GI) of various honey are reported to be within the range of 56-69 (Ischayek & Kern, 2006) with 65 for TH (Robert & Ismail, 2009). Thus, TH can be classified as intermediate GI foods. The GI of honey may vary, depend upon its floral variety and fructose-to-glucose ratio. However, it‘s found that no statistically significant difference between four US honey (clover, buckwheat, cotton, and tupelo) were apparent, nor was a relationship between GI and the fructose-to-glucose ratio detected, indicating that small difference in fructose-to-glucose ratios do not substantially impact honey GI (Ischayek & Kern, 2006).

Rodriguez-Pacheco et al. (2009) mentioned that it is good to practice honey ingestion in daily life and recommended to consume honey in gel form because they are easier to use and carry as well as having various flavours. Another study suggested that honey should be given in gel form with 300ml of plain water (Kreider, 2001) intake

every 20 minutes (Coyle 2004). The author suggested the amount of honey should be adapted to the body weight and to the ingestion time before exercise. Besides, the athlete should consume honey at 4 g/kg body weight 4 hours before exercise, ingest 1 g/kg body weight 1 hour before exercise, ingest 0.5 g/kg body weight 10 minutes before exercise or ingest 30 to 60 g during exercises (each hour of exercise) (Kreider, 2001).

2.5.1 Honey nutritional content

Honey contains mainly carbohydrates (fructose, glucose, and sucrose), vitamins, and some minerals such as calcium, phosphorus, and magnesium, which are believed to be important for bone health (National Honey Board, 2013). Honey gets its sweetness from monosaccharides fructose and glucose and has approximately the same relative sweetness as that of granulated sugar (National Honey Board, 2013). Honey has been known to exert significant in vitro antioxidant activity, in part due to its phenolic content (Gheldof & Engeseth, 2002).

Biochemical parameter	Tualang honey	Gelam honey	Indian forest honey	Pineapple honey
Total phenolic content (mg GAE per 100 g)	83.96 ± 4.53^{a}	74.12 ± 2.52^{b}	45.63 ± 0.66^{c}	27.75 ± 0.80^{d}
Total flavonoid content (mg QE per 100 g)	50.45 ± 1.83^{a}	46.11 ± 0.71^{a}	36.69 ± 0.74^{b}	24.74 ± 0.35^{c}
Total antioxidant capacity (mg AAE per gram of honey)	53.06 ± 0.41^{a}	45.79 ± 1.16^{b}	23.05 ± 0.43^{c}	16.12 ± 0.63^{d}
Ascorbic acid content (milligram per 100 g of honey)	36.09 ± 0.41^{a}	32.14 ± 0.68^{b}	26.09 ± 0.27^{c}	14.64 ± 0.72^{d}
Protein content (milligram BSA per 100 g of honey)	78.53 ± 0.40^{a}	72.87 ± 0.25^{b}	52.27 ± 0.35^{c}	40.87 ± 2.86^{d}
DPPH radical scavenging activity (IC_{50} values mg/mL)	5.80 ± 0.12^{a}	6.68 ± 0.28^{b}	10.32 ± 0.17^{c}	10.86 ± 0.38^{d}
FRAP (μmol Fe [II] per 100 g of honey)	121.89 ± 3.87^{a}	115.61 ± 3.86^{a}	73.35 ± 4.04^{b}	47.92 ± 1.76^{c}

Figure 2.3: Biochemical parameter of Tualang honey, Gelam honey, Indian forest honey and Pineapple honey. Data are expressed as mean ± SD. Significant *p*-values are presented ($p<0.05$). Significantly different values are represented by different letters. GAE indicates gallic acid equivalents; QE, quercetin equivalents; AAE, ascorbic acid equivalents; BSA, bovine serum albumin equivalents (adopted from Kishore et al., 2011).

In Malaysia, research found that TH had the highest TPC, followed by Gelam, Indian forest, and Pineapple honeys (Kishore et al., 2011) (**Figure 2.3**). Higher TPC in honey could increase absorption through the gut barrier by passive diffusion (Scalbert & Williamson, 2000) which are very important to remove oxidative stress in the blood quickly. Additionally, the authors also found a strong correlation (r = 0.987) between the total phenolic and total antioxidant capacity in TH. TH also had the highest 2,2-diphenyl-1-picrylhydrazyl (DPPH) activity, suggesting that TH may contain effective free-radical scavenging compounds. It appears that high level of ascorbic acid in TH may be responsible for the elevated scavenging of the reactive oxygen and nitrogen species. Those antioxidant content could exert anti-inflammatory effect which can increase free radical scavenger, reducing the oxidative stress level as well as inflammatory cytokine (Mohd Effendy, Mohamed, Muhammad, Mohamad, & Shuid, 2012). This will result in survival of osteoblasts, reduced osteoclastogenic activity, and consequently reduced bone loss (Mohd Effendy et al., 2012). TPC of TH is comparable to that of Slovenian honey (Chestnut, Fir, Forest, Multifloral, and Spruce honey) and Romanian honey like Acacia, Chestnut, Honeydew, Lime, and Sunflower honey. Other than that, it is clear that potassium and sodium were also the most abundant minerals in all honey samples from Malaysia covering from 74.18–74.51% and 20.4–28.7%, in 100g of honey, respectively (Chua, Abdul-Rahaman, Sarmidi, & Aziz, 2012). Sample of Malaysia honey also contain other major mineral such as aluminium, calcium, chromium, and zinc.

2.5.2 Mechanism of antioxidant in honey to reduce oxidative stress

Honey is composed of antioxidants including ascorbic acid, phenolics and flavonoids, amino acids, proteins, and some enzymes (Ahmed, Prabhu, Raghavan, & Ngadi, 2007). Among all types of honey in Malaysia, TH showed a similar antioxidant

activity as Manuka honey and Slovenian honey (Ahmed & Othman, 2013). Khalil, Mahaneem, Jamalullail, Alam, and Sulaiman (2011) found that based on nine different Malaysian honeys from different origins (using Manuka honey as a gold standard), TH had higher free radical scavenging and antioxidant activity than the other local and commercially available honeys. It is because TH had the highest concentration of TPC, flavonoids, DPPH, FRAP values as well as protein content indicating its strong antioxidant activities (Moniruzzaman, Khalil, Sulaiman, & Gan, 2013). Thus it was suggested that, elevated free-radical scavenging and antioxidant activity observed in TH is due to the increased level of antioxidant compound (TPC and flavonoid), antioxidant activities (DPPH and FRAP) and protein content.

Honey generally has been known to exert significantly *in vitro* antioxidant activity, in part due to its phenolic content. However, limited data is available on the *in vivo* antioxidant activity of honey. As a consequence, the exact dose of honey that is most protective against oxidative damage is still debatable. It was proven in a mechanistic study by Mohd Effendy et al. (2012) that 20g of TH daily was the appropriate dose as it contains sufficient amount of antioxidant to act as exogenous antioxidant and also for exerting anti-inflammatory effects. TH also could act as a free radical destroyer, reduced the oxidative stress level as well as inhibit proinflammatory cytokine. The mechanism is explained in **Figure 2.4**. **Table 2.2** shows the summary of honey studies that have evaluated antioxidant activity.

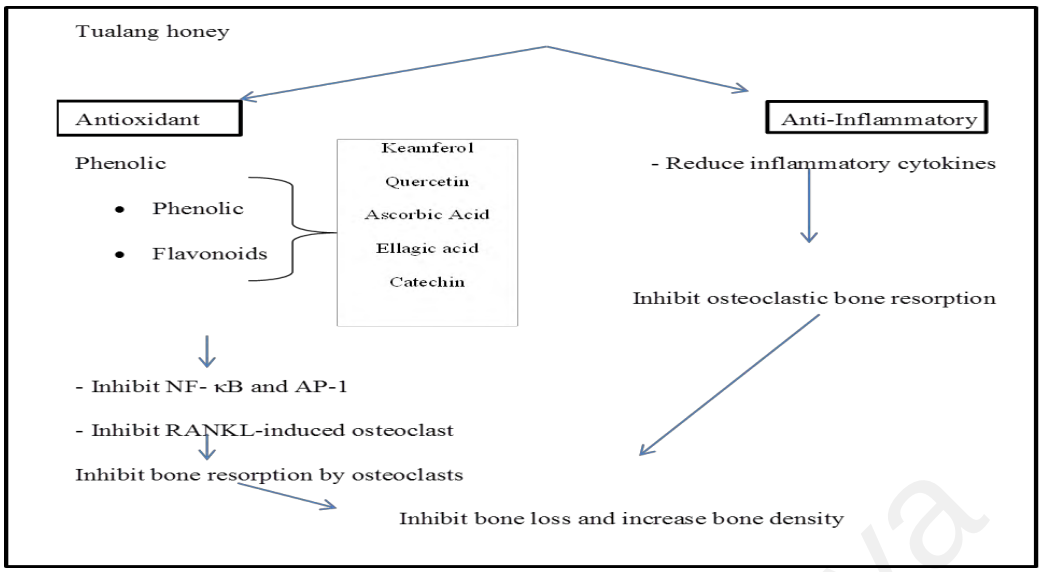

Figure 2.4: Schematic diagram of antioxidative and anti- inflammatory actions of Tualang honey on bone. Phenolic and flavonoid inhibit RANKL that induce osteoclast and subsequently inhibit bone resorption. It also reduces inflammatory cytokine consequently, reduce bone loss. (adopted Mohd Effendy et al., 2012)

Table 2.2: Honey dosage, intervention and antioxidant markers used

	Author and Year	Study target/ Subject target	Intervention/trial	Time for blood withdrawn	Antioxidant test
1	(Cao et al., 1998)	n= 8 healthy female subjects Double-blinded, randomize-controlled trial Acute intervention	• Breakfast beverages (Coconut drink) containing 4g/kg bw with either: a) Strawberries b) Vit C c) Spinach d) Red wine e) Control (coconut drink) • In 2 weeks apart during a period of 10 weeks • All drinks formulated to provide antioxidant capacity equal to 3.7mmol Trolox	• 10ml blood samples (0 baseline sample), followed by breakfast beverages i. • Blood samples were collected at 0.25, 0.5, 1, 2, and 4h • After 4h lunch meal was given and blood samples were obtained at 7, 9 and 11h • Dinner was given immediately after 11h, and last blood collection is at 24h after breakfast	i. ORAC assay ($ORAC_{TOTAL}$, $ORAC_{PCA}$, $ORAC_{ACETONE}$) ii. Trolox equivalent antioxidant capacity assay iii. FRAP assay iv. Plasma Vitamin C v. Serum urate vi. Bilirubin vii. Protein concentration
2	(Gheldof & Engeseth, 2002).	*n*= 5 healthy volunteers In vitro study	Serum will added with honey a) Clover honey b) Acacia honey c) NY Buckwheat honey d) IL Buckwheat honey e) Soy bean honey (2)		i. ORAC assay ii. Lipoprotein Oxidation in Serum iii. Total Phenolic Analysis

3	(Schramm et al., 2003)	*n*= 37 healthy human subjects Randomized-controlled trial Acute intervention	Consumed 1.5g/kg bw of low polyphenolic control meal (bread and water) for control group and plus either: a) Corn Syrup (*n*=7) b) Low Antioxidant buckwheat honey (*n*=10) c) High Antioxidant buckwheat honey (*n*=10)	Blood samples were drawn by venipuncture at: • baseline (immediately prior to meal consumption) • 1h , 2h, and 6 h time points after the consumption of the meal.	i. Assessment of Plasma Total Phenol Content ii. Plasma Antioxidant and Reducing Capacity iii. Plasma Reducing Capacity
4	(Gheldof et al., 2003).	*n*= 25 healthy men Cross over and Blinded Latin-rectangle design Acute intervention	Each volunteer was ingested two plain, low-fat bagels and 500 mL of one of the five test beverages, one at each visit (1 week apart) in random order, to consume within 30min: a) water b) water with buckwheat honey (160g/L) c) water with black tea d) water with black tea with sugar e) water with black tea with buckwheat honey (160g/L)	• Fasting blood samples were drawn at 0h • Immediately drawn 14ml blood for serum and plasma collection at 1.5h and 2h after the fasting blood drawn	i. ORAC assay, ii. Ex vivo susceptibility of ii. serum lipoprotein to Cu2+-induced oxidation, iii. Thiobarbituric acid reactive substances (TBARS) assay.

5	(Shafin, Othman, Zakaria, & Nik Hussain, 2014).	n= 78 healthy postmenopausal women Randomized-controlled trial 16 weeks intervention	a) Tualang Honey- 20g/day b) Estrogen Progestin Therapy- 1mg 17β-estradiol and 5mg dydrogestrone	Blood were taken every 8 weeks for both groups	i. Glutathione to oxidized glutathione ratio ii. Plasma glutathione peroxidase (GPx) iii. Catalase iv. Superoxide dismutase (SOD) v. Plasma 4-hydroxynonenal

2.5.3 Effects of honey on bone in animal studies

More recently, several studies has been focusing on honey as an alternative treatment for bone loss in animal rather than human studies. Honey generally have a variety of beneficial effects on rat health (Zaid et al., 2012) including improved bone health and reproductive system (Mosavat et al., 2014b). It is interesting to note that honey as low as 0.2g/day for 2 weeks have beneficial effects on menopausal (ovariectomised) rats leading to prevention of uterine atrophy, increased bone density and suppression of increased body weight (Zaid et al., 2012). The beneficial effect was also found in animal studies using 1g/kg body weight honey dosage combined with exercise (Mosavat et al., 2014a,Tavafzadeh et al., 2011, Ooi et al., 2014). As different studies using different dosages have been found to be beneficial, therefore the optimal dosage of honey remains debatable.

Recently, it has been reported that TH supplementation of 1 g/kg BW, 7 days/week combined with moderate jumping exercise intensity of 40 jumps/day, 5 days/week for 8 weeks may elicit beneficial effects on young female rats (Tavafzadeh et al., 2011). Meanwhile Mosavat et al. (2014a) showed that high intensity jumping exercise of 80 jumps/day, 5 days/week for 8 weeks combined with 1g/kg BW honey elicited a slightly higher beneficial effect on bone mass and bone metabolism markers when compared to moderate intensity. Thus, it appears that high intensity jumping exercise combined with honey supplementation resulted in more discernible effects on bone. The study also proved that honey could elicit protective effects on disturbance of reproductive hormone levels induced by high and low intensities of jumping exercise.

In terms of duration of feeding, findings of acute and chronic feeding studies revealed different results. Rats given acute feeding with 500 and 800 mg of honey

showed 25.5% and 33.6% increases in calcium absorption (p<0.05), respectively, compared to control group. However in the chronic feeding, groups fed honey did not show the negative effects on bone and had no advantage over the intervention group compared to control group (Ariefdjohan et al., 2008). It can be considered the bone has undergo the adaptation process after 8 weeks. Contrary, Chepulis and Starkey (2008) reported that in a group of young rodents fed with honey for 52 weeks, their BMD was significantly greater than the sugar-free diet-fed control without adaptation influences.

2.5.4 Effects of honey on bone in human studies

A study presented 14 years ago was the first to show honey has comparable effect as glucose in sustaining endurance and power in elite cyclists (Earnest et al., 2004). Nine cyclists received 15 grams of carbohydrate in gel form along with 250 ml of water prior to and every 16 km during the time trials. It was discovered that honey reduced time to complete a 64 km time trial by over 3 minutes (compared to placebo). Honey also produced over 6% greater cycling power during the time trial (compared to a placebo group). Interestingly, a previous study in humans, revealed that women taking TH as low as 20 mg/day for four months, had similar bone densitometry findings when compared with women on HRT (Hussain et al., 2012), thus supporting Zaid and colleagues (2010) suggestion to use honey as an alternative to HRT.

Recently a study was carried out in Malaysia to look at the effect of combined aerobic dance exercise and Gelam honey supplementation on bone turnover markers in young females for 6 weeks (Ooi, 2011). The author suggested that a combination of aerobic dance exercise and honey supplement (20g diluted in 300ml water) may elicit more beneficial effects on increasing bone formation markers compared to aerobic dance exercise or honey supplementation alone. Another study using buckwheat honey

suggested that honey feeding at 1.5g/kg body weight was observed to convey both phenolic antioxidants and increase plasma antioxidant capacity in healthy human subjects (Schramm et al., 2003). Apart from these, limited studies on humans, most of the studies showing the benefits of honey were performed on rodents. Additionally, none of the previous studies reported the underlying specific mechanism that could explain the increase in bone mass by honey feeding.

CHAPTER 3: BONE MINERAL DENSITY AND ASSOCIATED RISK FACTORS IN FEMALE ATHLETES: A CROSS-SECTIONAL STUDY

3.1 Introduction

Adequate nutrition is important not only for maintaining good health and optimal sports performance among female athletes, but also for bone health. It was found that inadequate energy intake decreased estrogen level which eventually resulted in low BMD (Quah et al., 2009). Energy intake of less than 2000 kcal/day could not support the nutritional demands during intense exercise which could reduce body weight and body fat of female athletes (McLean et al., 2001) leading to reduction of reproductive hormonal secretion especially estrogen which play an important role in bone formation (Chen & Brzyski, 1999). In addition, inadequate intake of nutrients such as carbohydrate, essential amino acids, fatty acids, calcium and vitamin C also affected bone health (McLean et al., 2001). Low energy intake coupled with high energy expenditure is associated with a condition known as low energy availability (McLean et al., 2001). There is evidence that energy availability below 30 kcal/kgFFM/day leading to low BMD was mediated by estrogen deficiency in amenorrhea athletes (Loucks, 2004). In contrast, it also has been shown that the reduction of energy availability reduced the rate of bone mineralization without deteriorating the estrogen concentration in the condition of energy deficiency (Ihle & Loucks, 2004).

Other than energy availability and hormonal factors, a large and growing body of literature has investigated the association between BMD and bone mechanical loading. Scofield and Hecht (2012) reported that weight-bearing exercises had high bone mechanical loading that could give an advantage for long-term bone health. Some examples of weight bearing exercise with high impact loading sports include

gymnastics, rugby, and volleyball which tend to produce a better overall osteogenic response than sports without impact loadings such as cycling, rowing and swimming (Nichols et al., 2007). However previous study has demonstrated a lower BMD among weight bearing sport that involved in low impact sport (e.g bowling) and long duration of sport activity (e.g marathon runners) compared to non-weight bearing sport (Scofield & Hecht, 2012).

The aim of this study was to explore potential factors that could be associated with low BMD among female athletes in Malaysia. Specifically, we investigated the association between BMD with energy availability, energy intake and energy expenditure, body weight, fat percentage, estrogen level and bone loading status and to what extend do these factors predict BMD.

3.2 Literature Review

Previous studies have reported that low energy intake was discovered among female college athletes (1939kcal/day) (Lenka et al., 2013), elite cross-country skiers (1988kcal/day) (Papadopoulou et al. 2012) and in elite skaters (1491kcal/day) (Dwyer et al. 2012). However, in Malaysia, it appears to be no published data regarding the nutritional status of Malaysian female athletes after 1997 (Wan Nudri et al., 2003). Previous study observed that athletes with dietary restraint undergo intense exercise (4.6 ±5.3 h/week), were more likely to lose body weight, report irregular menstrual cycles, prone to injury and stress fracture (McLean & Barr, 2003). In Malaysia it was found that the energy expenditure values were reported to be 2099 kcal in athletics, 2731 kcal in shot-put, 3098 kcal in basketball, 2522 kcal in pencak silat and 2295 kcal in swimmers female athletes aged 16 to 25 years old (Ismail et al. 1997).

Low energy availability occurs when the individual consumed low energy intake and exercise at high energy expenditure (McLean et al., 2001). Low energy availability happen when the athletes have lack of knowledge on energy needs which often cause energy deficit (Hidayah & Bariah, 2011). Low fat percentage due to poor energy balance could decrease adipose tissues which responsible for producing leptin hormone (Thong et al., 2000). Low leptin hormone cause reduced production of luteinizing hormone which consequently impairs estrogen hormone secretion in the blood (De Souza et al., 2008). This was proven in a study in 30 elite female athletes from leanness sports (gymnastics, karate, taekwondo, pencak silat) (Quah et al., 2009). Those sports are categorized as weight category sport that insisted for desired body weight and results the athletes to have energy restricted. Consequently, 47.6% experienced irregular menstrual cycles compared to athletes from non-leanness sports, 26.8% athletes fit the criteria for primary amenorrhea, 5.3% for secondary amenorrhea and 17.9% for oligomenorrhea (Quah et al. 2009). Low level of reproductive hormone (luteinizing and estrogen hormones) reduced osteoblast activity (bone formation) and increased osteoclast activity (bone resorption) (De Souza et al., 2008; Loucks, 2003). It is because estrogen acted as an endogenous antioxidant in the body that could reduce oxidative stress (Maggio et al., 2003). Other than hormone factor on bone, one study also found that women who took low calcium, vitamin B, iron, and zinc supplementation had higher incidence of bone loss, compared to women with higher nutrient intake, 29.3% vs 18.7% ($p=0.036$) (Lim et al., 2005).

To improve bone strength, it was found that high impact training associated with boxing and figure skating with the presence of osteogenic stimulus showed a higher beneficial effect compared to other sports (Dolan et al., 2012, Prelack et al., 2012). It was suggested that high load physical activity may have a positive effect on the

trabecular microarchitecture of bone (Modlesky, Majumdar, & Dudley, 2008). In contrast, adolescents and adults who participate in endurance sports, such as running, and non-weight-bearing sports, such as biking and swimming, often have significantly lower BMD than athletes participating in ball and power sports.

3.3 Methodology

3.3.1 Participants

Eighty-five female athletes aged between 18 and 30 years old who have been involved in competitive sports for at least five years were recruited from the University of Malaya. The details of the study and the procedures involved were explained to all participants. Inclusion criteria were female, aged between 18 - 30 years old and physically active (train or exercise three times per week for at least 60 min). All participants must be in good health and without any chronic diseases such as diabetes, stroke or high blood pressure. Exclusion criteria were: women who were pregnant, on medication, or taking supplements. Participants were given the study information sheet (Appendix A) and received explanations about the study procedures such as the experimental protocol and possible risks before being given the consent form (Appendix B). This study was approved by the University Malaya Research Ethics Committee (UMREC), UM.TNC2/RC/H&E/UMREC – 43 (Appendix C).

3.3.2 Experimental procedures

This cross-sectional study was conducted over two sessions with one week apart:

Session 1: Anthropometric measurements were performed on all participants. The participants were asked to complete a health screening form, menstrual history questionnaire (Appendix D) and 24-hour diet recall (Appendix E). Food records and

detailed instructions on how to complete the 3-day food record (Appendix F) were explained to participants.

Session 2: The participants' basal metabolic rate and BMD was measured. Fasting blood sample was taken from the mid-cubital vein by a qualified phlebotomist. Participants were required to complete the Bone-Specific Physical Activity Questionnaire (BPAQ) (Appendix G). In this session, they also returned their completed 3-day food record.

3.3.2.1 Anthropometry measures

Body weight and body fat percentage were measured using the bioelectrical impedance analysis (SC-330, TANITA, Japan). Height was measured using a portable stadiometer (SECA, Germany). The derived values for height and weight measurements were used to calculate BMI according to the formula, weight (kg) divided by the square of height (m) (kg/m^2) (Marfell-Jones, 2006).

3.3.2.2 Bone mineral density

BMD was measured using a portable ultrasound bone densitometer (Furuno CM-200, GB2424276, Japan) at the right side of calcaneus bone. The precise measurement has been accomplished with heel temperature compensation of SOS expressed in meter per second (m/s) and assisted by height adjustable footplate to accurately align a different size heel to the optimized position of the measuring device. Within 10 seconds, the results were printed out from an on-board printer and displayed on LCD. Z-score was used to categorize subjects into two groups, as recommended by Lewiecki et al. (2004): with those having low bone quality for chronological age classified as z-score < 0 and the normal group as z-score ≥ 0.

3.3.2.3 Energy expenditure measurement and calculation

BMR was measured by the indirect calorimetry ventilated hood system (Quark C-PET, COSMED, Italy) which analyses the oxygen consumption and carbon dioxide production from the expired air. The test was carried out in a metabolic room following overnight fast and 24 hours' abstention from exercise. For each 30 minutes BMR measurement, the first 10 minutes was excluded to ensure steady state values were used and the averaged BMR was calculated. The energy expenditure was estimated by multiplying the BMR value with PAL (Ismail, Wan-Nudri, & Zawiah, 1997)

PAL=1.7 for female athletes (FAO/WHO/UNU, 2004).

3.3.2.4 Energy availability

The mean value of energy intake, energy expenditure and fat free mass obtained were used to estimate the athletes' energy availability. Values of less than 30 kcal/day/kg FFM was described as low energy availability (McLean et al., 2001)

Energy Availability = (Energy Intake-Energy Expenditure)/kg FFM (Reed et al., 2015)

3.3.2.5 Fitness test

Twenty meters (20-m) shuttle run test (Beep test) is one of the field tests to measure the participant's fitness level. The level/stage achieved and maximum heart rate (palpitation count) was recorded. If maximum heart rate was less than 150 beat/min, the test had to be repeated on the next day. VO_{2max} was then calculated based on shuttle run score using the VO_{2max} online calculator, meanwhile PAL was estimated using a list of the physical activities a person performed within a 24 hour period and the amount of time spent on each activity, e.g. walking to work, light housework, swimming, carrying bricks at work, or whatever applied to an individual person. The range of PAL values listed was depended on the intensity of habitual physical activity (FAO/WHO/UNU,

2004). For example, light activity lifestyle (1.4-1.69), moderately active lifestyle (1.7-1.9) and vigorously active lifestyle (2.0-2.4).

3.3.6 Dietary intake assessment

3.3.6.1 24-hour food recall

The participants were required to recall a (Appendix E) and record all the food and beverages that were consumed within the last 24 hours. A food recall kit (bowl size- L, M, and S, plate, teaspoon, tablespoon, and scoop) was provided to help the participants to determine the amount of food taken.

3.3.6.2 3-day food record

Participants were required to record the details of the food and beverages consumed for the three days (2 weekdays and 1 weekend) and advised not to change their usual eating patterns (Appendix F). Their dietary intakes were reviewed by the researcher to clarify any issues on the record

Dietary intake of energy, macronutrients and some micronutrients from all participants were analyzed using Nutritionist Pro (Axxya System, Texas).

3.3.7 Blood collection

Five (5) ml of blood samples were collected into plain tube and centrifuged at 3, 000 rpm for 15 minutes at 4^{o}C. Following centrifugation, aliquots of plasma were transferred to labeled 1.5 ml eppendorf tubes and stored at -80^{0}C for later analysis of estrogen at Medical Laboratory, University Malaya Medical Centre. Blood was withdrawn from all participants during their luteal phase of menstrual cycle (14-28 days after the first day of period).

3.3.8 Questionnaires

3.3.8.1 Menstrual history questionnaire

The menstrual history questionnaire (Appendix D) was used to assess the athlete's menstrual status during the past year. Participants were asked for their age of first menarche, the number of menstruations in the past year and the estimated length of menstrual cycle.

3.3.8.2 Bone- specific physical activity questionnaire

BPAQ (Appendix G) was used to obtain a comprehensive account of lifetime physical activity. Participants were required to record the type, frequency and years of physical activity involvement. Independent sections for past (from one year of age) and current (previous 12 months) activity was recorded and necessary to facilitate the examination of the temporal and age-specific effects of mechanical loading on the skeleton. Type of activity involved was measured by Ground reaction force (GRF) which was available in a previous study by Weeks and Beck (2008). For example GRF was ≤ 1.0 for non-body weight exercise, 4.88 for track and field, 22.07 for badminton and 13.62 for soccer. Based on GRF (effective load stimulus), BPAQ score was calculated, with high BPAQ scores indicating that the bone received high mechanical stimuli during exercise.

$$BPAQ = [R + 0.2R (n-1)] \times a \text{ (Weeks \& Beck, 2008)}$$

R = effective load stimulus (derived from GRF testing)

n = frequency of participation (per week)

a = age weighting factor (age weightings: <10 years = 1.2; 10-15 years = 1.5; 15-35 years = 1.1; >35 years = 1.0)

3.3.9 Statistical analyses

Results are presented as means ± standard errors of the mean (SEM) unless otherwise stated. The distribution of data for normality was assessed using the Shapiro-Wilk test before statistical analysis. The association between body weight, fat percentage, energy intake, energy expenditure, energy availability, estrogen level and BPAQ score with BMD was determined using the Stepwise Linear Regression to determine which factor best to predict BMD. Standardized β-coefficients, *t* and *p*-values were reported. Data were analyzed using Statistical Package for the Social Sciences version 22.0 (SPSS, Inc., Chicago, IL). The sample size was estimated using G-Power version 3.1.9 and based on a previous study conducted by Ismail et al. (1997). The power of the study was set at 80%, with a 95% confident interval and effect size was 0.99. The calculated sample size for this study was 60 participants. However, 85 participants were recruited after taking into account 20% drop out rate.

3.4 Results

3.4.1 Participant characteristics, menstrual status, estrogen, BPAQ score and BMD

Participant characteristics are shown in **Table 3.1**. Menstrual history questionnaire classified all participants as eumenorrheic with age of menarche, 12 ± 1 years old, cycle frequency, 12 ± 1 times/year and cycle flow, 7 ± 2 days/ month. Participants are female athletes who involved in sports more than 10 years with mean VO_{2max} fall into ‗Good' category based on maximal oxygen uptake norm (ACSM, 2010). Based on the bone analyses 53% (*n*=45) of participants had low bone density (z-score <0) and 47% showed a normal bone density (z-score ≥0).

3.4.2 Energy Intake, energy expenditure and energy availability

Dietary intake analysis obtained from 24-hour recall (Appendix E) and 3-day food records (Appendix F) were averaged. Mean energy intake was 1291 ± 33 kcal/day with macronutrient distribution 53% carbohydrate, 17% protein and 34% fat (**Table 3.2**). The percentage of carbohydrate intake was slightly lower than the RNI for Malaysia (NCCFN, 2005). Meanwhile both protein and fat percentages were consistent with RNI macronutrient distribution ranges. Mean intake of vitamin D and calcium which are important for competing athletes were lower than the recommended RNI. Mean carbohydrate intake (6g/kg/day) meet the minimum requirement for the athletes whilst protein intake (1g/kg/day) was below recommendations (>1.2 g/kg/day). Energy expenditure (1807 ± 34 kcal /day) was higher than their energy intake (1291 ± 33 kcal/day) which produced low energy availability (29 ± 1 kcal/day/kg FFM).

Table 3.1: Characteristics of participants.

Variables	Mean ± SD
Age (years)	21 ± 3
Anthropometry measures	
Body weight (kg)	56 ± 9
Height (cm)	159 ± 0
Body mass index	22 ± 3
Body fat (%)	27 ± 6
Menstrual Cycle and Hormone Status	
Age of menarche (years old)	12 ± 1
Cycle frequency (times/year)	12 ± 1
Cycle flow (days/month)	7 ± 2
Estrogen level (pmol/L)	500 ± 40
Training and Fitness Status	
Frequency of exercise (days/week)	5 ± 2
Year of sport participation (year)	11 ± 4
VO_{2max} (ml/kg/min)	42 ± 6
Bone Status	
BMD z-score > 0	1.06 ± 0.99
BMD z-score < 0	-0.59 ± 0.06
BPAQ score	26 ± 19

Values are mean ± standard deviation (SD), *n*=85

Table 3.2: Dietary Intake of the female athletes.

Nutrient Intake	Mean ± SEM	RNI
CHO (g)	167 ± 4.7	209
Fat (g)	50 ± 1.6	50
Protein (g)	55 ± 1.8	55
Vitamin D (μg/d)	0.5 ± 0.2	5
Calcium (mg/d)	416 ± 17.8	800
Phosphorus (mg/d)	894 ± 35.0	550
Energy Intake (kcal)	1291± 33.0	2000
Energy from CHO (%)	53 ± 0.7	55-65
Energy from Fat (%)	34 ± 0.5	25-35
Energy from Protein (%)	17 ± 0.3	15-20

Values are mean ± standard error (SEM), *n*=85. RNI, Recommended Nutrient Intake

3.4.3 Association between body weight, fat percentage, energy intake, energy expenditure, energy availability, estrogen level and BPAQ score with BMD

The association between variety of factors and BMD are shown in **Table 3.3**. Body weight was significantly positive associated with BMD (r=0.3, p=0.01) which suggests that low body weight increases the chances of low BMD. Meanwhile, there was no different between and BMD. The female athletes‘ mean energy intake and availability was 1291 ± 33 kcal/day and 29 ± 1 kcal/day/kg respectively. However, there was no significant correlation between energy intake and energy availability and BMD. Energy expenditure among female athletes were high and Pearson correlation showed a statistically significant negative correlation (r= -0.4, p<0.001) between energy expenditure and BMD scores. The estrogen level was 500 ± 40 pmol/L which was lower compared to the normal estrogen level (700 - 1079 pmol/L). However, Pearson

correlation showed no significant correlation between estrogen level and BMD. The mean score for the measurement of BPAQ was 26 ± 19. Scores below 40 are considered low (Weeks & Beck, 2008). Pearson Correlation showed a significant positive correlation (r= 0.4, p<0.001) between BPAQ score and BMD scores.

Table 3.3: Correlation analysis exploring the association of energy intake, energy expenditure, energy availability, body weight, body fat, BPAQ score and estrogen level with BMD

Variable	Correlation (r)	*p*-value
Energy intake	0.159	0.074
Energy expenditure	-0.445	0.000
Energy availability	0.039	0.363
Body weight	0.276	0.005
Body Fat	0.124	0.129
BPAQ score	0.403	0.000
Estrogen level	-0.105	0.169

3.4.4 Stepwise regression analysis

Using stepwise regression analysis, the variables of energy expenditure and BPAQ score were found to be significant predictors of BMD (**Table 3.4**). Energy expenditure was the single best predictor of BMD, which accounted for 19.8% of the variation in BMD. With the inclusion of BPAQ score, an additional 7.3% of variation in BMD was accounted and adjusted R^2 was 0.254.

Table 3.4: Stepwise regression analysis models predict the association of variety factors with BMD.

Variable	Coefficient	SE	T	P
(Constant)	-2.499	0.620	-4.031	0.000
Energy expenditure	0.001	0.000	3.498	0.001
BPAQ Score	0.017	0.006	2.884	0.005

Adjusted R^2=0.254

3.5 Discussion

This study found that 53% of female athletes had z-score less than zero which was categorized as low BMD. BMD among the female athletes was found to be significantly associated with energy expenditure, body weight and BPAQ score. However, no correlation was found between BMD and energy intake, energy availability, body weight, body fat and estrogen level. By using stepwise regression, only energy expenditure and BPAQ score were the main factors that could predict BMD.

The body weight (56 ± 9kg), body fat (27 ± 6%) and estrogen level (500 ± 40pmol/L) among participants were in optimal level. However, BPAQ score (26 ± 9) was lower than normal value which revealed that the participants were exposed to low mechanical bone loading during physical activity. Energy availability was 29 ± 1 kcal/day/kg FFM and energy intake (1291 ± 33 kcal/day) was below than RNI values. Energy expenditure (1807 ± 34 kcal /day) among the female athletes was reported to be higher compared to energy intake which explained that female athletes in this study had low energy availability (below than 30 kcal/day /kg FFM). It was because the athletes usually reported to involve in intense exercise but consumed low calories intake (Nichols et al., 2007). Poor energy intake, food selection and dieting habit contributed

to insufficient intake of carbohydrate (Manore, 2002) and consequently reduced the athletes‘ body weight. Low body weight reduces fat percentage and adipose cell which subsequently effect the reduction of estrogen level (Thong et al., 2000). Nevertheless, mean body weight and body fat of the participants in present study was categorised as ideal due to optimal intake of protein and fat. The estrogen level also seems to be normal because participants were eumenoherriec athletes. Nevertheless more than half of the participants had low BMD due to low calcium and vitamin D intakes below RNI requirement. This is consistent with a study which showed a high incidence of low BMD among females who did not taking calcium and vitamin D supplementation regularly as opposed to those who did (Lim et al., 2005). Low absorption of vitamin D was found to increase the level of bone resorption activity (Loucks, 2003). There are several studies suggested that improving energy balance leads to improvement on overall nutritional status thus returning the athlete to normal body weight, estrogen level and bone health especially in female athletes (Doyle-Lucas et al., 2010; Loucks, Kiens, & Wright, 2011).

Overall, this study found that there was a negative significant association between energy expenditure, BPAQ score and body weight with BMD. Even though there was no significant association between energy availability and BMD but, it was shown that high energy expenditure significantly had negative effect on BMD. Previous study showed that daily total energy expenditure for different type of sports had been reported to be significantly higher than energy intake in female athletes (Shriver et al., 2013). High energy expenditure with poor nutrient replacement during exercise can enhance low calcium level in the body which happened by removal of calcium from the bone and muscle contraction activity during exercise (Quah et al., 2009). Long duration of

physical activity also reported to increase calcium loss in the bone (Cavanagh, Licata, & Rice, 2007).

Low bone mechanical loading was also associated with low BMD revealed that low impact activities may cause low BMD among female athletes. This finding is consistent with a study by Scofield and Hecht (2012) who reported that high bone mechanical loading have been acknowledged widely to be advantageous for long-term bone health. This is because high mechanical loading sport produces osteogenic effects on bone. This effect will occur when mechanical stimuli are present to stimulate bone formation, and conversely, degradation will occur if such stimuli are absent. Similarly, one study using high impact exercise consisting of walking, running, jumping, aerobic dance and stair climbing had found increased bone serum biomarkers in both males and females (Adami et al., 2008; Eliakim et al., 1997). It is believed that high impact exercise involves body weight or known as weight bearing exercise may help to strengthen the bone (Erickson & Vukovich, 2010; Lester et al., 2009; Tavafzadeh et al., 2015). Thus, the athletes involved in anti-gravitational or long distance sports are advised to do an extra training session that involved in high osteogenic activity particularly weight- bearing exercise.

There was a positive significant association between body weight and BMD revealed that underweight athletes have high risk of low BMD. Low body weight produces low ground reaction force and poor mechanical stimuli on the bone (Lester et al., 2009). Besides that, low body weight also might impair the fat percentage and subsequently lead to low estrogen secretion (De Souza et al., 2008). Declining estrogen levels can increase the lifespan of osteoclasts, leading to bone resorption in the female. It is because, estrogen is needed for proper development of epiphyseal growth plates in

bone (Kassem, 1997). Estrogen secretion depends on secretion of luteinizing hormone in the blood on which ovarian function critically depends (Loucks, 2003). GnRH in the hypothalamus of the brain control the secretion of the luteinizing hormone (Loucks, 2003). Low luteinizing hormone leads to reduce estrogen release which negatively affecting bone formation and resorption, and finally cause net bone loss (reduced osteoblast, increased osteoclast) in amenorrheic athletes (De Souza et al., 2008). In contrast, this study found no correlation between estrogen level and low BMD because all the participants were eumenorrheic. It showed that low BMD can affect not only among amenorrheic but also in eumenorrheic individual. This was supported the finding by Ihle &Loucks (2004) who mentioned that low BMD can also happen without deteriorating of estrogen concentration.

Using a stepwise regression analysis, it was found that only energy expenditure and BPAQ score are the main factors that could predict BMD among female athletes.

3.6 Conclusion

In conclusion, BMD was associated with energy expenditure, body weight and BPAQ scores in female athletes. Excessive energy expenditure and decreasing bone mechanical loading were the main factors to predict low BMD. More research is warranted looking at biochemical and pathophysiological which include hormone concentration and bone metabolism.

CHAPTER 4: A DOSE-RESPONSE EFFECT FROM TUALANG HONEY CONSUMPTION ON ANTIOXIDANT STATUS AND OXIDATIVE STRESS IN FEMALE ATHLETES

4.1 Introduction

Athletes developed oxidative stress easily during their physical activity, physical injury or emotional stress (Bloomer et al., 2005). Oxidative stress is essentially an imbalance between the production of free radicals and the ability of the body to counteract or detoxify their harmful effects through neutralization by antioxidants (Wang et al., 2006). Naturally, the human body has developed an antioxidant defense system called endogenous to counteract ROS as part of the metabolic process. Overproduction of ROS induces an imbalance between pro-oxidants and antioxidants in cells and tissues resulting in oxidative stress, which have been related to ageing, tissue inflammation and degeneration (Filaire & Toumi, 2012). Involvement in strenuous and intense training causes the endogenous antioxidants in the body unable to fight the oxidative stress (Kerksick & Willoughby, 2005). In view of this, supporting endogenous defences with exogenous antioxidant supplements may represent a suitable non-invasive tool in reducing oxidative stress. Vitamins such as ascorbic acid, tocopherol and polyphenol (flavonoid, quarcetin) are examples of exogenous antioxidants.

Among the natural products that have been researched as antioxidant food such as spinach, strawberries (Wang et al., 2000), onion, garlic, leek (Muhlbauer, 2001) and olives (Puel et al., 2006), honey is slowly gaining a lot of interest worldwide. Malaysia herself produces several types of honey namely Tualang, Gelam, longan, borneo, rubber tree, sourwood and pineapple honeys (Moniruzzaman, Amrah Sulaiman, & Gan, 2016). Honey is recommended to be consumed by athletes before exercise as one of the carbohydrate sources for energy (Earnest et al., 2004). It also contains antioxidants

which may have protective effect against oxidative stress due to exercise (Urso & Clarkson, 2003). Among all types of honey in Malaysia, TH showed similar antioxidant activity as Manuka honey and Slovenian honey (Ahmed & Othman, 2013). Khalil et al. (2011) also found that TH had highest free radical scavenging and antioxidant activity compared to nine different Malaysian honeys from different origins (using Manuka honey as a gold standard). Based on these findings, it is suggested that elevated free-radical scavenging and antioxidant activity observed in TH is due to the increased level of FRAP values.

Although honey generally has been known to exert significantly *in vitro* antioxidant activity, in part due to its phenolic content (Gheldof & Engeseth, 2002), there is limited data on the *in vivo* antioxidants activity of honey. As such, the exact dose of honey that is optimal for protection against oxidative damage is still debatable. A human study using low dose (20 g daily) of TH for 16 weeks had shown a reduction in blood oxidative stress (GPx, Catalase and GSH) levels among postmenopausal women compared to those who received HRT (Shafin et al., 2014). This finding is in line with a study by Tartibian and Maleki (2012), who found that 70 g/day of unprocessed honey could provide a beneficial effect in suppressing oxidative stress markers (ROS and MDA) and increasing antioxidant activity (TPC) in seminal plasma after eight weeks of cycling among male cyclists. Meanwhile, Schramm et al. (2003) proposed the dosage of 1.5g/kg BW of buckwheat honey as being able to increase antioxidants activity (TPC and FRAP) level in plasma as early as 6 hours after ingestion among healthy male subjects.

However, there is no study available on the acute effects of TH consumption on antioxidant activity and oxidative stress among female athletes. To ensure that all participants meet the sufficient antioxidant requirement to suppress oxidative stress in the body, in the present study, the consumption of TH with high dosage (1.5g/kg BW) and low dosage (0.75g/kg BW) to examine the optimal dosage of TH that can increase antioxidants (TPC and FRAP) and reduce oxidative stress (MDA and ROS).

4.2 Literature Review

Exercise can produce an imbalance between ROS and antioxidants (Ji, 1999) and several pathways have been suggested by which exercise can increase ROS production. The primary pathway involves electron leakage from mitochondria during aerobic exercise, whereby there is an increase in oxygen flux, resulting in an increase in free radical production (Deaton & Marlin, 2003). Furthermore, auto-oxidation of catecholamines, which are released from the adrenal glands in greater quantities during exercise, induces an increase in free radicals production (Finaud et al., 2006).

High level of free radical production cannot be supported by endogenous antioxidant in the body. Although an adequate intake of vitamins and minerals as exogenous antioxidant source through a varied and balanced diet remains the best approach to maintain an optimal antioxidant status, it may be necessary in particular conditions, such as when athletes are exposed to high oxidative stress and unable to meet dietary antioxidant requirements or consumed low energy intake (less than 2000kcal/day). Urso and Clarkson (2003) supported that dietary antioxidant supplements are marketed and used by athletes as a means to counteract the oxidative stress of exercise. Many studies have reported on the protective effects of high-antioxidant food (containing polyphenol, quarcetin and flavonoid) against oxidative

stress (Alía, Horcajo, Bravo, & Goya, 2003; Borek, 2001, 2004). Several antioxidant foods at different dosages were examined on the effect on antioxidant capacity and oxidative stress in human. For example, flavonoid-rich chocolate, spinach, strawberries, and red wine when consumed in quantities of 4 g/kg body weight have been observed to increase plasma antioxidant capacity by 7-25% in sedentary subjects (Cao et al., 1998; Wang et al., 2000). Meanwhile, recent study found that combination of quarcetin (500 mg) and vitamin C (250mg) supplementation for 8 weeks was effective in reducing oxidative stress and inflammation among young and physically active individuals (Pingitore et al., 2015). In contrast, supplementation at higher doses of quercetin (1 g/day) for 3 weeks in marathon runners did not change levels of inflammatory and oxidative indices (Nieman et al., 2007).

Antioxidants such as vitamin C, phenolics, flavonoids, quarcetin, amino acids, proteins, and some enzymes also contained in honey (Ahmed et al., 2007). Honey could help in exercise performance and body protection from oxidative stress. In the first study by Earnest et al., (2004) of honey on cycling performance, it was recommended to consume 15g of honey before exercise as one of the carbohydrate sources for energy. Earnest et al. (2004) found that honey could improve in time over the last 16 km of a 64-km stimulated time trial compared to placebo. In an animal study, TH alone provided protection against oxidative stress in streptozotocin-induced diabetic rats (Omotayo et al., 2010). It is because TH had the highest concentration of phenolic compounds, flavonoids, DPPH, FRAP values as well as protein content compared to acacia, pineapple and borneo honey indicating its strong antioxidant activities (Moniruzzaman et al., 2013). Plasma antioxidants levels are sensitive and is a reliable method that is often used to assess the ability of the body to protect against oxidative stress (Kishimoto, Lynch, Reiger, & Yingling, 2012).

4.3 Methodology

4.3.1 Participants

Twenty female athletes aged between 18 and 25 years old who have been involved in sports competition for at least 5 years were recruited at the University of Malaya; without previous history of allergies to any medications or supplements. This study was conducted with the approval of University Malaya Research Ethics Committee and the participants provided written consent. This study was approved by the University Malaya Research Ethics Committee (UMREC), UM.TNC2/RC/H&E/UMREC – 43.

The sample size was estimated using G-Power version 3.1.9 and based on a previous study conducted by Schramm et al. (2003). The power of the study was set at 80%, with a 95% confident interval. The calculated sample size was 8 participants. However after considering 20% drop out rate, the participants per group was 10 and total participants were 20.

4.3.2 Study design

The participants, in randomised-order double blind study, participated in a dietary intervention trials lasting for 5 hours: they consumed either 1.5g/ kg BW (high dose, HH) or 0.75g/kg BW (low dose, LH) of honey. Stratified randomization process was performed based on their age and body weight to ensure the amount of honey ingested for both groups is equal at baseline. The participants were asked to refrain from participating in any vigorous physical activity and from taking any medications, vitamins, and food rich in antioxidants (such as coffee, honey, tea, wine, fruit, juice, fruits, vegetables, cocoa products) 24-h prior to the intervention trial.

4.3.3 Experimental trial

Participants arrived at the Sports Nutrition laboratory after a 12-hour overnight fast, at approximately 08:30 hours. They were weighed using Bioimpedance Analysis Machine (Tanita, Japan) and a cannula (G-15, Venflon) was inserted in an anticubital vein. Participants then rested in a seated position for 10 minutes before the baseline blood sample (6ml into heparin tube) was drawn. Then the participants consumed either 0.75 g/kg or 1.5 g/kg of honey within 10 minutes. Blood samples were obtained at 0.5, 1.0, 2.0 and 3.0 hours after the baseline blood sample using the same blood sampling procedure. The participants were asked to stay within the testing area, executing only sedate behaviour like sitting, reading and studying.

4.3.4 Honey

TH (*Koompassia excelsa*) from a single batch of honey was provided by the Federal Agriculture Marketing Authority (FAMA), Malaysia. The pure honey used in this study was extracted from the original source without any additional processing and treatment prior to administration. TH sample was sent to Unipeq Laboratory, Universiti Kebangsaan Malaysia for analysis of total phenolic, antioxidant activities, ascorbic acid, and protein content (Appendix H).

4.3.5 Blood Collection

Blood samples were collected into a 6 ml heparinised tubes (BD vacutainer) and centrifuged at 30000 RPM for 15 min at 4°C. After centrifugation, aliquots of plasma were transferred into 1.5 ml Eppendorf tubes and stored at -80^{o}C before the analysis of plasma TPC, FRAP, MDA and ROS.

4.3.6 Analysis of phenolic content and antioxidant activities in plasma

4.3.6.1 Total phenol content (TPC)

The TPC was measured using the Folin-Ciocalteu assay which was modified from Cao, Alessio, and Cutler (1993) analysis method. Plasma samples (50 µl) was mixed with 25 µl of 1 N Folin-Ciocalteu reagent. The mixture was incubated at room temperature (27°C) for 5 min, following which 100 µl of saturated sodium carbonate (Na_2CO_3) solution and 75 µl distilled water was added to the mixture. The reaction was incubated for 2 hours after which the absorbance was read at 760 nm using a microplate reader (Spectrophotometer, USA). Gallic acid (concentration range between 400- 0 GAE µg/ml was used to build the standard curve. TPC of plasma samples were expressed as µg of gallic acid equivalents (GAEs) per ml of plasma.

4.3.6.2 Ferric reducing antioxidant activity (FRAP)

Reagent for the FRAP assay was initially prepared; 300 mM of acetate buffer, 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ), and 40 mM HCl and 20 mM $FeCl_3$. Working FRAP reagent was prepared as required by mixing 10 ml acetate buffer, 1 ml TPTZ solution, and 1 ml $FeCl_3$ solution. For the assay, 300 µl freshly prepared FRAP reagent was mixed with 5 µl of sample and incubated at 37°C for 30 min. Absorbance reading was taken at 593 nm (Benzie & Strain, 1996).

4.3.6.3 Malondialdehyde (MDA)

To assess lipid peroxidation in the plasma samples, malondialdehyde (MDA), an end product of lipid peroxidation, was measured by TBARS. TBARS are formed as by-products of lipid peroxidation (i.e. as degradation products of fats). MDA was analysed using the TBARS assay. Reagent for this assay comprised thiobarbituric acid, trichloroacetic acid, and hydrochloric acid as a reagent in the ratio of 1:1:1. For

measurements, 100 µl of TBARS were added with 50 µl of plasma. A total volume of 150 µl of the mixture was put in a 1.5 ml tube and vortex for 1-2 minutes. Then, the mixture were heated on a heating block at 90°C for 20 minutes before added with another 150 µl of butanol. The mixture was centrifuged and 100 µl of the supernatant was read using a microplate reader at 532 nm (Oteiza et al., 1997).

4.3.6.4 Reactive oxygen species (ROS)

A 5 µl of plasma was added with 100 µl dichlorofluorescein diacetate (DCF-DA) in a black plate. The samples were shaken using a shaker for 1 minute. Then, the plate was incubated 30 minutes in a water bath (37^{o}C). Fluorescence reading was taken with the excitation (EX) and emission (EM) wavelengths set at 485 nm and 530 nm (Spectrophotometer, USA) which was modified from Apel and Hirt (2004) analysis method.

4.3.7 Area under the curve (AUC) calculations

The honey consumed over 180 minutes was calculated from the area under antioxidant markers (TPC and FRAP) and oxidative stress markers (MDA and ROS). The integrated area under the postprandial antioxidant and oxidative stress curve was calculated using the trapezoidal method (Wolever & Jenkins, 1986) and was used to assess the plasma responses for the whole period of observation. In the response for antioxidant and oxidative stress markers plasma concentration versus time, the graph area was split into vertical segments. The total AUC for plasma antioxidant and oxidative stress markers responses was calculated by adding all the segments together.

4.3.8 Statistical analysis

Data were expressed as mean ± standard deviation (SD) and mean ± standard error mean (SEM). The distribution of data normality was assessed using the Shapiro-Wilk test before statistical analysis. All statistical analysis was performed using the Statistical Package for Social Sciences (SPSS) Version 22.0 (SPSS, Inc., Chicago, IL). Descriptive analysis was used on honey content (phenolic content, antioxidant activities, ascorbic acid and protein content), participants‘ characteristics and details of honey consumption. The total area under curve (AUC) was compared using independent t-test. Two-way mixed ANOVA (Mixed between-within subjects) was performed on TPC, FRAP, MDA and ROS analyses to determine group and time difference Significant main effects and interactions were further analysed using Tukey‘s post hoc test. Differences were considered significant if $p < 0.05$.

4.4 Results

4.4.2 Participants

The participants (*n=20*), 10 per group in the LH and HH groups completed the experiment. The physical characteristics of the participants and the detail of honey consumption in both groups are presented in **Table 4.1.**

Table 4.1: Physical characteristics of participants and total honey consumption

Variables	**Group**	
	LH	**HH**
Age (y)	20.9 ± 2.33	21.7 ± 1.77
Body weight (kg)	53.62 ± 4.62	54.52 ± 6.78
Honey dosage (g/kg BW)	0.75	1.5
Total honey consumption (g)	40.215 ± 3.47	81.78 ± 10.18

Values are mean ± standard deviation (SD), *n*=20

4.4.1 Honey content

The phenolic content, antioxidant activities, ascorbic acid and protein content in the TH is shown in **Table 4.2**. The presence of phenolic compounds and ascorbic acid in honey could contribute to the observed antioxidant activities. The variability in protein content may depend on the floral preference of the honeybee, from which protein and colloids are derived, and the presence of enzymes, which are from the honeybees themselves (Alvarez-Suarez et al., 2010).

Table 4.2: Total phenolic content, DPPH-radical scavenging activity, ferric reducing antioxidant power, ascorbic acid content and protein content of TH.

Biochemical parameter	**Tualang honey**
Total phenolic content (mg GAE per 100 g)	20.09 ± 0.13
DPPH radical scavenging activity (% inhibition)	35.75 ± 0.58
FRAP (μmol Fe [II] / 100g of honey)	255.5 ± 6.01
Ascorbic acid content (mg/100 g of honey)	1.04 ± 0.00
Protein content (g/100 g of honey)	0.73 ± 0.05

GAE indicates gallic acid equivalents
Data are expressed as mean ± SD.

4.4.3 Area under the curve (AUC) calculations

The time-averaged incremental area under antioxidant versus time curve over 3 hours (180 minutes) after consumption LH and HH of honey, n=20. MDA showed the HH group was significantly (p<0.05) higher than LH group (**Table 4.3**). However, no significant difference was observed for TPC, FRAP and MDA between the two groups.

Table 4.3: Area under the curve (AUC) of the markers

	Group		
Markers	LH	HH	*p*-value
TPC	849.7 ± 190.9	659.8 ±138.8	0.094
FRAP	3697.8 ± 260.4	3685.4 ± 405.6	0.351
MDA	70660.8 ± 8574.9	86131.3 ± 19204.8	0.030
ROS	0.0056 ± 0.0012	0.0050 ± 0.0012	0.628

4.4.4 Phenol content, FRAP, MDA and ROS values

Antioxidant activities and oxidative stress markers were measured in both LH and HH groups. Independent t-test was conducted individually for all parameters for baseline only. It was found that, there were significant difference for baseline values in TPC, FRAP, and ROS parameters between the groups. Two-way mixed ANOVA had been run and the baseline for those markers becomes a covariate. There were no significant main effect between the two groups (LH and HH) in TPC ($F_{1,17}$ =0.03, p=0.863), FRAP($F_{1,17}$ =0.060, p=0.809), MDA ($F_{1,18}$= 0.713, p=0.410) and ROS ($F_{1,17}$ =1.323 p =0.266) level/activity. There were also no significant main effects within time in TPC ($F_{3,51}$=0.089, p =0.966) and FRAP($F_{3,51}$ =0.615, p =0.608) but showed a significant main effects in MDA ($F_{1.8,32.4}$=7.2, p <0.05) and ROS ($F_{1.7,29.2}$ =13.489, p <0.05).

Following the consumption of honey, plasma TPC in LH and HH increased over time until 2 hours and slightly decreased after that (**Figure 4.1 and Figure 4.2**). No significant difference among time points between groups at every time point. Phenolic content in honey could sustain at the optimal level within 1 to 2 hours and will slowly reduce after its optimum point (**Figure 4.1**). FRAP concentration was higher at both 1

hour and 2 hour time points relative to baseline values (**Figure 4.2**). Both TPC and FRAP shows the same trend suggested that acute ingestion of honey was absorbed and responded in blood. Generally, both LH and HH groups were effective in increasing not only TPC but also the FRAP in female trained athletes.

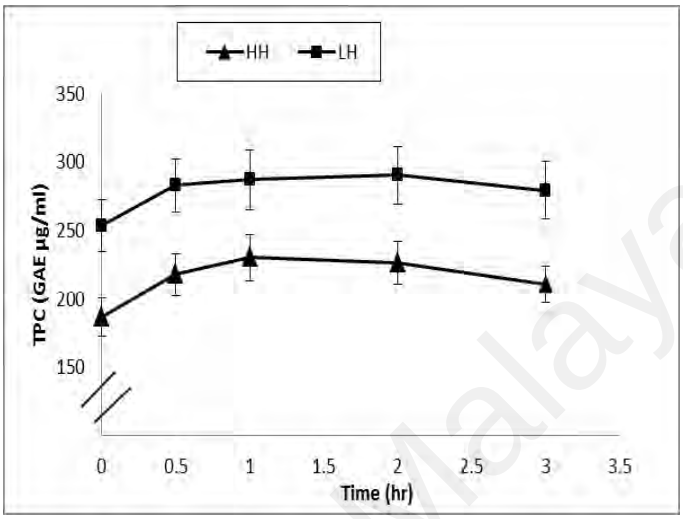

Figure 4.1: Plasma Total Phenolic Content (TPC) before (baseline) and at 0.5, 1, 2 and 3 hours after honey consumption in the LH (0.75g/kg BW) and HH (1.5g/kg BW) groups.
Values are mean ± standard error (SEM), *n*=20

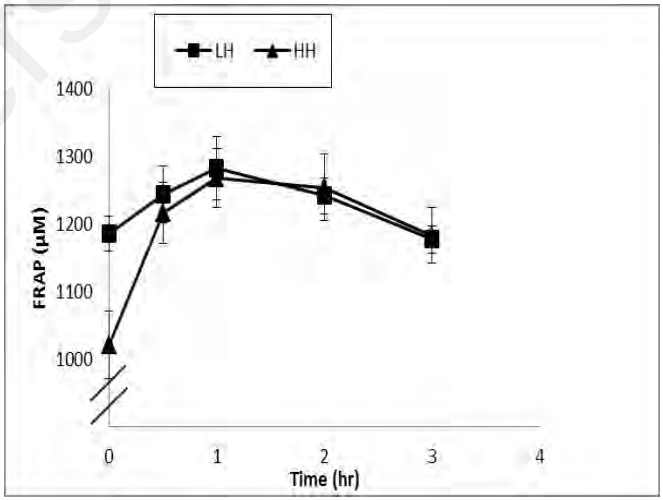

Figure 4.2: Plasma Ferric Reducing Activity (FRAP) before (baseline) and at 0.5, 1, 2 and 3 hours after honey consumption in the LH (0.75g/kg BW) and HH (1.5g/kg BW) groups.
Values are mean ± standard error (SEM), *n*=20

The changes of plasma MDA and ROS were presented in **Figure 4.3 and Figure 4.4**. TH consumption demonstrated a reduction in plasma MDA and ROS level starting at 1 hour and 0.5 hour, respectively. There is a significant (p<0.05) reduction in MDA in HH at 2 hours compared to 0.5 hour and 1 hour (**Figure 4.3**). Both LH and HH groups demonstrated a similar reduction following honey consumption until the optimal level of honey antioxidant activity (maximum at 2 hours). After the optimum point (3 hours), the result of MDA and ROS were detected to be high. There is a significant (p<0.05) increment in HH group at 3 hours for ROS compared to 0.5 hour, 1 hour and 2 hours (**Figure 4.4**) and MDA when compared to 2 hours (**Figure 4.3**). However, no significant difference between LH and HH groups were observed.

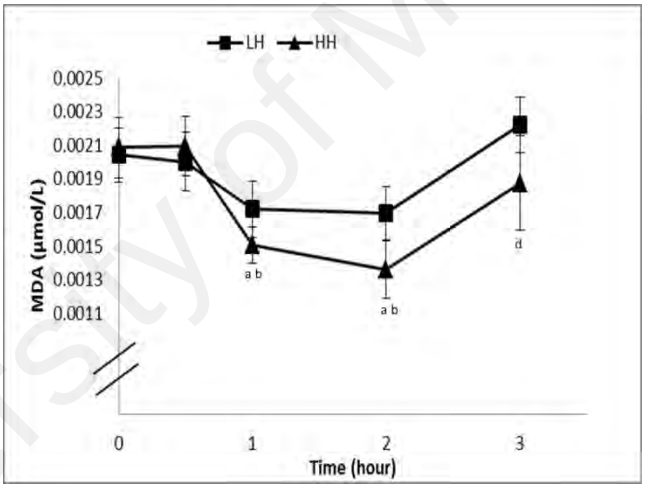

Figure 4.3: Plasma Malondialdehyde (MDA) before (baseline) and at 0.5, 1, 2 and 3 hours after honey consumption in the LH (0.75g/kg BW) and HH (1.5g/kg BW) groups.

Values are mean ± standard error (SEM), n=20
[a]p ≤ 0.05 significantly different from baseline in HH group
[b]p ≤ 0.05 significantly different from 0.5 hours in HH group
[d]p ≤ 0.05 significantly different from 2 hours in HH group

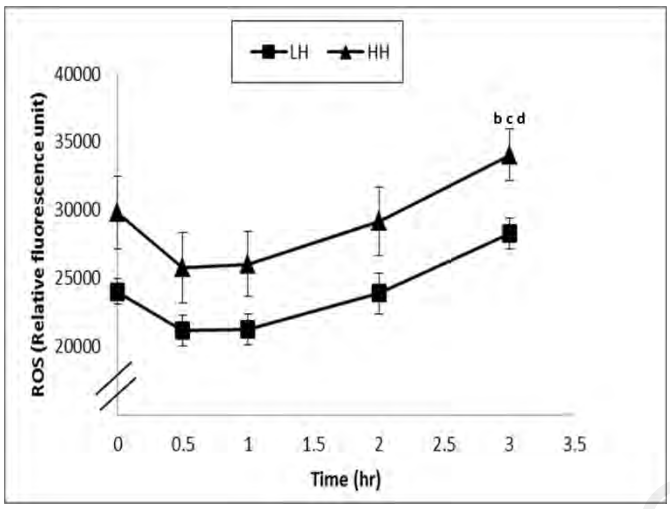

Figure 4.4: Plasma Reactive Oxygen Species (ROS) before (baseline) and at 0.5, 1, 2 and 3 hours after honey consumption in the LH (0.75g/kg BW) and HH (1.5g/kg BW) groups.

Values are mean ± standard error (SEM), n=20
[b]$p \leq 0.05$ significantly different from 0.5 hours in HH group
[c]$p \leq 0.05$ significantly different from 1 hour in HH group
[d]$p \leq 0.05$ significantly different from 2 hours in HH group

4.4.5 Percentage of phenolic content, FRAP, MDA and ROS values

The percentage of difference in comparison to baseline values for antioxidant and blood oxidative stress level of female athletes between two groups at different time points were presented in **Table 4.4**. There were no significant main effect between the two groups (LH and HH) in the mean TPC ($F_{1,18}$ =1.659, p = 0.214), FRAP ($F_{1,18}$ = 0.289, p=0.598), MDA ($F_{1,18}$ = 0.074, p = 0.789) and ROS ($F_{1,18}$ = 0.002 p = 0.965) level/activity. However, there were significant main effects within time in TPC ($F_{4,72}$ =14.128, p <0.05), FRAP ($F_{4,72}$ = 14.645, p <0.05) and ROS ($F_{1.5,27}$ =11.839, p <0.05) except for MDA showed no significant main effects ($F_{2.3,40.6}$ = 3.325 p = 0.085).

Table 4.4: The percentage difference of antioxidant and blood oxidative stress level/activity of female athletes at different time points.

Marker	Time				
	Baseline	0.5 hour	1 hour	2 hours	3 hours
TPC (%)					
LH	0 ± 0	12.3 ± 2.7[a]	14.6 ± 5.4	15.8 ± 4.8[a]	10.4 ± 2.7[ad]
HH	0 ± 0	17.9 ± 4.8[a]	24.5 ± 4.5[a]	22.4 ± 4.6[a]	14.9 ± 5.2[a]
FRAP (%)					
LH	0 ± 0	10.2 ± 3.8[a]	13.8 ± 3.6[a]	10.2 ± 3.4[a]	4.8 ± 3.8[cd]
HH	0 ± 0	10.4 ± 2.3[a]	15.1 ± 3.3[a]	13.7 ± 1.1[a]	7.5 ± 2.5[cd]
MDA (%)					
LH	0 ± 0	63.5 ± 3.6	-61.9 ± 4.4	-46.4 ± 8.3	86.4 ± 21.5
HH	0 ± 0	1.2 ± 92.1	-25.7 ± 29.3	-31.2 ± 23.8	9.2 ± 56.6
ROS (%)					
LH	0 ± 0	-12.0 ± 2.6[a]	-11.4 ± 3.4[a]	0.1 ± 6.0	19.7 ± 7.2[bcd]
HH	0 ± 0	-13.6 ± 2.6[a]	-12.2 ± 2.7[a]	1.7 ± 10.4	21.7 ± 12.6[bcd]

Values are mean ± standard error (SEM), n =20

[a]$p < 0.05$ significantly different from baseline in LH and HH group
[b]$p < 0.05$ significantly different from 0.5 hours in LH and HH groups
[c]$p < 0.05$ significantly different from 1 hour in LH and HH groups
[d]$p < 0.05$ significantly different from 2 hours in LH and HH groups

4.5 Discussion

The results of this study indicated that consumption of TH with high (1.5g/kg BW) and low (0.75g/kg BW) dosages enhance the antioxidant capacity (TPC and FRAP) and reduce oxidative stress (MDA and ROS) of human blood within 3 hours. No significant difference between groups showed that, both dosages of TH possess better antioxidant effects against a reactive oxygen species by reducing MDA and ROS level. In the present study, an amount of LH and HH of TH was consumed per person, providing ~8 g and 16 g respectively of phenolic content. High antioxidant content in blood was found after TH consumption but was resulted no significant difference between both groups. A similar finding between LH and HH could be explained by amount of TH (LH: 40.215 ± 3.47g, HH: 81.78 ± 10.18g) (**Table 4.2**) consumed in

single dosage. This amount was not enough to give a big different in acute study. A daily intake of honey was recommended to ensure the sustainable effects of honey as an antioxidant agent. Therefore, longer term trials with repeated consumption of honey may result in accumulation of the antioxidant components, which may in turn result in sufficiently active phenolic concentrations to influence the blood antioxidant status. Other than that the inter-individual variations in the antioxidant capacity response also gave a small difference outcome between the dosages. In the present study, the athletes showed a variation in antioxidant activity. This can be seen when fasting blood for baseline values in selected parameter appeared a significant results. Some athletes had high antioxidant activity and some of them had low antioxidant activity in the body before consume honey supplementation. Previous study by Gheldof et al. (2003) also found that inter-variation between participant response influences the antioxidant capacity results in buckwheat honey.

A higher antioxidant level in plasma after TH consumption in present study is assumed that body will have a better protective capacity towards free radicals. These finding showed that TH contains high amount of antioxidant properties **(Table 4.1)** and it was enough to suppress MDA and ROS activity in the blood**.** It is because honey contains a wide range of components, of which the phenolic acids and flavonoids play a significant role in the antioxidant capacity of honey (Gheldof, Wang, & Engeseth, 2002). It is important to note that the phenolic contents in TH is the highest TPC compared to other types of Malaysian honey, the nearest to the TPC in Manuka honey. TH in the present study was within the range of TH in previous study conducted by Khalil, Alam, Moniruzzaman, Sulaiman, and Gan (2011) which was between 15.21 - 42.23 mg/kg and FRAP values between 92.15 - 363.38 μM Fe [II]/kg. Kishore et al.

(2011) also found that TH had the highest TPC, followed by gelam, indian forest, and pineapple honey.

LH and HH groups present a greater antioxidant effects in blood plasma starting form 0.5 hours until 2 hours (**Figure 4.1 and 4.2**). It is speculated that honey could react to free radical presence in blood as quickly as 30 min after TH ingestion and also could perform as a protective agents for up to 2 hours. In general, the results of TPC concentration significantly increased by more than 24% and FRAP by 15% (**Table 4.4**) after 1 hour consumption of TH. The higher phenolic activity measured in blood plasma could due to quick absorption of phenolic content in honey through the gut barrier by passive diffusion (Scalbert & Williamson, 2000). Pharmacokinetic study explained that the absorption of honey nutrient was more rapid because honey consists of high quercetin content compared to other antioxidant rich food (Erlund et al., 2000).

However, after 2 hours, the antioxidant activities (TPC and FRAP) tend to decreased and consequently raised MDA and ROS levels (**Figure 4.3 and 4.4**). It is because the optimal level for antioxidant content in honey could sustain were within 1 to 2 hours and will slowly reduce after its optimum point. McKay & Blumberg, (2002) study found that the maximum rise in plasma antioxidant activity is most often reached within 1 to 2 hours after antioxidant rich food was ingested. That was the cause when TPC and FRAP reduced, MDA and ROS were increased after optimum point. The raise of oxidative stress might also due to high carbohydrate content in TH ingested especially in HH group which significantly increase the oxidative stress differentiation after 2 hours. A study showed that people with high blood glucose level tend to have high oxidative stress compared to normal individual (Yasunari, Maeda, Nakamura, &

Yoshikawa, 2002). These factors could be the reason of high oxidative stress observed at the end of 3 hours of intervention.

As there were no significant difference of both dosages in reducing ROS and MDA levels and increasing in TPC and FRAP, low dosage was selected for being the sufficient dosage to provide protection to female athletes. Additionally, low dosage is more suitable to be consuming for a long duration after considering the side effect of toxicity, sugar content and allergy effects on the participants (Shaw, Leon, Kolev, & Murray, 1997). Future investigations should conduct direct comparisons between honey and other high antioxidant foods which will provide additional insight into the relative efficiency of antioxidant transfer from these various antioxidant rich foods.

4.6 Conclusion

In the current investigation, honey feeding at 0.75 and 1.5 g/kg BW was observed to convey both phenolic antioxidants, increased plasma antioxidant capacity and reduced oxidative stress or free radical in healthy female athletes. On the other word, both honey dosages showed the same effect on antioxidant activity and oxidative stress in female athletes. The data also suggested that the phenolic content and FRAP activity in TH are responsible for free-radical scavenging and antioxidant activity that produce beneficial effects in human health. This study highlights the relevance of TH as a healthy food and a source of antioxidants for reducing oxidative stress in female athletes. To our knowledge, this is the first study to investigate a different dosage of honey on antioxidant defence in humans. Since both high and low dosage of honey tends to increase antioxidant activity which may lead to reduce oxidative stress with no difference between high and low dosage responses. Therefore, low TH will be used as the dosage for subsequent intervention study.

CHAPTER 5: EFFECTS OF HONEY SUPPLEMENTATION AND JUMPING EXERCISE ON BONE METABOLISM, ESTROGEN LEVEL, AND OXIDATIVE STRESS IN FEMALE ATHLETES WITH LOW BONE DENSITY

5.1 Introduction

It has been suggested that the intake of antioxidants may influence BMD by acting as free radical scavengers and preventing oxidation-induced damage to bone cells and was negatively associated with oxidative stress (Rivas, 2012). Other than antioxidant supplementation, it has been reported that high impact exercise could produce peak forces and increase bone loads (MacKelvie et al., 2003). Jumping exercise are considered as high impact exercise and also produces great osteogenic effects that stimulate bone formation (osteoblast activity) and reduce bone resorption (osteoclast differentiation) (Matthews et al., 2006).

Honey contains antioxidant phytochemicals i.e. phenolic acid and flavonoid which has been shown to play an important role in bone metabolism (Prior & Cao, 2000). Honey consumption resulted in prolong survival of osteoblasts and reduction of osteoclastogenic activity, and consequently, reduction in bone loss (Mohd Effendy et al., 2012). This is because honey had the ability to enhance collagen synthesis and stimulate BAP, a marker for osteoblast formation (Morton et al., 2001) and reduce ROS (Mohd Effendy et al., 2012). Among the different types of honey (e.g. Tualang, Gelam, Indian forest, Pineapple honey), TH had the highest TPC which allows it to act as a trabecular protector (Kuiper et al., 1997), produce immunostimulatory effects (Lakshmi et al., 2003) and help enzymatic glucose oxidase reactions (Wu et al., 2005) in bone. Furthermore, antioxidant in TH was able to elicit protective effects on disturbance of reproductive hormone especially estrogen (Mosavat et al., 2014b). In pre-menopausal females, bone loss progresses much more rapidly due to estrogen deficiency

(MacKelvie et al., 2002). Estrogen acts as natural antioxidant in the body and abnormal estrogen secretion could trigger the disruption of bone cycle process (osteoblast and osteoclast) (Mosavat et al., 2014b).

Previous study reported an increase in BMD in postmenopausal females fed with TH for 16 weeks (Shafin et al., 2014). TH was also found to have beneficial effects on bone metabolism and reproductive hormones after TH consumption with or without jumping exercise for 8 weeks, however this study was performed on rats (Mosavat et al., 2014a; Tavafzadeh et al., 2011). Thus far, there are no data on the effects of honey consumption among female athletes with low bone density and used jumping as an exercise prescription.

With regards to honey dosage, our study in Chapter 4 had suggested that acute low TH dosage (0.75g/kg BW) was sufficient to increase antioxidant level and suppress oxidative stress as the high TH dosage (1.5g/kgBW). As the 0.75g/kg BW of TH was sufficient to act as free radical scavengers, this dosage was chosen as a dosage in this present study to see the effects of TH consumed for 8 weeks on BMD, estrogen level, antioxidant level (TPC and FRAP) and oxidative stress (MDA and ROS) in female athletes.

5.2 Literature Review

High energy expenditure and BMR together with low-calorie intake may decrease percentage of body fat in female athletes (Quah et al., 2009). Low body fat percentage may result in reduction of leptin hormone secretion (Misra, 2008; Thong et al., 2000). Low levels of leptin subsequently may diminish the secretion of GnRH and signal the hypothalamus in the brain to reduce the production of luteinizing hormone

pulsatility in the blood (Loucks, 2003). Luteinizing hormone is responsible for estrogen production in the ovaries which are important for the reproductive system and bone health (Loucks, 2003). Reduction of estrogen hormone decreases osteoblast activity (bone formation) and increases osteoclast activity (bone resorption) which consequently resulting in increased bone loss (De Souza et al., 2008).

Estrogen can be considered as a natural antioxidant in the human body. It exhibit antioxidant protection of lipoproteins in the aqueous system and was shown to increase the expression of GPx in osteoclasts (Badeau et al., 2005). A decrease in estrogen level increases oxidative stress on the body resulting in lipid accumulation which is expressed as MDA. MDA will promote osteoblast apoptosis and simultaneously upregulate ROS production, particularly H_2O_2 and superoxide anion (Almeida, Han, Martin-Millan, Plotkin, et al., 2007). ROS may increase bone resorption through RANK which plays an important role in osteoclastogenesis (Wauquier et al., 2009). It has been demonstrated that in both in-vitro (Baek et al., 2010) and animal (Hamada, Fujii, & Fukagawa, 2009) studies, oxidative stress decreases the level of bone formation by modulating the differentiation and survival of osteoblasts (Bai et al., 2004).

Among all types of honey in Malaysia, TH has highest free radical scavenging and antioxidant activity than the other local and commercially available honeys (Khalil et al., 2011) probably due to the high level of atioxidant compounds (phenolic and flavonoid), antioxidant activity (DPPH and FRAP) and protein content (Moniruzzaman et al., 2013). Furthermore, TH had been scientifically proven for their effects on reproductive hormonal metabolism (Zaid et al., 2010), oxidative stress (Shafin et al., 2014) and bone metabolism (Mohd Effendy et al., 2012).

Other than honey consumption, high impact exercise is also important to help strengthen bone density. Jumping is an activity that is categorized as high impact exercise. High impact exercises are believed to generate peak force and bone load which stimulate osteogenic effect on bone (Kato et al., 2006; Turner & Robling, 2003). Furthermore, high stimulation on bone will increase the bone mass (Mosavat et al., 2014a; Rubin et al., 2002; Tavafzadeh et al., 2011). A training program following the gradual progressive approach in exercise was recommended to adapt with physiological change on bone (Erickson & Vukovich, 2010). Jumping session was approached to stimulate ground reaction force on bone. However, specific duration to prescribe the exercise was crucial and need for further investigation.

5.3 Methodology

5.3.1 Participants

Forty five female athletes with low BMD and energy intake of less than 2000kcal/day identified from Chapter 3 study participated in the present study. The participants were involved in regular exercise for at least 3 times per week, and involved in sports competition at the university or state level. Inclusion criteria were female, aged between 18 - 30 years old, physically active (train or exercise three times per week for at least 30 min), with low BMD and low energy intake (<2000kcal/day). All participants must be in good health and without any chronic diseases such as diabetes, stroke or high blood pressure. They were excluded if they engaged in jumping activities on a regular basis (two times per week or more), women who were pregnant, on medication, or taking supplements. Participants were given the study information sheet (Appendix A) and received explanations about the study procedures such as the experimental protocol and possible risks before being given the consent form (Appendix

B). This study was approved by the University Malaya Research Ethics Committee (UMREC), UM.TNC2/RC/H&E/UMREC – 43 (Appendix C).

5.3.2 Honey supplementation

Tualang (*Koompassia excelsa*) honey was used in this study. TH from a single batch honey was provided by FAMA, Malaysia. The honey was extracted from the same source and was used without additional processing and treatment prior to administration.

5.3.3 Study design

Participants were randomly assigned into three groups; Honey (H), Honey and Jumping (HJ) and Jumping (C). A computer generated block randomization of three was used to create a randomization schedule. Treatment assignment was conducted by the trial manager. Participants of the supplementation condition (Group H and HJ) consumed 0.75g/kg BW TH for 8 weeks. Both participants in the HJ and Control groups were prescribed with jumping exercise performed twice a day 3 times per week for 8 weeks. BMD was measured, and blood was analyzed for bone biomarkers (BAP and ICTP), estrogen level and oxidative stress biomarkers (MDA and ROS) at week 0 and 8.

5.3.4 Preliminary measurement

5.3.4.1 Anthropometric measurement

Baseline testing for HJ, H and C groups included anthropometric, body composition and BMD measurement. The Participants‘ weight (kg), height (cm), and body fat (%) were collected using a Bioimpedance Analysis Machine (Tanita, Japan) during this session. Meanwhile, BMD was measured using the ultrasound bone densitometer

machine (Furuno CM-200, GB2424276, Japan). BMI where derived from a calculation BMI= BW/height^2

5.3.4.2 Dietary intake assessment

Three-day food diary can refer to Section 3.3.6.2 (Chapter 3)

5.3.4.3 Bone mass density

BMD measurement can refer to Section 3.3.2.2. (Chapter 3)

5.3.5 Main trial

The intervention in the present study was conducted for 8 weeks. Measurement for fitness level, vertical jumping height and blood collection was done at week 0. At week 8, the participants were asked to come again and had BMD measured and blood collected. Bone density was measured using a portable ultrasound bone densitometer (Furuno CM-200, GB2424276, Japan) at the calcaneus. Meanwhile, blood was analysed for estrogen, BAP, ICTP, MDA and ROS

During the 8 weeks intervention, participants were asked to refrain from consuming any products containing honey and to refrain from participating in any physical training and/or tournaments. To monitor the participant‘s diet and physical activity throughout the weeks, the participants need to record their diet intake and physical activity. Besides that, at each session, participants from the H group were reminded to consume honey with 500ml of plain water between 8.00am to 9.00am. Meanwhile, participants in the HJ group consumed honey and 500ml of plain water approximately 60 min before starting their jumping exercise. Participants in the C group were asked to drink 500ml plain water 60 min before jumping exercise. Five minutes before starting the training program, participants in the HJ and C groups performed

standardised warm-up activities. Then participants were required to jump for 2 sessions per day on 3 alternate days per week. The training protocol consisting of an 8-week intervention period following a progressive overload model (**Table 5.1)** was provided to participants in HJ and C groups. For the first 4 weeks, the number of repetition was increased week by week. The load was increased progressively from week 4 for body adaptation to meet greater physiological demands.

Table 5.1: Training program for participants in C and HJ group

Week (W)	Session 1	Rest	Session 2
W 1	2 set of 5 jumps	6 hours	2 set of 5 jumps
W 2	2 set of 7 jumps	6 hours	2 set of 8 jumps
W 3	2 set of 10 jumps	6 hours	2 set of 10 jumps
W 4	2 set of 15 jumps	6 hours	2 set of 15 jumps
W 5	3 set of 8jumps	6 hours	3 set of 8 jumps
W 6- W 8	3 set of 10 jumps	6 hours	3 set of 10 jumps

5.3.6 Measurements at week 0 and week 8

5.3.6.1 Fitness assessment

At 0 week, participant‘s fitness level was estimated using indirect VO_{2max} test, whereby a 20-m shuttle run (Beep test) was performed. Participants were asked to run 20 meters to the marker on the opposite side and attempted to get there before the next beep sounded. Participants waited there until the next beep sounded before running back to the starting markers. VO_{2max} was then calculated based on shuttle run score using the VO_{2max} online calculator.

5.3.6.2 Vertical jump height determination

Maximum vertical jump and ground reaction force was also recorded at 0 week by using force platform (AMTI, USA) for all participants. By using a force platform, maximum jump height was demonstrated by ground reaction force (GRF) in AMTI software. The method of calculating jump height was based on time in the air (TIA). TIA was identified as the period between take-off and contact after the flight. The time was then used in the following equation of uniform acceleration.

$$\text{TIA jump height} = \tfrac{1}{2} g\,(t/2)^2 \qquad \text{(Aragón, 2000)}$$

where g = 9.81 m · sec−2, t = time

5.3.6.3 Jumping exercise

During the 8 weeks intervention, participants in HJ and C groups performed jumping exercise based on jumping height calculated by force platform and AMTI software. Following identifying the jump height, they were given an individual measuring card to be pasted on the wall at home or hostel accommodation. Participants were required to tap the card every time they performed the jumping activity. This measuring card is important to ensure all participants trained the desired height. The height of the measuring cards is different for each participant depending on participant‘s performance during the jumping test on force platform. Prior to the actual jumping exercise for HJ and C participants were briefed about the training schedule for the jumping activity (3 alternated days/ per week for 8 weeks). For the first week of jump training, participants in the HJ and C groups were required to run through a familiarisation session under the researcher‘s supervision. Participants performed two-legged maximum vertical jumps using arm swing in counter-movement style on 3 alternated days/week. The same jump style was performed at home for another 7 weeks on a relatively hard floor with proper

sports shoes. The interval of each jump was between 8–12 s, so the training session took less than 2 min. Home record cards was supplied during familiarisation sessions to monitor compliance. Additionally, participants were reminded to record their jumping activity in the card given through short-message-service (SMS) or phone call before each session.

5.3.6.4 Honey dosage calculation

Participant in H and HJ groups need to consume honey every day for 8 weeks. Honey dosage for H and HJ groups was consumed using a 10 ml syringe to help the participants to measure the amount of honey based on participant‘s body weight. Measured of honey dosage based on estimation calculation below:

Participant BW: 50kg

Honey dosage: 0.75g /kg BW

Honey dosage per participant: 0.75g of honey x 50kg BW

= 37.5g of honey

By using a syringe, 20g honey equal to 15ml

Thus the participant need to consume: (37.5g/20g) * 15 ml

= 28 ml of honey

5.3.6.5 Diet and physical activity records

All participants were required to record their three-day food diary (two weekdays and one weekend) and physical activity diaries every week for 8 weeks to monitor and ensure no major changes in energy and nutrient intake. Both diaries were collected at the end of intervention (after 8 weeks). Meanwhile, the checklist record as prescribed on jumping activity and honey consumption were sent to the researcher every 4 weeks for

monitoring. Dietary records from all participants were analysed using Nutritionist Pro (Axxya System, Texas).

5.3.6.6 Blood collection and preparation

At week 0 week (baseline) and week 8 (post-intervention) of the study, 6ml of blood was collected from participants' median cubital vein following 10-hours overnight fasting (only plain water was allowed). Samples were collected into a 4 ml plain tube and a 2 ml heparinised tubes. The blood samples in plain tube was allowed to clot completely for at least 2 hours and subsequently were centrifuged at 3000 revolutions per minutes for 15 min at 4^{o}C (Heraeus^{TM} Multifuge^{TM} X1R Centrifug, USA) within 2 hours of blood draw. The serum was divided into equal portions and stored in labeled eppendorf tubes at -40^{o}C for subsequent analysis of serum estrogen, BAP and ICTP. Meanwhile, the blood in heparinised tube was mixed well and centrifuged at 4000 RPM for 15 min at 4°C. After centrifugation, aliquots of plasma were transferred using a disposable plastic pasteur pipette into labeled 1.5 ml tubes. The aliquots plasma was stored at -40^{o}C before the analysis of oxidative stress levels (MDA and ROS).

5.3.7 Blood analysis

5.3.7.1 Bone metabolism markers

BAP and ICTP were measured using a commercially available kit (CUSABIO, China). The kit uses a double-antibody sandwich enzyme-linked immunosorbent one-step process assay ELISA kit to assay the level of BAP in samples. The test was performed according to the manufacturers' guide. The assay was measured at 450nm absorbance on a plate reader within 30 minutes of stopping the reaction.

5.3.7.2 Estrogen

Blood samples were sent to University Malaya Medical Centre for analysis of estradiol level. Estradiol concentration was determined using a double-antibody procedure. The serum sample was preincubated with antiestradiol antiserum. Sodium iodide I 125–labeled estradiol, which competes with estradiol for binding sites, was then incubated with the sample for a fixed time. Bound and free estradiol was separated by the polyethylene glycol–accelerated double-antibody method, the antibody-bound fraction was precipitated and counted, and the concentration of estradiol in the sample was read from a calibration curve. The intra-assay coefficient of variation is 6.5% (SD, 2.1 pmol/L [0.6 pg/mL]) at an estradiol concentration of 33 pmol/L (9.0 pg/mL) (Cummings et al., 2002).

5.3.7.3 Oxidative stress markers

Plasma MDA level was measured to assess lipid peroxidation, MDA, an end product of lipid peroxidation, by TBARS assay. TBARS are formed as a by-product of lipid peroxidation (i.e. as oxidative degradation products of fats) which can be detected by the TBARS assay using thiobarbituric acid, trichloroacetic acid, and hydrochloric acid as a reagent in a ratio of 1:1:1. By determining TBARS, the extent of lipid peroxidation could be measured. For measurements, 100µl of TBARS were added with 50µl of plasma. A total of 150 µl of samples was added and vortexes for 1-2 min. Then, the samples were heated at 90°C for 20 min on a heating block. Then the samples were stood for 5 min to cold down to room temperature before another 150 µl of butanol was added. The samples were vortexes and centrifuged prior to aliquot 100 µl of supernatant from each sample to a microplate, and the absorbance was read at 532 nm (Oteiza et al., 1997). Meanwhile, ROS was measured by 5µl of plasma was added with 100µl dichlorofluoresceindeacetate (DCF-DA) in a black plate. The samples were gently

shaken for 1 min. Then, the plate was incubated for 30 min in a water bath (37^{o}C). Finally, the reading was taken at 485 [Excitation (EX)] and 530 [Emission (EM)].

5.3.8 Statistical analyses

5.3.8.1 Sample size

Sample size was estimated using G-Power version 3.1.9 and data from a previous study conducted by Ooi (2011). The power of the study was set at 80%, with a 95% confident interval, and an effect size of 1.22. The calculated sample size was 12 per group. However, after taking into account 20% drop out rate the participants per group is 15 and the total participants in the study were 45 participants.

5.3.8.2 Data analyses

Statistical Package for Social Sciences Version 22.0 (SPSS, Inc., Chicago, IL) was used for the statistical analyses. The distribution of data for normality was assessed using the Shapiro-Wilk test. The two-way mixed ANOVA were performed on participants‘ characteristic, all blood parameters and BMD respectively. Significant level was set at $p<0.05$. Significant difference demonstrated in the two-way mixed ANOVA was followed by a post-hoc Bonferroni test. One way ANOVA and paired t-test were used as a follow up test if the data was significantly different between group and within group respectively. All data were reported as means ± standard deviations for participant characteristics and mean ± SEM for BMD and all blood parameters (bone metabolism, estrogen and oxidative stress markers).

5.5 Results

5.5.1 Anthropometric characteristics and energy intake of the participants

A total of 85 female athletes from study 1 were screened in this study. From those, 45 were identified to have low BMD (z-score: -0.61 ± 0.1) and energy intake (1291 ±

304 kcal/day). The remaining 40 participants were excluded due to normal BMD score (z-score ≥ 0). Participant characteristics across groups are displayed in **Table 5.2**. Eligible participants were randomized into H, HJ, and C groups. There were no significant difference between groups for age, weight, height, BMI, the percentage of body fat and VO_{2max}.

Table 5.2: Participants characteristic

Variables	**Honey (H)**	**Honey and Jumping (HJ)**	**Jumping (C)**
Number of subjects	15	15	15
Age (years)	21.6 ± 2.9	22.0 ± 2.3	19.7 ± 0.9
Weight (kg)	53.2 ± 4.6	54.4 ± 5.2	48.6 ± 5.8
Height (cm)	158.5 ± 0.04	160.5 ± 0.07	159.9 ± 0.04
BMI	21.2 ± 3.7	21.1 ± 1.6	19.2 ±2.3
Body fat (%)	29.7 ±6.1	26.8 ± 3.6	24.5 ± 5.5
VO_{2max} (ml/kg/min)	28.0 ± 4.4	28.6 ± 4.7	28.7 ± 2.9

Values are mean ± standard deviation (SD), n=15. BMI,body mass index

5.5.2 Nutritional content of TH

The nutritional content of TH was analyzed at the Unipeq laboratory (Appendix H), Universiti Kebangsaan Malaysia and was presented in **Table 5.3.**

Table 5.3: Nutritional contents of Tualang Honey

Ingredient	Amount in 100g
Energy (kcal)	307
Carbohydrate (g)	76.01
-Fructose (g)	39.71
-Glucose (g)	35.33
-Sucrose (g)	0.58
Proteins (g)	0.73
Fats (g)	<0.1
Minerals (mg)	
-Potassium (K)	1868.93
-Calcium (Ca)	36.59
-Sodium (Na)	976.60
-Magnesium (Mg)	510.61
-Phosphorus (P)	109.17
-Selenium (Se)	0.02
-Manganese (Mn)	1.42

5.5.3 Bone mineral density measurement

The BMD measurement showed no significant difference between H, HJ and C after 8 weeks. Within each group, there were also no significant difference when compared between week 0 and week 8 (**Table 5.4**). There was 7% and 13.5% increment in BMD results after 8 weeks of intervention in H and HJ groups, respectively. Meanwhile, participants in the C group showed a reduction (**Table 5.4**).

Table 5.4: Mean bone mass density (z-score) at 0 week and 8 weeks

Groups	0 week	8 weeks	Percentage of changes
Honey (H)	-0.59 ± 0.1	-0.55 ± 0.2	7.0
Honey and Jumping (HJ)	-0.66 ± 0.1	-0.57 ± 0.2	13.5
Jumping (C)	-0.62 ± 0.1	-0.65 ± 0.1	-5.4

Values are mean ± standard error (SEM), *n* =15

5.5.4 Bone formation markers

The BAP measurement showed that there were no significant difference between H, HJ and C after 8 weeks. Within each group, there were also no significant difference when comparing week 0 and week 8 (**Table 5.5**).

Table 5.5: Mean Bone Alkaline Phosphatase (BAP) Concentration at 0 week and 8 weeks

Groups	0 week (U/L)	8 weeks (U/L)	Percentage of changes
Honey (H)	46.9 ± 1.7	47.1 ± 2.4	0.5
Honey and Jumping (HJ)	47.2 ± 2.2	47.2 ± 2.1	-0.1
Jumping (C)	46.9 ± 1.0	46.8 ± 0.8	-0.2

Values are mean ± standard error (SEM), *n* =15

5.5.5 Bone resorption markers

The 1CTP measurement revealed no significant difference between H, HJ and C after 8 weeks intervention. There were no significant difference between 0 and 8 weeks for each H, HJ and C groups (**Table 5.6**).

Table 5.6: Mean Serum C-terminal telopeptide of type I collagen (ICTP) Concentration at 0 week and 8 weeks

Groups	0 week (U/L)	8 weeks (U/L)	Percentage of changes
Honey (H)	7.0 ± 0.1	7.0 ± 0.2	-0.7
Honey and Jumping (HJ)	7.0 ± 0.1	7.0 ± 0.2	-0.4
Jumping (C)	7.0 ± 0.0	7.0 ± 0.0	0.04

Values are mean ± standard error (SEM), *n* =15

5.5.6 Estrogen level

Table 5.7 shows the mean serum estrogen levels for all groups at week 0 and 8 and the percentage of change across time. After 8 weeks of intervention, there was no significant difference in estrogen level between H, HJ and C groups. Within each group, there were also no significant difference when comparing week 0 and week 8. Although insignificant, estrogen level recorded 29.8% and 24.4% increment in H and HJ groups respectively after 8 weeks (**Table 5.7**). Meanwhile, participants in the C group showed a reduction of 12.4% from week 0 to week 8.

Table 5.7: Mean serum estrogen level at 0 week and 8 week

Groups	0week (pmol/L)	8 weeks (pmol/L)	Percentage of change
Honey (H)	419 ± 104.6	544 ± 95.2	29.8
Honey and Jumping (HJ)	426.6 ± 89.9	530.5 ± 76.7	24.4
Jumping (C)	412.8 ± 71.7	361.7 ± 79.8	-12.4

Values are mean ± standard error (SEM), *n* =15

5.5.7 Oxidative stress markers

MDA were significantly reduced (**Figure 5.1**; $p<0.05$) in both H (12.6 ± 2.3%) and HJ (5.7 ± 3.0%) groups but significantly increased in C (3.5 ± 4.0%) from week 0 to week 8. Nevertheless, there was no significant difference between H, HJ and C groups after 8 weeks of intervention. As for ROS, significant group difference were detected between H and C as well as between HJ and C (**Figure 5.2**; $p<0.05$). In addition, there was a significant reduction in H (20.2 ± 5.5%) and HJ (15.3 ± 6.1%) and a significant increment in C (15 ± 3.3%) from week 0 to week 8 (**Figure 5.2**; $p<0.05$).

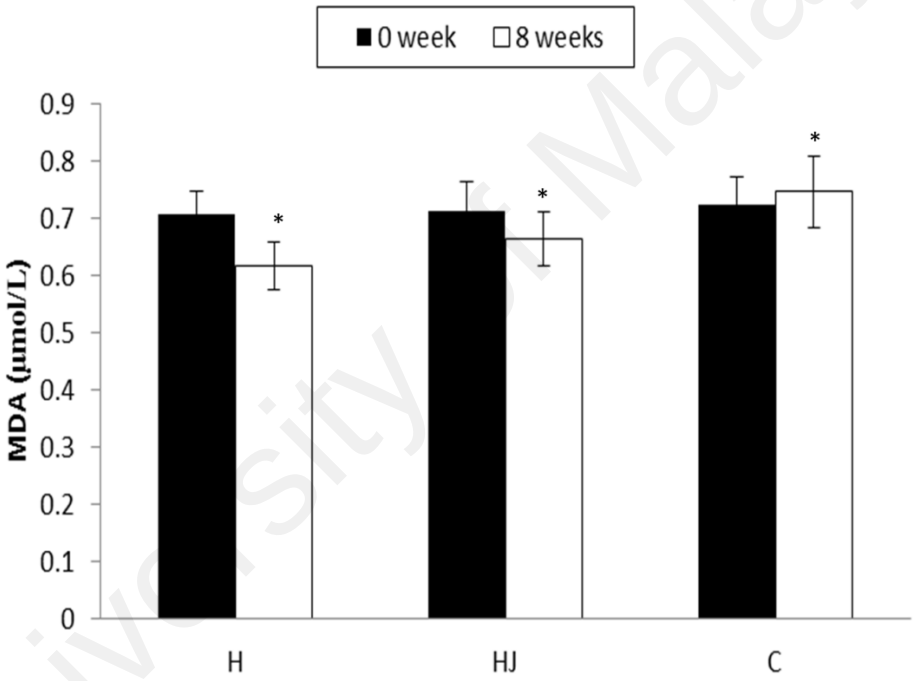

Figure 5.1: Mean of Malondialdehyde (MDA) at 0 weeks (■) and after 8 weeks (□) in Honey (H), Honey and Jumping (HJ) and Jumping (C) groups.

Values are mean ± standard error (SEM), n= 15

*H, HJ and C significantly different ($p<0.05$) compared to 0 week

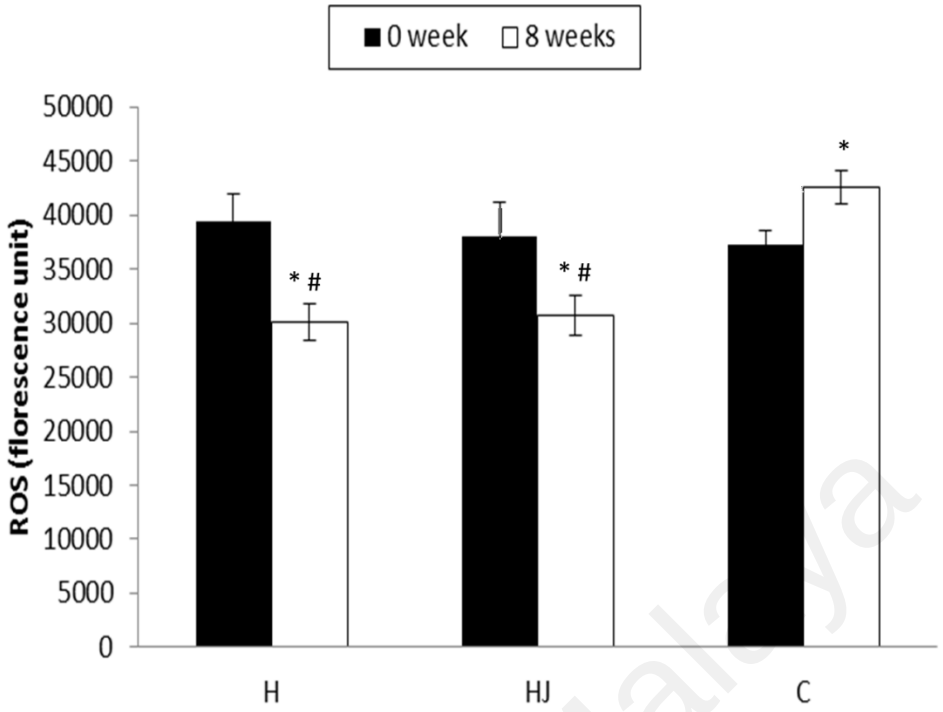

Figure 5.2: Mean of reactive oxygen species (ROS) at 0 weeks (■) and after 8 weeks (□) for Honey (H), Honey and Jumping (HJ) and Jumping (C) groups.

Values are mean ± standard error (SEM), *n*= 15

* H, HJ and C significantly different ($p<0.05$) compared to 0 week

H and HJ significantly different ($p< 0.05$) compared to C group

5.6 Discussion

The main finding of the present study was the consumption of TH supplementation with 0.75g/kg BW for 8 weeks significantly ($p<0.05$) reduced MDA and ROS compared to consumption of TH combined with jumping exercise. No difference was observed for BAP, ICTP, estrogen and BMD results between and within the groups. Although there were no statistically significant changes compared to C, those variables showed an improvement in H and HJ after 8 weeks. To our knowledge this is the first study investigating the effects of honey on BMD in female athletes with low bone density. No significant difference in BMD values between H and HJ group could be attributed to

the small increase or improvement in bone development and insufficient time for recovery in low BMD individuals fed with honey. It takes some time to recover with honey consumption to repair and develop a new cell in osteopenia (low BMD) female patient to be compact and strong (Bachrach, Katzman, Litt, Guido, & Marcus, 1991). In contrast, other honey supplementation and jumping studies reported a significant improvement in less than 8 weeks in healthy males (Kreider, Ferreira, Greenwood, Wilson, & Almada, 2002), non-athletes (females) (Ooi, 2011) and males athletes (Abbey & Rankin, 2009; Earnest et al., 2004) with normal BMD and not among low BMD individual.

Nutrient contained in TH such as calcium, vitamin D could suppress bone resorption and increased bone growth and mineralization (Chepulis & Starkey, 2008). Meanwhile, antioxidants content such as flavonoid act as a trabecular protector (Kuiper et al., 1997), polyphenolics produce immunostimulatory effects on bone (Lakshmi et al., 2003). These researchers showed that calcium content in honey increased the bone growth and mineralisation. Aside from supplements, jumping exercise may have to contribute to bone strength (Mosavat et al., 2014a; Umemura, Ishiko, Yamauchi, Kurono, & Mashiko, 1997). While the combination of TH feeding with jumping appeared to be beneficial on bone, jumping exercises alone (C group) displayed a negative effect. This finding is in line with old study by Cavanaugh and Cann (1988) who discovered that there was increased calcium loss through exercise. In the HJ group, additional feeding of honey helped to maintain nutrient demand of the bone during jumping exercises. A study by Welch et al., (2008) demonstrated that high impact exercise produce net bone loss, thus the bone need to be balance by increasing dietary calcium intake. In terms of nutritional intake, participants in the present study consumed energy intake of less than 2000kcal/day with low intake calcium (below RNI

value). Their active lifestyle may require them to have additional nutrient intake or extra calories to keep up with bone and body demands. Thus, jumping alone without honey supplementation could worsen the bone health when combined with poor nutrient intake (McNaughton, Wattanapenpaiboon, Wark, & Nowson, 2011).

In a normal body, excessive exercise produces high oxidative stress that may cause reduction in bone formation, and enhancement in bone resorption which could lead to low BMD (Wauquier et al., 2009). Alternately, antioxidant in honey (i.e. phenolic) can inhibit bone resorption and subsequently inhibit bone loss (Ozgocmen, Kaya, Fadillioglu, Aydogan, & Yilmaz, 2007). However, based on the bone formation and bone resorption markers in this study, it appeared that 8 weeks honey consumption did not affect bone metabolism as no significant difference between or within the groups were observed. It could be speculated that, the duration of 8 weeks was not long enough to observe any significant changes in bone metabolism. In contrast, one study has demonstrated significant changes in bone alkaline phosphate of healthy female individuals in less than 8 weeks (Swaminathan, 2001). Hence, the duration of an 8 week intervention may be sufficient for healthy individuals but not female athletes with low BMD. Another possibility for not detecting any group difference could be because the reaction on the bone was impaired due to insufficient dosage of honey for low BMD athletes. Based on current scientific evidence, there are no available studies of honey supplementation for participants with low BMD, hence it was not possible to gain clarity on the recommended dosage. In other studies though, most researchers had chosen a dose of TH not more than 1g/kg BW of longer intervention period in human (16 weeks) (Shafin et al., 2014) and animal studies (8 weeks) (Mosavat et al., 2014a; Ooi, 2011; Tavafzadeh et al., 2015). The 0.75g/kg BW dosage used in this study was implemented in view of the safety of the participants. The toxicity of honey supplement

intake should be an important point of consideration to avoid harmful effects on the human body such as allergy, diabetes or liver problem (Shaw et al., 1997). For example, several cross-sectional surveys in Hong Kong found a relationship between the consumption of Royal Jelly and hypersensitivity reaction to skin, asthma and anaphylaxis (Leung, Ho, Chan, Choy, & Lai, 1997; Thien et al., 1996).

Despite of low energy intake and low BMD, all the participants had normal estrogen level. There appeared to be no significant improvement in the levels among H, HJ and C groups. Poor nutrition is a factor that can lead to changes in hormone that reduce the estrogen secretion. It is undeniable that low energy intake (below RNI) contributes to low fat percentage which could affect reproductive hormone secretion (Loucks, 2003; Sundgot-Borgen & Torstveit, 2007). Low levels of adipose tissues will reduce leptin hormone which subsequently could trigger the reduction of gonadotropin hormone (Misra, 2008; Thong et al., 2000). Imbalance of those hormones will send the signals the hypothalamus in brain to reduce the luteinizing hormone pulsatility in the blood (Christo et al., 2008). Low level of luteinizing hormone could then reduce the secretion of estrogen hormone by ovaries (Cobb et al., 2003).

In the present study, almost 30% increment of estrogen level was observed in participants consuming TH alone or TH combined with jumping. This is an indication that honey may affect reproductive hormone secretion. The finding of the study is in agreement with Masovat et al., (2013) who found antioxidant content in honey could elevate the estrogen level. In the C group, it is possible that estrogen levels were reduced by 12.4% because participant might suffer from imbalance hormone secretion during long and high frequency of exercise intervention. This finding was supported by McTiernan et al. (2004) who found that active individual experienced decline in

estrogen level of 8.2% compared to individuals in the control group in a 12 week exercise intervention study. Production of estrogen is very important as a regulator for osteoblast activities (Qu et al., 1998) because estrogen could act as endogenous antioxidant (Yagi, 1997) and enhance vitamin D absorption in body to avoid bone loss (Loucks, 2003). The association between insignificant of BMD and bone metabolism finding can be partly explained by effects on serum estrogen.

The consumption of TH and TH combined with jumping for 8 weeks reduced oxidative stress. In contrast, jumping exercise alone showed an increased in ROS level over time. The decrease of oxidative stress markers could be explained by the presence of antioxidants compound (TPC, DPPH) and antioxidant activity (FRAP) in TH as TH has been found to have high FRAP value (255.5 μmol Fe [II] / 100g of honey) (Table 4.2, Section 4.4.1) when comparing with TH (FRAP: 121.89 μmol Fe [II] / 100g of honey) used in Kishore et al., (2011) study (Figure 2.3, Section 2.5.1). The variation of the TH content depends on various factors including geographical regions, floral source, climate, temperature, handling, and storage. High antioxidant content could reduce and neutralize ROS and MDA generated during exercise (Schramm et al., 2003). Our findings are consistent with several studies which showed a reduction in MDA after honey supplementation in healthy (McKibben & Engeseth, 2002; Omotayo et al., 2010; Yao et al., 2011) and unhealthy (diabetic and albino) (Adeleye, Nwozor, Okafor, & Oguwike, 2012; Erejuwa et al., 2010) rats. Meanwhile, the different response observed among participants in the C group could be explained by the absence of honey supplementation and the possibility that jumping exercises may have aggravated more oxidative stress. As a result of excessive exercise, body produces high oxidative stress as a result from unbalanced of bone coupling that could reduce osteoblasts activities, whereas enhanced osteoclast activities (Wauquier et al., 2009). There is growing

evidence, that many athletic populations especially in endurance athletes (Loucks, 2007), aesthetic (Koutedakis & Jamurtas, 2004) and weight category sports are suffering from negative energy balance with low calories intake (Sundgot-Borgen & Garthe, 2011). Thus these sport groups have a higher risk of generating more oxidative stress and suffering from bone loss. The results of the oxidative stress markers in this study provide support that TH will not only able to provide additional nutrients but also serve as antioxidants for bone. The use of antioxidant supplements is already widespread among females for aging (Fusco, Colloca, Monaco, & Cesari, 2007) and breast cancer (Fauzi, Norazmi, & Yaacob, 2011). In term of specific mechanism, it is suggested that honey appeared to work on bone by first, reducing oxidative stress, secondly, increasing estrogen level and thirdly, increasing bone formation and before reducing bone resorption. The possible action of TH on improving BMD is summarised in **Figure 5.3**.

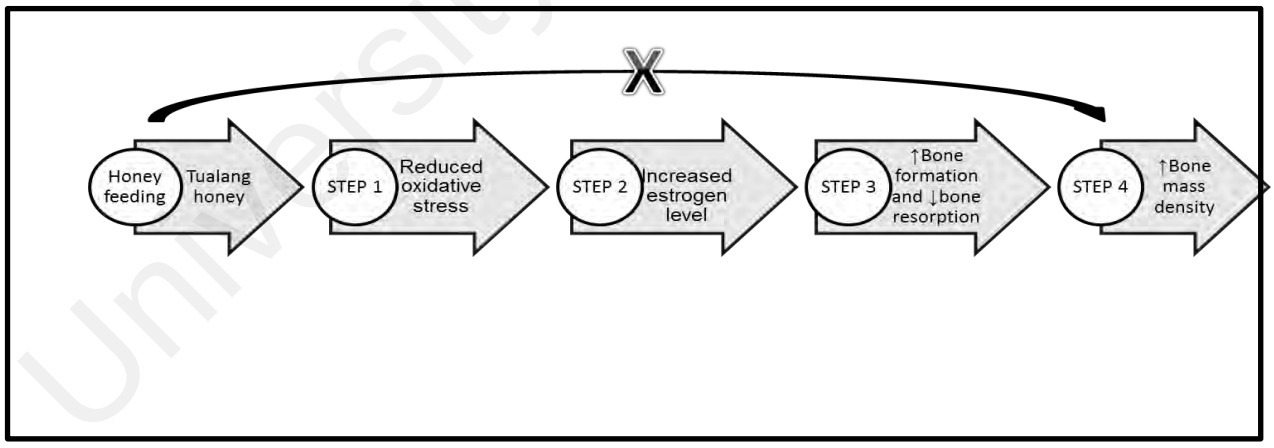

Figure 5.3: Suggested mechanism of honey ingestion on bone health

Among the limitation of the study was the small number of variables. This study primarily focused on the effects of honey on oxidative stress, estrogen, bone metabolism markers and BMD in trained female. Subsequent studies should include a wider panel of antioxidant response in blood plasma to raise options to identify rationales and

mechanisms. Parameter like total phenolic compound and FRAP could indicate the antioxidant response in blood after 8 weeks consumption of honey. These parameters performed more rapid changes within 6 hours antioxidant test in Schramm et al. (2003) study. Besides that, the used of biomarkers is an estimate of a systematic response and does not indicate actual site-specific adaptations to the high impact loading.

5.7 Conclusion

We observed an increasing trend in the BMD, bone formation and resorption, estrogen level and reduction of oxidative stress in H and HJ groups after 8 weeks of TH consumption. As we did not find any significant result on all parameters except for the level of oxidative stress markers, our duration of study might not be long enough to show the desired changes in BMD, bone biomarkers and estrogen level.

Thus, consumption of TH with a dosage of 0.75g/kg BW alone or combined with jumping were found to have beneficial effects and could be introduce as an alternative treatment for female athletes with low BMD.

CHAPTER 6: CONCLUSION

Excessive energy expenditure and decreased bone mechanical loading are the main factors that lead to low BMD among the female athletes in Malaysia. Low body weight was also associated with low BMD. To minimise the risks of low BMD, TH was recommended as an alternative treatment and as it was found to have beneficial effects on BMD due to its high level of antioxidant content acting as a free-radical scavenger. A dosage of 0.75 and 1.5 g/kg BW of TH produced the same effect on antioxidant activity and oxidative stress in female athletes. In view of this finding, 0.75 g/kg BW of TH was chosen as the appropriate dosage in the subsequent study investigating the role of TH as a protector against oxidative damage. As a result, it was observed that there was a significant reduction in oxidative stress and an increasing trend in BMD, bone biomarkers, and estrogen level after 8 weeks of supplementation of a low dosage of TH alone or in combination with an exercise intervention program. It is possible that the insignificant results were due to the length of intervention whereby the duration of an 8-week intervention program might not sufficient to show the desired changes in BMD, bone biomarkers and estrogen level amongst the participants with low BMD. Thus, future studies with a similar design and intervention program should consider a longer period of intervention if it‘s going to involve the same population. In conclusion, the consumption of TH with a dosage of 0.75g/kg BW alone or combined with jumping exercises were found to have beneficial effects on bone health, estrogen level and oxidative stress and could be introduce as an alternative treatment for female athletes with low BMD.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

Publication:

Ahmad, N.S., Hamid, M.S., Cheong, J.P.G., Hamzah, S.H. (2017). Bone Mineral Density and Associated Risk Factors among Female Athletes: A Cross-Sectional Study. This paper has been accepted for publication in Sains Malaysiana.

Ahmad, N.S., Aziz, A.A., Kong, K.W., Hamid, M.S., Cheong, J.P.G., Hamzah, S.H. (2017). Dose-Response Effect of Tualang Honey on Postprandial Antioxidant Activity and Oxidative Stress in Female Athletes: A Pilot Study. This paper has been accepted for publication in Journal of Alternative and Complementary Medicine.

Ahmad, N.S., Hamid, M.S., Cheong, J.P.G., Hamzah, S.H. (2017). Diet and Bone Status in Eumenorrheic Female Athletes. 3rd International Conference on Movement, Health and Exercise. *IFMBE Proceedings, 58*, 144-147.

Conferences:

2016 Beijing International Forum on Industry of Sports and Health Nutrition. The First Asian Sports and Health Nutrition Science Conference

(Beijing, China,13-14 September 2016)

Abstract: Tualang Honey Supplementation Prevents Bone Loss, Oxidative Stress and Improve Estrogen Level on Female Athletes with Low Bone Density.

Award: Excellent Poster Presentation

3rd International Conference on Movement, Health and Exercise

(Melaka, 28-30 September 2016)

Abstract: Diet and Bone Status in Eumenorrheic Female Athletes

FORM 2: SUBJECTS INFORMATION SHEET

Research Title:

Effects of Honey Consumption on Bone Metabolism in Moderately Trained Female Athletes

Introduction:

Honey is a sweet food made by bees using nectar from flower. The use of honey in medical area is well published as antimicrobial, anti-inflammatory, antioxidant, antimutagenic, antitumor, and antidiabetic properties, and for wound-healing attributes (Ahmed & Othman 2013). Other than that it also can be used to prevent osteoporosis and enhance calcium absorption (Erejuwa et al., 2012). Tualang honey contains antioxidant which can act as free radical scavenger, reducing the oxidative stress level as well as inflammatory cytokine (Nadia et al. 2012). This will result in survival of osteoblasts, reduced osteoclastogenic activity, and consequently reduced bone loss (Nadia et al. 2012) induces by oxidative stress. Natural antioxidant in body that could reduce bone stress is estrogen. However, if estrogen secretion is reduced then an alternative ergogenic aid is needed to improve bone formation and bone resorption among female athletes. Thus, in the present study it is anticipated that the positive effects of Tualang honey may test specifically on bone metabolism (osteoclast and osteoblast activity) and gonadotropin hormone production in female athletes with low bone density athletes.

What is the purpose of the study?

1) To assess the energy intake, eating attitude, menstrual status and the relationship between body fatness and gonadotropin hormone secretion of female athletes.
2) To examine relationship between energy intakes, gonadotropin hormone and bone mass density of female athletes.
3) To identify the antioxidant properties content of Tualang honey and determine the acute changes of antioxidants activities in blood plasma with different Tualang honey dosages.
4) To identify the impact of Tualang used alone and in combination with running exercise, on improving bone metabolism among moderately trained female athletes
5) To determine the protective effect of Tualang honey on disturbance of reproductive hormones.

Why am I eligible?

You are eligible to take part in this study because you are aged between 18-30 years, you are active (exercise at least 3 times a week), healthy female and no chronic diseases. You are involved actively in specific sport for more than 12months at least in university level. You will be remind to not taking any medication (i.e contraceptive pills), refrain from ingesting any products containing honey and self-admit that you are not pregnant. You will be asked to not allow training or getting involved in training or tournament throughout eight weeks of intervention program.

What will happen to me if I take part?

You will be involved in this study for approximately eight weeks. During this time, you will be required to come to the laboratory for seven study visits. An explanation of what will happen at each of these visits is given below

APPENDIX B- CONSENT FORM

First, second, third visit

At the first visit, you will be asked to come to the laboratory to fill in a demographic and anthropometric form, measuring body composition and answering three questionnaires (menstrual history, 24-hour diet recall and 3 days food record). You will have to complete a three day food record at home. After one week you will be asked to come again to the laboratory for a second visit. During this time, you must returns the completed 3 days food record and fill in another two questionnaires (EAT-26 and BPAQ Questionnaire). Bone mass density (BMD) will be measured using FURUNO CM-200 Ultrasound Bone Densitometer and fasting blood will be collected for gonadotropin hormones and bone biomarkers test (baseline measurements). In addition, VO_{2max} will be estimated by Multi-stage Fitness Test. All the results will be recorded and the one who has energy intake less than 2000kcal/day and low BMD (-0.1<Z-score<-2) will be eligible to be the recruited as a participant and needs to come for another three visits. At third study visit, 20 participants (normal BMD, Z-score> 1) are needed to get involved in antioxidant activity test. You will be divided into two groups which are low and high dose of Tualang honey group (LHG) and (HHG), respectively. You will be asked to consume honey with 0.75g/kg body weight or 1.5g/kg body weight and blood will be taken at 0hr, 2 hr and 6 hr to test your antioxidative activities,

Fourth, fifth and sixth visits

At your fourth visit, only 60 selected participant (low BMD and low energy intake) you will be asked to come to the laboratory. Then you will randomly assigned into four different groups which are Control group (CG), Tualang honey group (THG), Tualang honey plus exercise group (THEG) or exercise group (EG). If you are from the THEG and EG groups, you will take part in a running session and have to do familiarisation prior to the actual exercise test. In actual intervention program, participants from THG will be reminded to consume honey in standard time which is 8.00-9.00 am daily for eight weeks at home. Diet and physical activity need to be recorded throughout intervention program for all groups. Meanwhile, at laboratory participants from THEG group will measure their nude body weight and will eat two pieces of plain bread, honey fluid and plain water (500ml). Participants from EG group will eat plain bread and plain water one hour before start an intervention. CG will not involve in intervention and supplement. Then five minute before starting their training program, a standardized dynamic warm-up is use to begin each training session. Then participants will be required to run 65%MHR, 3 sessions per week (only for THEG and EG group). Blood and bone density for all of you will be measured after 4 weeks in fifth visit for mid-test and after 8 weeks in sixth visit for post-test .

Benefit of the study:

1. To develop a norm of nutritional status on Malaysian female athlete
2. To promote the most effective exercise that is enjoyable, low cost and beneficial to improve bone mineral density in 8 weeks.
3. Getting a free supply honey supplementation.
4. Getting an opportunity to participate in a sports science project and contribution to the development of sports nutrition.
5. Information obtained from this study will benefit the researchers and university.

- If you have any question about this study or your rights, please contact the investigator, Nur Syamsina binti Ahmad at telephone number 013-5091585.

Confidentiality :

- Your answer and information will be kept confidential by the investigators and will not be made public unless disclosure is required by law.
- By signing this consent form, you will authorize the review of records, analysis and use of the data arising from this study.

APPENDIX C- ETHICAL APPROVAL LETTER

UNIVERSITI MALAYA

UM. TNC 2/RC/H&E/UMREC

24 November 2014

Nur Syamsina Binti Ahmad
Sport Centre
University of Malaya

Dear Sir/Madam,

RESEARCH ETHICS CLEARANCE APPLICATION

On behalf of the University of Malaya Research Ethics Committee (UMREC), we are pleased to inform you that your application as detailed below has been reviewed:

Name of PI : **Nur Syamsina Binti Ahmad**
Title of proposal : **Effects of Honey Consumption on Bone Metabolism in Moderately Trained Female Athletes.**
Reference Number : **UM.TNC2/RC/H&E/UMREC - 43**

The committee has approved the application subject to the following conditions:

1) **Minor revision : Please provide details on blood collection procedure.**

Please make the necessary ammendments and resubmit the application to the panel for final approval.

For more information about University of Malaya Research Ethics, please visit: http://umresearch.um.edu.my/.

Thank you.

Yours sincerely,

PROFESSOR DR. AZIRAH HASHIM
Dean,
Humanities Research Cluster/
Chairperson UMREC-Non Clinical

APPENDIX D- MENSTRUAL HISTORY QUESTIONNAIRE

Menstrual History Questionnaire

1. **How old were you when you started having menstrual periods?**
   Age: _____ 1a. If you cannot remember your exact age, were you:
   - ☐ Younger than 10
   - ☐ 10-12 yrs old
   - ☐ 13-15 yrs old
   - ☐ 16 or older
   - ☐ Don't Know

2. **At present which statement best describes your menstrual cycle?**

   - ☐ I'm still having regular periods: The date of my last period was: ___/___/_____
   - ☐ My periods are irregular: The date of my last period was: ___/___/_____
   - ☐ I'm pregnant, or my last pregnancy ended within the past 2 months, or I'm breast feeding

   - ☐ My periods have stopped on their own. (I've had menopause.)
   - ☐ I've had menopause, but now have periods because I am taking hormones.
   - ☐ I've had an operation (surgery) which stopped my periods.
     If your menstrual periods ceased because of surgery, what did you have removed?
     - ☐ One ovary only
     - ☐ Both ovaries
     - ☐ Uterus only
     - ☐ Uterus and one ovary
     - ☐ Uterus and both ovaries
     - ☐ Don't know
   - ☐ I've taken medication which has stopped my periods.
     If your periods stopped because of medication, which medication were you taking? Medication name: _____

   - ☐ I've had chemotherapy which has stopped my periods.
   - ☐ I've had radiation therapy which has stopped my periods.
   - ☐ Other: _____

3. **If your menstrual periods have stopped, how old were you when your menstrual periods stopped?** (Please provide us with the age at which your menstrual periods stopped regardless of why they have stopped – naturally, due to surgery, medication, chemotherapy, or radiation therapy. If your periods have stopped, but you now have periods because of taking hormones, answer with the age at which your periods first stopped.)
   Were you:
   - ☐ Younger than 20
   - ☐ 20-29 yrs old
   - ☐ 30-39 yrs old
   - ☐ 40-44 yrs old
   - ☐ 45-49 yrs old
   - ☐ 50-54 yrs old
   - ☐ 55 – 59 yrs old
   - ☐ 60 or older

   OR ☐ My menstrual periods have not stopped.

4. **If your menstrual periods have stopped, how old were you when you first experienced symptoms of menopause such as hot flashes or night sweats?**
   _____ Years old
   - ☐ Did not experience symptoms
   - ☐ Don't Know

   OR ☐ My menstrual periods have not stopped.

P:\FERNALD\Questionnaires\2007 Menstrual History Questionnaire.doc

All women should answer the next two questions, whether they currently have menstrual periods or not.

5. When you are (were) having regular menstrual cycles, how many days are (were) there between periods? _____ Days between periods

For how many days do (did) you have your period? _____ Days

6. Between the ages of 18 and 40, excluding times when you may have been on the pill, pregnant, or nursing, which of the following statements BEST describes your menstrual periods? They are (were)...

- ☐ Nearly always regular, that is, you could usually predict when you would start bleeding to within two or three days
- ☐ Fairly Regular
- ☐ Irregular
- ☐ Don't Know

APPENDIX E- 24-HOUR DIET RECALL

24-hour diet recall

Name: _____

Age:_____

Date:_____

Time	*Food & Beverage Description*	*Amount*

APPENDIX F- 3-DAYS FOOD DIARY

3-day food diary

Name: _____

Age:_____

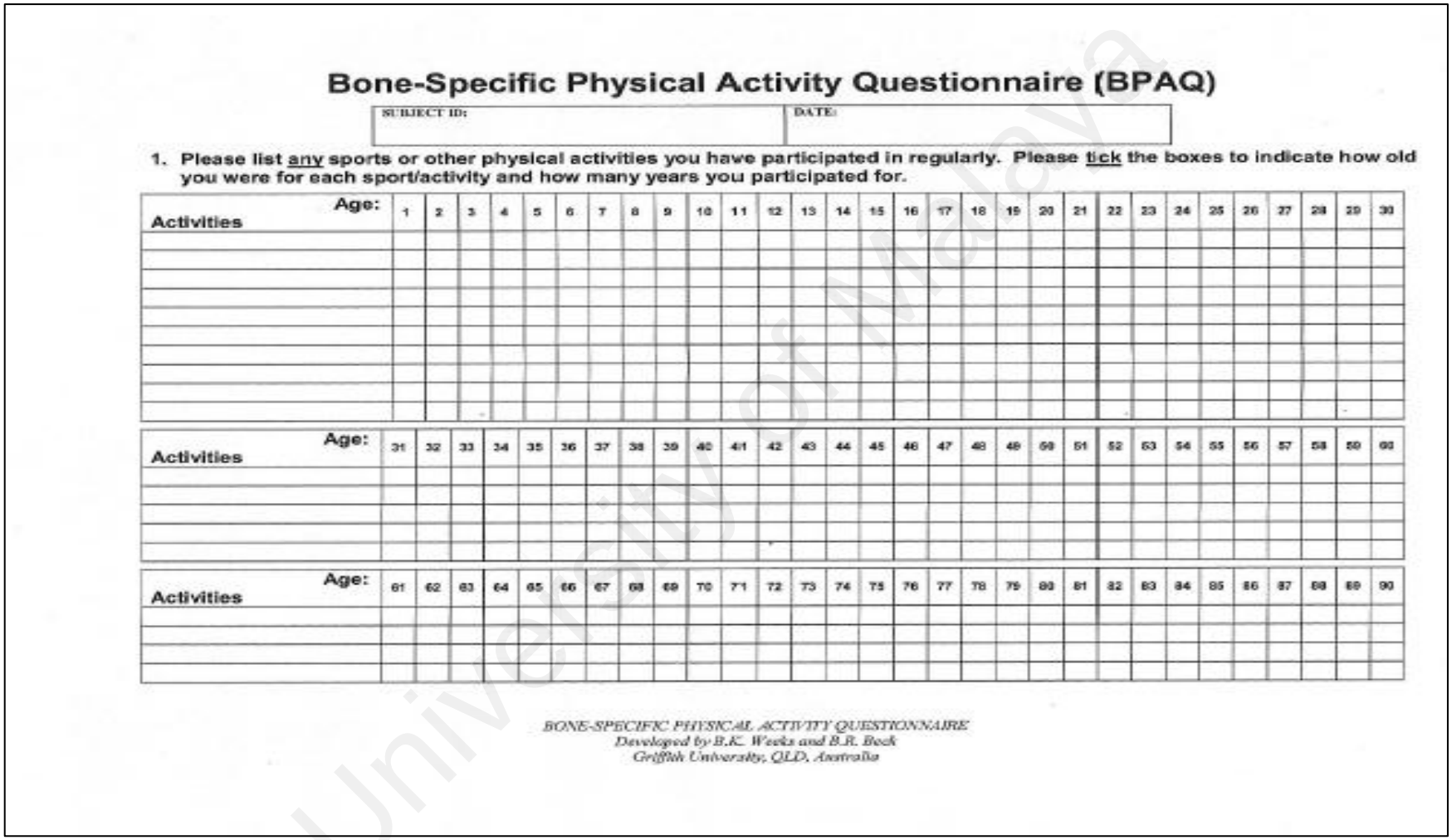
Date:_____

Day 1 Food Record		**Date:**
Time	*Food & Beverage Description*	*Amount*

Day 2 Food Record		**Date:**
Time	*Food & Beverage Description*	*Amount*

Day 3 Food Record		**Date:**
Time	*Food & Beverage Description*	*Amount*

APPENDIX G-BONE PHYSICAL ACTIVITY QUESTIONNAIRE



Bone-Specific Physical Activity Questionnaire (BPAQ)

SUBJECT ID:	DATE:

1. Please list any sports or other physical activities you have participated in regularly. Please tick the boxes to indicate how old you were for each sport/activity and how many years you participated for.

Activities / Age:	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30

Activities / Age:	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60

Activities / Age:	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90

BONE-SPECIFIC PHYSICAL ACTIVITY QUESTIONNAIRE
Developed by B.K. Weeks and B.R. Beck
Griffith University, QLD, Australia

Bone-Specific Physical Activity Questionnaire (BPAQ)

SUBJECT ID:	DATE:

2. **Please list the sports or other physical activities (be as specific as possible) you participated in regularly during the last 12 months and indicate the average frequency (sessions per week)?**

Activity: _____ Frequency (per week): _____

Activity: _____ Frequency (per week): _____

Activity: _____ Frequency (per week): _____

Activity: _____ Frequency (per week): _____

Activity: _____ Frequency (per week): _____

Activity: _____ Frequency (per week): _____

Activity: _____ Frequency (per week): _____

Activity: _____ Frequency (per week): _____

BONE-SPECIFIC PHYSICAL ACTIVITY QUESTIONNAIRE
Developed by B.K. Weeks and B.R. Beck
Griffith University, QLD, Australia

UKM UNIPEQ

Ref : ULUKM/1915/15
Date : 08/07/2015
Page : 1 of 1

CERTIFICATE OF ANALYSIS

Name of customer : **PUSAT SUKAN**
Address : Universiti Malaya, Lembah Pantai
50603 Kuala Lumpur.
(Attn : *Miss Sareena Hanim Binti Hamzah*)
Tel No. / Fax No. : 03-79673321
Sample Description : One (1) sample describe as ***Madu Tualang***
Ref. No : U150015
Date of Receipt : 11/06/2015

ANALYSIS RESULTS
(As per sample)

Parameter, Unit	Result		Test Method
Protein, g/100g	0.76	0.70	In house method No. STP/Chem/A03 based on AOAC 16th Edi. 981.10
Fat, g/100g	<0.1	<0.1	In-house Method No: STP/Chem/A02 (ii) based on The Chemical Analysis of Food, David Pearson, 6th Edition: Page 13
Carbohydrate, g/100g	75.96	76.06	In house method No. STP/Chem/A06 based on Promerance Food Analysis: Theory and Practice, 2nd Ed. (pg 637)
Ash, g/100g	0.28	0.24	In-house method no. STP/Honey02 based on Harmonised Methods of The International Honey Commission 1.3
Moisture, g/100g	23.0	23.0	In-house method no. STP/Honey/01 based on ATAGO Hand Refrectometer Honey Manual
Energy, Kcal/100g	307 (1289kJ)	307 (1289kJ)	In house method No. STP/Chem/A01 based on Pearson's The Chemical Analysis of Foods (6th Edition, page 578)

Remarks:
a. %Total Carbohydrate = 100 – (%Ash + %Moisture + %Protein + %Fat)
b. *Opened / balance samples will be discarded two weeks after issuance of Certificate of Analysis.*

Authorised by:

NOOR AZINAH MAAMIN
CHEMIST (A M.I.C)
IKM No. A/1973/4360/03

ilac-MRA
STANDARDS MALAYSIA
MS ISO/IEC 17025
TESTING
SAMM NO. 330

UKM UNIPEQ

Ref : ULUKM/1917/15
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Page : 1 of 1

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Ref. No : U150015
Date of Receipt : 11/06/2015

ANALYSIS RESULTS
(As per sample)

PARAMETER, UNIT	Result		Test Method
Total Phenolic Content, as GAE, (mg/100g)	20.18	20.00	Journal of Food Chemistry 95 (2006) , Pg 319-327
DPPH (% Inhibition)	35.34	36.16	Journal of Food Chemistry 95 (2006) , Pg 319-327
ABTS, (mg/100g)	0.47	0.46	Journal of Food Chemistry 95 (2006) , Pg 319-327
FRAP (in 10mg/ml)	29.80	21.30	Journal of Food Chemistry 103 (2007) , Pg 1003-1008

Remarks:
a. *Opened / balance samples will be discarded two weeks after issuance of Certificate of Analysis.*

Authorised by:

NOOR AZINAH MAAMIN
CHEMIST (A.M.I.C)
IKM No. A/1973/4360/03