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EFFECTS OF ADDING ANIMAL-BASED (WHOLE) MILK AND PLANT-BASED (SOY, ALMOND) MILK ON TOTAL POLYPHENOLS CONTENT AND ANTIOXIDANT ACTIVITY IN DARK CHOCOLATE

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APPROVAL

This project entitled "Effects of adding animal-based (whole) milk and plant-based (soy, almond) milk on total polyphenols content and antioxidant activity in dark chocolate" was prepared by They Sin Siaw and submitted to the Faculty of Medicine and Health Sciences as a partial fulfillment of the requirement for the degree of Bachelor of Science (Nutrition and Community Health) from the Faculty of Medicine and Health Sciences, Universiti Putra Malaysia.

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TPC	Total polyphenols content
F-C	Folin-Ciocalteu
AA	Antioxidant activity
DPPH	2, 2-Diphenyl-1-Picrylhydrazyl
FRAP	Ferric reducing antioxidant power
AO	Antioxidants
DC	Dark chocolate
WM	Whole milk
SM	Soy milk
AM	Almond milk
DCWM	Dark chocolate with whole milk
DCSM	Dark chocolate with soy milk
DCAM	Dark chocolate with almond milk
GAE	Gallic acid equivalent
TE	Trolox equivalent

M ± *SD*	Mean plus minus standard deviation
NFCS	Non-fat cocoa solids
Igs	Immunoglobulins
LDL	Low-density lipoprotein
HDL	High-density lipoprotein
RDA	Recommended dietary allowance
CVD	Cardiovascular diseases
CHD	Coronary heart disease
NO	Nitric oxides
MUFA	Mono-unsaturated fatty acids
AUC	Area under the curve
β-Lg	B-lactoglobulin
HPLC	High performance liquid chromatography
Fe_3-TPTZ	Ferric tripyridyltriazine
Fe_2-TPTZ	Ferrous tripyridyltriazine
TPTZ	Tripyridyltriazine
Na_2CO_3	Sodium carbonate
HCl	Hydrochloric acid
$FeCl_3$	Ferric chloride
m	Milli
M	Molar
L	Liter
g	Gram
µ	Micro
rpm	Revolutions per minute
A_{cont}	Absorbance of control
A_{samp}	Absorbance of sample

ABSTRACT

EFFECTS OF ADDING ANIMAL-BASED (WHOLE) MILK AND PLANT-BASED (SOY, ALMOND) MILK ON TOTAL POLYPHENOLS CONTENT AND ANTIOXIDANT ACTIVITY IN DARK CHOCOLATE

They Sin Siaw

Dark chocolate with ≥70% cocoa solids has been claimed as functional food due to its polyphenols-rich properties. Chocolate is commonly incorporated with milk to be consumed as chocolate milk. However, inconsistent findings were reported regarding effects of adding milk into chocolate on its antioxidants properties. Moreover, types of milk used were also unclear. Hence, this study aims to determine the effects of adding whole milk (DCWM), soy milk (DCSM) or almond milk (DCAM) on the total polyphenols content and antioxidant activity in 85% dark chocolate. The total polyphenols content (TPC) of each sample was estimated through the Folin-Ciocalteu method, while antioxidant activities (AA) were determined through 2,2-diphenyl-1-picrylhydrazyl (DPPH) and Ferric Reducing Antioxidant Power (FRAP) assays. Results showed that TPC in dark chocolate (8.06±0.11 mg GAE/ml) is significantly enhanced after adding whole milk (15.68±0.17 mg GAE/ml), almond milk (13.16±0.09 mg GAE/ml), and soy milk (11.48 ±0.06 mg GAE/ml) (p <0.001). In terms of AA, DCWM (90.0±0.75%), DCSM (82.6±1.54%), and DCAM (94.9±1.13%) possess higher DPPH scavenging ability than dark chocolate (64.0±2.39%) alone (p <0.001). The FRAP values of dark chocolate (95.94±2.94 µmol TE/L) is also increased after adding whole milk (133.67±1.55 µmol TE/L; p <0.001), almond milk (325.67±14.91 µmol TE/L; p <0.001), and soy milk (98.61±2.23 µmol TE/L; p =0.999). Strong and positive correlations are found between TPC with DPPH scavenging ability (r =0.714, p <0.01) and FRAP values (r =0.741, p <0.01) in chocolate milk samples. The TPC and AA among milk from highest to lowest are almond milk > whole milk > soy milk. Overall, our study revealed that both animal-based and plant-based milk could enhance antioxidants properties when combined with 85% dark chocolate, as existing antioxidants in milk complement that of dark chocolate. Therefore, combining dark chocolate with milk and consuming it in modest quantities may increase antioxidants uptake.

ABSTRAK

KESAN PENAMBAHAN SUSU (PENUH KRIM) BERASASKAN HAIWAN DAN SUSU (SOYA, BADAM) BERASASKAN TUMBUHAN TERHADAP JUMLAH KANDUNGAN POLIFENOL DAN AKTIVITI ANTIOKSIDAN DALAM COKLAT GELAP

They Sin Siaw

Coklat gelap dengan ≥70% koko tergolong sebagai makanan berfungsi kerana ia kaya dengan polifenol. Coklat biasanya ditambahkan dengan susu dan diminum sebagai susu coklat. Namun, kajian sebelum ini mengenai kesan penambahan susu terhadap antioksidan dalam coklat adalah tidak konsisten. Jenis susu yang digunakan juga tidak jelas. Oleh itu, kajian ini bertujuan untuk mengenalpasti kesan penambahan susu penuh krim (DCWM), susu soya (DCSM) atau susu badam (DCAM) terhadap jumlah kandungan polifenol dan aktiviti antioksidan dalam 85% coklat gelap. Jumlah kandungan polifenol (TPC) dalam sampel kajian susu coklat telah dianalisis menggunakan kaedah Folin-Ciocalteu, manakala aktiviti antioksidan (AA) telah diuji melalui kaedah 2,2-diphenyl-1-picrylhydrazyl (DPPH) dan FRAP (Ferric Reducing Antioxidant Power). Hasil kajian melaporkan bahawa TPC dalam coklat gelap (8.06±0.11 mg GAE/ml) telah meningkat dengan ketara setelah ditambahkan dengan susu penuh krim (15.68±0.17 mg GAE/ml), susu badam (13.16±0.09 mg GAE/ml) dan susu soya (11.48 ±0.06 mg GAE/ml) (p <0.001). Dari segi AA, DCWM (90.0±0.75%), DCSM (82.6±1.54%) dan DCAM (94.9±1.13%) memiliki daya mengurangkan DPPH yang lebih tinggi daripada coklat gelap (64.0±2.39%) sahaja (p <0.001). Nilai FRAP coklat gelap (95.94±2.94 µmol TE/L) juga telah meningkat setelah ditambah susu penuh krim (133.67±1.55 µmol TE/L; p <0.001), susu badam (325.67±14.91 µmol TE/L; p <0.001) dan susu soya (98.61±2.23 µmol TE/L; p =0.999). Selain itu, korelasi yang kuat dan positif telah terbukti antara TPC dengan nilai DPPH (r =0.714, p <0.01) dan FRAP (r =0.741, p <0.01) dalam sampel susu coklat. Didapati bahawa TPC dan AA antara susu daripada tertinggi hingga terendah adalah susu badam > susu penuh krim > susu soya. Kesimpulannya, susu berasaskan haiwan ataupun tumbuhan dapat meningkatkan sifat antioksidan apabila dicampurkan dengan 85% coklat gelap, kerana antioksidan yang sedia ada dalam susu dapat melengkapi antioksidan dalam coklat gelap. Oleh itu, meminum coklat gelap bersama susu dalam kuantiti yang sederhana berkemungkinan dapat meningkatkan pengambilan antioksidan.

CHAPTER 1

INTRODUCTION

1.1 Background

Chocolate is a well-known confectionery processed from the beans of cocoa tree or *Theobroma cacao* which means “foods of the Gods” in Greek (McShea et al., 2009, as cited in Katz et al., 2011). Chocolate was first cultivated thousands of years ago by the Maya, an indigenous population in South America (Verna, R., 2013). During ancient times, chocolate was first appeared as a bitter and strong-tasted drink, prepared by dissolving the grounded dried cocoa beans into hot water and added with cinnamon and pepper for flavouring purpose; after evolutions, chocolate bars were eventually introduced in the 19th century after the production of mechanical mills to squeeze cocoa butter out from the cocoa mass (Ackar et al., 2013; Rogovská & Čukanová, 2015). Cocoa butter gives a smooth and creamy taste to the chocolate (Visioli et al., 2009). Cocoa liquor which contains both cocoa butter and non-fat cocoa solids (NFCS) are produced after a series of processing of the cocoa beans; where the cocoa liquor is then combined again with cocoa butter and sugar to yield solid chocolate. Percentage of the cocoa liquor present in a particular chocolate can be noticed and identified on the food packaging, which is usually referred to as the "percent cacao" (Katz et al., 2011).

There are several types of chocolate available on the market, including dark chocolate, milk chocolate, and white chocolate, with percentage of cocoa liquor normally ranging from 70-85%, 10-25%, and 0% respectively (Katz et al., 2011; Montagna et al., 2019). White chocolate does not consist of any cocoa solids as it is only made up of milk, sweeteners such as sugar, and at least 20% of cocoa butter by its weight; milk chocolate is different from white chocolate due to the presence of cocoa solids or cocoa liquor in its content; while dark chocolate consists of only cocoa butter and cocoa solids at higher proportion, and might sometimes added with low content of sugar as well, depending on the manufacturers and the cacao percentage present in that particular dark chocolate (Katz et al., 2011; Montagna et al., 2019).

Besides, chocolate, or specifically dark chocolate, has been claimed as a functional food due to the several functional ingredients available in its content; for examples, methylxanthines such as caffeine, and antioxidants such as polyphenols (Freeman, 2002; Albrecht et al., 2010; Ackar et al., 2013). Functional food refers to any food, either in whole, enriched, enhanced, or fortified form, which had been scientifically proven to be able to promote health benefits beyond its basic nutritional values (Hasler, 2002). Basic nutrients include carbohydrates, protein, fat, vitamins, and minerals. Apart from these nutrients, functional food also consists of at least one of other active ingredients or compounds in its content which could potentially reduce risk of diseases and improve human well-being as well, including phytochemicals such as polyphenols which are available in most of the plant-based foods; for examples, anthocyanins found in berries and catechins available in green tea (Musial et al., 2020; Olas, B., 2018; Albrecht et al., 2010).

Apart from dietary fiber, magnesium, potassium, copper, as well as iron, dark chocolate also contains a substantial amount of polyphenols especially phenolic acids and flavonoids (Katz et al., 2011). Flavonoids were further classified into several sub-categories, including flavanols or called flavan-3-ols, such as catechin, epicatechin, and procyanidins, which could be found abundantly in dark chocolate (Ding et al., 2006; Katz et al., 2011; Silva Medeiros et al., 2015). Dark chocolate is particularly rich in flavonoids; while procyanidins have been reported to play a major role in the antioxidant activity in cocoa products (Katz et al., 2011). Besides, polyphenols or chocolate flavonoids were found to have anti-inflammatory property to effectively fight against free radicals in human bodies, then helping to reduce risk of cancer, cardiovascular diseases, stroke, hypertension, and hypercholesterolemia, as well as improving insulin sensitivity to reduce risk of developing type-2 diabetes mellitus (Ding et al., 2006; Silva Medeiros et al., 2015; Ramos et al., 2017).

Studies had reported that dark chocolate consists of the highest amount of antioxidant, particularly polyphenols, among all types of chocolate, followed by milk chocolate (Albrecht et al., 2010; Zujko & Witkowska, 2014; Silva Medeiros et al., 2015; Kemsawasd et al., 2016; Database on Polyphenol Content in Foods, n.d.). Research demonstrated that the higher the percentage of non-fat cocoa solids (NFCS) or cocoa liquor present in a chocolate, the higher the total polyphenols content in that particular chocolate; while more cocoa liquor or cocoa solids will bring bitter taste to the chocolate (Silva Medeiros et al., 2015; Database on Polyphenol Content in Foods, n.d.). Milk chocolate contains lower amounts of antioxidants compared to dark chocolate; while white chocolate does not consist of any polyphenols or flavonoids due to the absence of cocoa solids in its content (Verna, R., 2013).

1.2 Problem Statement

Not only being consumed directly as a healthy snack, dark chocolate is also melted and commonly mixed with milk to become chocolate milk, one of the most popular beverages especially among the children (Hanks et al., 2014). Milk, or to be specific animal-based milk, is a nutritious fluid secreted by mammary glands of the mammals such as cows, goats, sheep, horses, or even donkeys, camels, and buffalos (Yigit, A. A., 2019). Animal-based milk is also known as dairy milk. Similar to dark chocolate, milk and milk products had also been claimed as functional foods due to their bioactive components such as antioxidants, immunoglobulins (Igs), prebiotics, and probiotics (Bhat, Z. F. & Bhat, H., 2011). Dairy milk contains higher amount of saturated fatty acids particularly stearic acid and palmitic acid, compared to unsaturated fatty acids in its content (Aydar et al., 2020). Dairy milk and milk products are also rich in protein, lactose, conjugated fatty acids such as linoleic acid, and other essential minerals and vitamins including highly absorbable calcium, phosphorus, magnesium, fat-soluble vitamins A, D, E, K, as well as water-soluble vitamin B such as riboflavin and cobalamin or vitamin B12 (Yigit, A. A., 2019; Dougkas et al., 2018). Lactose is a form of carbohydrate which could only be found in animal-based milk; while this naturally existing lactose plays a role in contributing to the palatable taste of the cow's milk (Aydar et al., 2020; Yigit, A. A., 2019).

Besides, cow's milk is the most consumed milk and represents 82.7% of milk production globally (Food and Agriculture Organization of the United Nations, 2016). Cow's milk is commonly sold on the market as whole (full cream) milk, semi-skimmed (low-fat) milk, and skim (fat-free) milk, with 3.3%, 1-2%, and 0.1% of lipid content respectively; whereby the higher the milk lipid content, the higher the amount of fat-soluble vitamins available in the milk (Yigit, A. A., 2019). Furthermore, whole milk, semi-skimmed milk, and skim milk are not much differ in their protein content, which is around 3.3% or to be exact, 7.86 g, 8.22 g, and 8.26 g of protein per cup (244 g) respectively (Tremblay et al., 2003; Milk Facts, n.d.). Meanwhile, casein and whey proteins are milk proteins which could be found in milk at around 82% and 18% respectively.

Despite the excellent nutritional values of dairy milk; however, due to the concerns in individuals with lactose intolerance, milk allergies, veganism practices, hypercholesterolemia, and increased awareness in environmental issues such as the emissions of greenhouse gas in agriculture, plant-based milk, also known as milk substitute has rapidly gained its popularity recently as an alternative to the animal-based milk or dairy milk, for examples soy milk, almond milk, coconut milk, and rice milk (Aydar et al., 2020; Haas et al., 2019; Yigit, A. A., 2019; Vanga & Raghavan, 2018). Raw materials such as nuts, soy, and coconut underwent a series of processes such as de-hulling, roasting, grinding, extraction, fermentation, blanching, wet milling, filtration, fortification, sterilization, and homogenization before being produced as a milk substitute. Besides, protein, calcium, and other vitamins are also often fortified or enriched into milk substitutes to improve the quality and bioavailability of these nutrients (Haas et al., 2019). Soy milk and almond milk are among the most consumed plant-based milk, and have contributed to the major non-dairy beverage industries on the market (Paul et al., 2019).

Moreover, plant-based milk alternatives have higher content of unsaturated fatty acids except for coconut milk which contains more saturated fatty acids; while these unsaturated fatty acids such as linoleic acid, α-linolenic acid, and oleic acid could help in lowering blood lipid concentration, and to promote neuroprotective benefit as well to prevent Alzheimer's disease (Aydar et al., 2020). Phytosterols or plant-sterols available in plant-based milk could also help to reduce cholesterol and low-density lipoprotein (LDL) in blood by competing with and limiting the absorption of cholesterol into the digestive system or bloodstream (Decloedt et al., 2017). Although plant-based milk has been claimed and believed to possess greater health benefits due to its cholesterol-free property, higher amount of unsaturated fatty acids and fiber as compared to cow's milk, and also most importantly, the presence of health-promoting and disease-preventing phytochemicals from its raw materials such as nuts and cereals; however, milk substitute was found to have lower protein content except in soy milk, and was normally consisting of added sugar (ranged from 4-8 g per 100 ml) in sweetened milk substitutes in order to recreate the pleasant flavor as in cow's milk (Aydar et al., 2020; Yigit, A. A., 2019).

Several studies had reported that milk could adversely affect and reduce antioxidants in chocolate due to the interaction between milk proteins and chocolate polyphenols (Gallo et al., 2013; Neilson et al., 2010; Tabernero et al., 2006; Serafini et al., 2003); while another study claimed that milk could enhance and increase uptake of antioxidants in chocolate (Otemuyiwa et al., 2017). There were also several other studies indicating that there was actually no significant effect on the antioxidant content and antioxidant activity in chocolate upon the addition of milk or milk powder (Keogh et al., 2007; Roura et al. 2007; Schramm et al, 2003; Richelle et al., 2001). Hence, there were inconclusive and contradictory findings on this topic in the previous studies, and also lacking recent findings as well. Moreover, although milk chocolate was significantly reported to have lower antioxidant content compared to dark chocolate, it was due to its lower content of cocoa solids; there were only few studies indicating that it was due to the milk addition during its manufacturing process (Serafini et al., 2003; Database on Polyphenol Content in Foods, n.d.).

Hence, this study aims to fill the gap to determine whether the antioxidant content and antioxidant activity in chocolate, particularly dark chocolate, could be affected upon the addition of milk. In this study, both animal-based dairy milk and plant-based milk substitutes will be used as different samples and combined with dark chocolate to become chocolate milk, since the sources or types of milk involved in the previous studies were not stated clearly as well. Whole milk (cow's milk) represents the dairy milk in this study, while soy milk and almond milk represent the milk substitute. Cow's milk is chosen as it is the most consumed milk worldwide; while soy milk and almond milk are selected as these two types of milk substitutes are among the most consumed plant-based milk (Paul et al., 2019). Besides, 85% dark chocolate has been selected as the functional food to be involved in this study since dark chocolate with at least 70% of cocoa solids was significantly reported to contain higher amount of polyphenols (Kemsawasd et al., 2016; Silva Medeiros et al., 2015; Zujko & Witkowska, 2014; Database on Polyphenol Content in Foods, n.d.); while dark chocolate with much higher cacao percent such as 99% might be too bitter to be consumed, hence 85% dark chocolate as the percentage that lies in between 70% to 99% of cocoa solids has been selected.

1.3 Significance of Study

Dark chocolate is available on the market worldwide and can easily be getting accessed to. As discussed earlier, besides being consumed directly, dark chocolate is also commonly incorporated into milk to make chocolate milk. Some people consume this beverage due to its palatable and aromatic flavour; while some others might consume dark chocolate milk due to its nutritional values. Hence, the potential changes in the health benefits of dark chocolate upon its combination with milk should be investigated, so that the public could consume these functional foods in the most effective way to obtain the maximum beneficial effects.

Besides, the nutrient compositions were varied among animal-based milk and plant-based milk. Plant-based milk has become very popular recently due to the cholesterol-free and phytochemicals available in its content which has been believed to be able to reduce risk of diseases and could promote health benefits greater than animal-based milk. Although milk substitutes generally have lower content of protein and lower bioavailability of other vitamins and minerals, these issues can be overcome by fortification or enrichment of these nutrients to further enhance the quality and to improve the nutritional values of plant-based milk. Plant-based milk substitutes also have their lipid content mostly made up of unsaturated fatty acids, except for coconut milk.

Based on the elaborations mentioned above, plant-based milk is expected to possess higher commercial value compared to conventional animal-based milk in the future. Hence, it is also important to investigate whether milk with different sources, types, and properties possess different interactions with antioxidants in dark chocolate; and whether dark chocolate after being combined with milk substitutes could still have higher antioxidant content and antioxidant activity compared to when dark chocolate is being combined with dairy milk. Moreover, the sources and types of milk involved in the previous studies were not stated clearly, hence the results in this study could be provided as a baseline data for the future studies on whether different types and sources of milk, either from animal sources or plant sources, with different nutritional compositions, could influence the total polyphenols content and antioxidant activity in dark chocolate differently when appeared as chocolate milk. The findings in this study can also be provided as preliminary data or as reference for related research in the future.

1.4 Objectives

1.4.1 General Objective

To determine the effects of adding dairy milk (whole milk) or plant-based milk (soy milk, almond milk) on the total polyphenols content and antioxidant activity in dark chocolate with 85% of cocoa solids.

1.4.2 Specific Objectives

1. To determine the effect of adding whole milk, soy milk, or almond milk on

the total polyphenols content in dark chocolate by using Folin-Ciocalteu method.

2. To determine the effect of adding whole milk, soy milk, or almond milk on the antioxidant activity in dark chocolate by using DPPH assay.

3. To determine the effect of adding whole milk, soy milk, or almond milk on the antioxidant activity in dark chocolate by using FRAP assay.

4. To compare the total polyphenols content and antioxidant activity between dark chocolates either added with drinking water, whole milk, soy milk or almond milk by using one-way ANOVA Tukey's post-hoc statistical test.

5. To determine the correlation between the total polyphenols content and antioxidant activity in dark chocolate added with drinking water, whole milk, soy milk, and almond milk, by using Pearson's correlation statistical test.

1.5 Hypotheses

1. There is a significant difference in the total polyphenols content between dark chocolate added with drinking water or with whole milk, soy milk or almond milk.

2. There is a significant difference in the DPPH radical scavenging activity and ferric reducing antioxidant power (FRAP) between dark chocolate added

with drinking water or with whole milk, soy milk or almond milk.

3. There is a positive correlation between the total polyphenols content and antioxidant activity in dark chocolate added with drinking water, whole milk, soy milk, and almond milk.

CHAPTER 2

LITERATURE REVIEW

2.1 *Theobroma Cacao*

Theobroma cacao or also known as cocoa tree is a tall tropical tree initially grown in "cocoa belt", a region with high humidity and hot climate located within 20° latitude north to south across the equator; and was believed to have been first cultivated in South America by Mayan people in the 4th century (Verna, R., 2013). The fruits of *Theobroma cacao* took around five years to be borne, and the maximum yield are to be obtained after ten years; while the fruits of *Theobroma cacao* could either appear in red, yellow, brown, or purple colour (Verna, R., 2013). The fruits of *Theobroma cacao* were also known as cabossides or pods. The cocoa pods had been believed as a symbol of fertility by Mayan people (Rogovská & Čukanová, 2015).

Every year, around twenty (20) to fifty (50) pods are produced by each *Theobroma cacao* plant; with each pod containing about twenty (20) to forty (40) cocoa beans, which could be further processed to yield and manufacture cocoa products such as chocolates and cocoa powder (Verna, R., 2013). For instance, one kilogram of cocoa beans could be obtained from around ten (10) cabossides or pods of cocoa trees. Besides, Verna, R. (2013) also demonstrated that the quality of soil, quantity or intensity of rainwater and sunlight received, environmental temperature, and the plant varieties, could also influence the taste of the cocoa beans.

During ancient times, the initial cocoa drink invented by Mayans and Aztecs was named as "xocolati", means "bitter" and "water" in Aztec language, prepared by dissolving dried grounded cocoa beans into water, and added with pepper and cinnamon as flavour enhancers (Montagna et al., 2019; Verna, R., 2013). Cocoa was first brought to Europe from America in 1492; however, it did not successfully gain popularity among Europeans until 1528 when Hernán Cortés brought the cocoa samples to King Charles of Spain (Dillinger et al., 2000, as cited in Montagna et al., 2019). After modification of "xocolati" recipe from Aztecs by Spaniards who further added cocoa drink with sugar, vanilla, cloves, and nutmegs; the cocoa or chocolate drink eventually turned out as a sweet and soft beverage (Rogovská & Čukanová, 2015; Verna, R., 2013). Besides, the scientific name of cocoa tree, *Theobroma cacao* was given by a Swedish scientist, Carl Linnaeus in 1753 based on the Latin word "Theobroma" which means "foods of the Gods" to indicate the preciousness of the cocoa (Montagna et al., 2019). In the 13th century, the cocoa drink or "xocolati" had particularly become a favourite drink of Emperor Moctezuma; even the cocoa beans themselves were so valuable until being identified and used as a form of currency and were as precious as gold during that time (Verna, R., 2013).

To be manufactured into cocoa products such as chocolate and cocoa powder, the cocoa beans or seeds had to further undergo a series of processes such as drying, roasting, fermentation, de-hulling, and grinding (Verna, R., 2013). The fully fermented dried cocoa beans are also known as cacao or cocoa (McShea et al., 2009, as cited in Katz et al., 2011). On the other hand, cocoa liquor consisting of both cocoa butter and non-fat cocoa solids was produced and obtained from hotter roasting of cocoa beans. By eliminating some cocoa butter from the cocoa liquor through additional pressure and heat, cocoa powder was produced; while further combination of sugar and cocoa butter with cocoa liquor could yield solid chocolate (Verna, R., 2013; Katz et al., 2011). Chocolate as the most popular cocoa product has been consumed worldwide (Del Prete & Samoggia, 2020). The types of chocolate were determined by the proportion and percentage of cocoa liquor or non-fat cocoa solids (NFCS) present in each chocolate; for instance, chocolate with at least 70% of cocoa solids can be considered and classified as dark chocolate (Katz et al., 2011).

Besides, Katz et al. (2011) also stated that non-fat cocoa solids (NFCS) derived from the cocoa liquor consists of fiber, vitamins, minerals such as magnesium, potassium, copper, zinc, phosphorus, and iron, as well as bioactive components such as methylxanthines and polyphenols; in which one whole cocoa bean consists of 10%

of polyphenols of its dry weight and has been claimed as a good source of dietary polyphenols (Rusconi & Conti, 2010, as cited in Montagna et al., 2019). One of a group of polyphenols, known as flavonoids such as catechin, anthocyanidin, and proanthocyanidin are phytonutrients which could be found abundantly in cocoa bean, which contribute up to 37%, 4%, and 58% respectively in a single cocoa bean (Montagna et al., 2019). Despite the rich source of polyphenols in cocoa, however, the polyphenols content will somehow be reduced after a series of processing and manufacturing before the cocoa products are being available commercially on the market (Montagna et al., 2019). Besides, other than cocoa beans, the extract of cocoa pods was found to possess high antioxidant activity as well, which could help to protect skins against sun exposure and promote skin-whitening and anti-wrinkle properties (Karim et al., 2014).

2.2 Dark Chocolate

Dark chocolate is a type of commercially available chocolate consisting of at least 70% of cocoa solids in its content (Katz et al., 2011). Based on previous studies, dark chocolate has been significantly reported to contain the highest polyphenols content and antioxidant activity among all types of chocolates when compared to soy chocolate, milk chocolate, and semisweet chocolate; white chocolate and milk chocolate; as well as semisweet chocolate, milk chocolate, and white chocolate (Silva Medeiros et al., 2015; Kemsawasd et al., 2016; Zujko & Witkowska, 2014).

Neveu et al. (2010) also demonstrated in 'Database on Polyphenol Content in Foods' that dark chocolate consists of the highest total polyphenols content due to its highest non-fat cocoa solids (NFCS) percentage among all types of chocolates. The same database also reported that dark chocolate consists of up to 622 mg of polyphenols per 30 g of its serving, when measured through Folin-Ciocalteu method, and has been claimed as one of the rich sources of dietary polyphenols (Neveu et al., 2010).

As mentioned earlier, a series of manufacturing processes could reduce the nutrients and other bioactive components in cocoa; however, dark chocolate is still able to retain a relatively high amount of nutrients, for example, about 1.7 g of fiber is available in 100 kcal of dark chocolate (Katz et al., 2011). These fibers are mostly insoluble fiber, which could reduce risk of developing type-2 diabetes mellitus (Weickert & Pfeiffer, 2008, as cited in Katz et al., 2011). Besides, dark chocolate also provides minerals such as magnesium, copper, potassium, and iron, which have respectively contributed to about 9% (36 mg), 31%, 2% (114 mg), and 25% (1.9 mg) of the United States Recommended Dietary Allowance (RDA) for middle-aged men per 100 kcal serving of dark chocolate (Katz et al., 2011).

Due to the presence of cocoa butter, dark chocolate contains unsaturated fatty acids such as oleic acid (33%), as well as saturated fatty acids particularly stearic acid (33%) and palmitic acid (25%) (Montagna et al., 2019). Different from most of other saturated fatty acids, stearic acid has been reported to be non-atherogenic and possess no adverse cholesterolemic effect in human bodies (Bracco, U., 1994, as cited in Katz et al., 2011). In fact, studies had found that stearic acid could induce mitochondrial functions such as lipid β-oxidation, and could lower low-density lipoprotein (LDL) cholesterol in human bodies as well thus lowering risks of developing heart diseases (Rooijen et al., 2021; Rooijen & Mensink, 2020; Senyilmaz-Tiebe et al., 2018). Besides, stearic acid contributes to about one-third of fat content in cocoa butter; hence, dark chocolate is still considered as healthy despite its relatively higher lipid content (Katz et al., 2011).

2.3 Polyphenols and Disease Prevention

Polyphenols as a kind of antioxidant are largely found in plant-based foods such as cereals, fruits, and vegetables, as well as dark chocolate, teas, coffee, and red wine. Dark chocolate contains a substantial amount of polyphenols, especially the flavonoids, which have a chemical structure of C_6-C_3-C_6 (Corti et al., 2009). There are substantial amounts of flavonoids available in dark chocolate, particularly flavanols in both monomeric and oligomeric forms. Catechins and epicatechins are monomers of flavanols; while proanthocyanidins are the polymers (Magrone et al., 2017). Flavanols or flavan-3-ols had even been identified as one of the most powerful antioxidants (Visioli et al., 2009). Besides, the oligomeric procyanidin is known as condensed tannin as well, and it brings bitter taste to the cocoa through the complexes formed upon its interaction with salivary protein (Corti et al., 2009). Dark chocolate also consists of phenolic acids as another category of polyphenols, but in minor amounts.

Cocoa and cocoa products have high bioavailability of polyphenols especially the monomeric and polymeric flavanols which could be retained in human bodies for up to six hours; while they reach their peak concentrations in the bloodstream after two hours of ingestion since they are rapidly absorbed into the digestive system or small intestine (Magrone et al., 2017). With large amount of polyphenols, dark chocolate is able to scavenge free radicals such as reactive oxygen species (ROS) synthesized through oxidation in human bodies, by donating protons to these free radicals (Ackar et al., 2013); which could in turn promoting health and reducing risk of various chronic diseases as antioxidants possess anti-inflammatory property (Ding et al., 2006; Albrecht et al., 2010; Silva Medeiros et al., 2015).

Despite polyphenols are not essential in human daily diets such like those basic nutrients, however, regular consumption of dark chocolates that are particularly rich in polyphenols was shown to develop positive effects on insulin sensitivity, blood pressure, platelets function, and vascular functions as well to reduce risk of cardiovascular diseases (CVD) such as stroke and coronary heart disease (CHD); however, despite of the significant health outcomes provided, the oxidative stress biomarkers in healthy subjects were not changed upon the consumption of cocoa polyphenols due to their existing optimum antioxidant status (Corti et al., 2009; Jalil, A. M. M. & Ismail, A., 2008). In addition, Ackar et al. (2013) reported that polyphenols in dark chocolate could help to control blood cholesterol levels by suppressing the low-density lipoprotein (LDL) oxidation by up to 75%, reducing LDL cholesterol by 7.5% and total cholesterol level by 6.5%, as well as raising high-density lipoprotein (HDL) cholesterol by 9% after twelve weeks of dark chocolate consumption. These statements indicated that polyphenols possess anti-atherosclerotic and anti-inflammatory properties in order to reduce risk of hypercholesterolemia (Jalil, A. M. M. & Ismail, A., 2008).

From the aspect of blood pressure, flavanols significantly reduced systolic and diastolic blood pressures among pre-hypertensive and hypertensive individuals by about 2 to 3 mmHg according to a research by Ried et al. (2010). In addition, dark chocolate administration even in short term duration could improve the problem of insulin resistance and is able to enhance the insulin sensitivity in both healthy and normal individuals as well as in hypertensive individuals with glucose intolerance (Grassi et al., 2005; Gassi et al., 2008). Furthermore, the monomer form of flavanols in dark chocolate, particularly catechins, could inhibit the activation, aggregation, and conjugation of platelets and leukocytes which are involved in the inflammation (Visioli et al., 2009). Most of the diseases such as atherosclerosis and hypertension are due to the inflammation in human body cells; where anti-inflammatory properties of polyphenols could indirectly prevent the incidence of these chronic diseases (Katz et al., 2011).

On the other hand, in the prevention of cardiovascular diseases, flavanols in dark chocolate could help to protect vascular endothelium and improve bioavailability of nitric oxide (Ludovici et al., 2017; Fraga et al., 2011; Gassi et al., 2008). Nitric oxides (NO) are strongly associated with the regulations of blood pressure and insulin response by enhancing the antioxidant enzyme activities in endothelium; while prevention of endothelial dysfunction could directly prevent the major risk factors associated with atherosclerosis or inflammations (Ackar et al., 2013; Katz et al., 2011). Excessive oxidative stress or free radicals such as superoxide anion in human bodies could reduce nitric oxide bioavailability, inactivate the anti-platelet and vasodilator properties in nitric oxide, and could also impair the endothelial functions (Forstermann et al., 2017; Pierini & Bryan, 2015; Mann et al., 2007). However, polyphenols in dark chocolate could help to reduce these free radicals production, promote vasodilation of the blood vessels' wall and improve arterial dilatation by maintaining a healthy endothelium as a result of adequate production and bioavailability of nitric oxide (Katz et al., 2011; Visioli et al., 2009).

2.4 Cow Milk

Cow's milk is the most consumed animal-based dairy milk, and contributes to about 82.7% of milk production worldwide (Food and Agriculture Organization of the United Nations, 2016). Cow's milk is secreted from the mammary glands of cattle, and is a wholesome yet nutritious complete food (Yigit, A. A., 2019). Cow's milk is particularly rich in protein; it even consists of up to four times more protein than in human breast milk. Cow's milk is also rich in calcium and bioactive components such as immunoglobulins (Igs) and probiotics, but with lower content of iron (Kumar et al., 2014; Bhat, Z. F. & Bhat, H., 2011). In addition, the nutritious properties of cow's milk is due to the greatest efficiency of dairy cattle among all ruminants or farm livestock in converting the phytonutrients in eaten fibrous plants and protein in feeds into nutrients in their wholesome food products such as cow's milk and milk products (Hodgson, H. J., 1979; Yigit, A. A., 2019).

However, lactose as a kind of milk sugar which could only be found in animal-based milk, is not easily digestible by human's digestive system; individuals who have insufficient lactase, an enzyme to digest and break down lactose into glucose and galactose, are known to be lactose intolerant (Vanga & Raghavan, 2018; Lucey, J. A., 2015; Kumar et al., 2014). After the consumption of milk or milk products, individuals with lactose intolerance will experience diarrhea, flatulence, or other digestive symptoms (Lucey, J. A., 2015). Other than lactose intolerance, individuals with cow's milk allergy are also not suitable to consume dairy milk. Cow's milk allergy is an immunoglobulin E (IgE)-mediated allergic reaction usually triggered by protein such as cow's milk protein, and is normally occurred among infants and children; however, their allergenicities towards dairy milk will usually be outgrown after they have growing up (Vanga & Raghavan, 2018; Lucey, J. A., 2015).

2.5 Soy Milk

Despite plant-based milk substitute generally contains lower amount of protein as compared to animal-based dairy milk, soy milk is an exception; it is rich in protein which consists of up to 3 g of protein per 100 g of soy milk, and has become the most consumed milk substitute on the market (Vanga & Raghavan, 2018; Paul et al., 2019). Soy milk has the highest protein content among all types of plant-based milk substitutes. Besides, soy milk is inexpensive, yet able to provide various nutrients and bioactive components that are beneficial for human health, for examples unsaturated fatty acids such as oleic acids, fiber, minerals particularly calcium, iron, and zinc, as well as isoflavones, a kind of polyphenols available in soy or soy products, particularly genistein, glycitein, and daidzein; whereas fermentation could enhance isoflavones concentration in soy milk (Paul et al., 2019). These bioactive components improve health especially on the cardiovascular aspect (Paul et al., 2019).

Soy and soy products had become an important source of protein for vegans and vegetarians, and soy milk is particularly favoured by those who suffer from lactose intolerance and cow's milk allergy; however, soy protein is lacking of essential amino acids such as methionine, and with lower content of lysine, despite the high amount of total proteins available in soy or soy milk (Friedman & Brandon, 2001). The soy bean itself even consists of up to 35-45% of protein (Paul et al., 2019). Besides, individuals with poor functionality of the digestive system and those who are allergic to soy protein are not suitable to consume soy products including soy milk, tempeh, tofu, and soy bean paste (Friedman & Brandon, 2001).

2.6 Almond Milk

Apart from soy milk, almond milk is another most-consumed milk substitute (Vanga & Raghavan, 2018). Almonds are soaked, grinded with water, and filtered out to obtain almond milk; sometimes homogenization and pasteurization are applied in order to prolong the shelf life and increase stability of the commercially available almond milk (Bernat et al., 2014, as cited in Vanga & Raghavan, 2018). Almond milk is free of cholesterol and is particularly rich in mono-unsaturated fatty acids (MUFA) such as oleic acid which could help in lowering LDL cholesterol and controlling blood cholesterol level; almond milk also contains lower calories than other types of milk hence it is suitable for individuals who aim to lose weight (Vanga & Raghavan, 2018; Yigit, A. A., 2019).

Almonds, the raw materials of almond milk, have been identified to be able to suppress inflammation, tumour formation, and hyperlipidemia (Paul et al., 2019). Besides, calcium is always fortified into almond milk to mimic the calcium-rich content of cow’s milk (Vanga & Raghavan, 2018). Other than that, almond milk contains more vitamins compared to other milk substitutes, especially vitamin A and vitamin E, are good sources of antioxidants as well (Yigit, A. A., 2019). Moreover, almond milk, particularly unsweetened, is stated to be more suitable for individuals with lactose intolerance compared to soy milk; however, those who are allergic to tree nuts are not recommended to consume almond milk (Paul et al., 2019).

2.7 Milk and Antioxidant in Chocolate

A research conducted by Serafini et al. (2003) reported that *in vivo* antioxidant absorptions among participants who consumed dark chocolate with milk, or only milk chocolate, were significantly lower than those who ingested dark chocolate alone, when area under the curve (AUC) of their plasma (-)epicatechin were plotted against the time after 4 hours of ingestion. The same study showed that participants who ingested dark chocolate alone showed significantly higher *in vitro* plasma antioxidant capacity when measured with Ferric Reducing Antioxidant Power (FRAP) assay (Serafini et al., 2003). They stated that this condition happened due to the milk proteins that interacted with flavonoids in chocolate and formed secondary complexes, which could then affect the bio-accessibility of flavonoids and decline the antioxidant capacity as well. The statement of protein-polyphenols interaction was further supported by Gallo et al. (2013) in their research which reported as well that milk could adversely affect antioxidants in chocolate. It was found that casein, one of the protein fractions in milk, could significantly reduce antioxidant activity of polyphenols especially catechins and epicatechins, when determined through ABTS assay after the mixture of casein and polyphenols was incubated for 24 hours (Gallo et al., 2013). Other milk proteins such as β-Lactoglobulin (β-Lg), a whey protein was also found to interact with polyphenols through the covalent bindings (Gallo et al., 2003).

Besides, Tabernero et al. (2006) reported that antioxidant capacities of the cocoa products were significantly reduced by around 30% upon the addition of milk. Another research conducted by Neilson et al. (2010) also suggested that milk could modulate the bioavailability of chocolate flavonoids. Plasma concentrations of catechin and epicatechin in Sprague−Dawley rats administered with milk chocolate was significantly lower than other rats administered with dark chocolate, when determined through high performance liquid chromatography (HPLC) after 8 hours of administration (Neilson et al., 2010). It was most probably due to the lower cocoa solids content in milk chocolate compared to dark chocolate; while the findings shown by other studies indicating that it was the milk protein that interfere with polyphenols in chocolate thus affecting its antioxidant activity or capacity.

However, there was research found the addition of milk could significantly enhance the *in vitro* phenolic availability in chocolate and cocoa drinks (Otemuyiwa et al., 2017). On the other hand, a few other studies stated that milk actually did not affect chocolate flavanols absorption (Roura et al., 2007; Schramm et al., 2003). This statement was supported by another research conducted by Richelle et al. (2001) reporting that antioxidant activity of cocoa and other polyphenolic beverages were not affected by milk addition. Keogh et al. (2007) also indicated that addition of milk powder did not significantly affect the average polyphenols concentration, specifically catechin and epicatechin, in cocoa or cocoa products such as chocolate.

2.8 Determination of Polyphenols Content and Antioxidant Activity

Folin-Ciocalteu (F-C) assay is applied in this study as it is one of the most commonly used methods to determine the total polyphenols content in plants and cocoa products (Lamuela-Raventós, 2017). Folin-Ciocalteu reagent is made up of phosphotungstates and phosphomolybdates in acidic medium and the basic condition is adjusted by sodium carbonate solution (Schaich, K. M., 2016). The principle of this method is based on the oxidation-reduction reaction, in which the electrons will be transferred from the polyphenols to molybdenum if there is presence of antioxidants (Adriana & Cornelia, 2013). When the molybdenum in Folin-Ciocalteu reagent is reduced, it will form blue coloured complexes; the absorbance of the sample is also measured using a spectrophotometer in order to determine the exact total polyphenols content in each sample (Singleton & Rossi, 1965). This method had been widely used in most of other studies as well to access the antioxidants or polyphenols content in plants, foods, or other extracts (Otemuyiwa et al., 2017; Kemsawasd et al., 2016; Silva Medeiros et al., 2015; Vertuani et al., 2014).

For the determination of antioxidant activity, 2,2-diphenyl-1-picrylhydrazyl (DPPH) method was commonly used in many researches, in which the ability of the sample to scavenge free radicals or its antioxidant potential against the stable synthetic free radicals DPPH is measured (Otemuyiwa et al., 2017; Kemsawasd et al., 2016; Silva Medeiros et al., 2015; Yashin et al., 2013). DPPH method is suitable to be used to determine the antioxidant activity in both solid and liquid samples, and it works based on the principle of electron-transfer and colour formation as well; where in the presence of antioxidant, the violet DPPH solution will turn into light yellow colour due to the reduction of free radicals DPPH (Plank et al., 2012). Besides, Ferric reducing antioxidant power (FRAP) assay is another sensitive yet precise way to determine the antioxidant capacity in various samples, and had also been widely applied in most of the previous studies (Kemsawasd et al., 2016; Zujko & Witkowska, 2014; Vertuani et al., 2014; Yashin et al., 2013; Serafini et al., 2003). The principle of the FRAP method is also based on the oxidation-reduction reaction and colour formation as well. In the presence of antioxidant, the ferric tripyridyltriazine (Fe_3-TPTZ) in the FRAP reagent will be reduced to ferrous tripyridyltriazine (Fe_2-TPTZ) complexes and an intense blue colour can then be observed (Benzie & Strain, 1999).

CHAPTER 3

METHODOLOGY

3.1 Materials

Dark chocolates with 85% cocoa solids (Lindt Excellence Dark 85% Cocoa; Belgian Dark 85% Chocolate), drinking water, whole milk (Goodday; Dutch Lady), soy milk (Australia's Own organic unsweetened), and almond milk (137 Degrees original unsweetened) were purchased from the local markets in Selangor Darul Ehsan. Convenient sampling method was applied when purchasing the materials needed. Several materials bought from two brands were mixed together respectively in order to obtain more homogenized yet representative samples. After purchasing, the dark chocolates were stored in the refrigerator until used.

3.2 Chemicals and Reagents

Chemicals which had been used in this study were Folin-Ciocalteu (F-C) reagent, sodium carbonate (Na_2CO_3), gallic acid, α, α-Diphenyl-β-Picrylhydrazyl (DPPH), absolute ethanol, sodium acetate, glycial acetic acid, tripyridyltriazine (TPTZ), hydrochloric acid (HCl), ferric chloride ($FeCl_3$), and trolox.

3.3 Apparatus and Equipments

Apparatus and equipment required in this study were pipettes and micropipettes (Eppendorf, Germany), pipette tips, spatulas, beakers, measuring cylinders, reagent bottles, plastic cuvettes, UV-visible spectrophotometer (Secomam Prim Light, France), centrifuge tubes, screw cap test tubes, test tube rack, hot plate stirrer (Heidolph, Germany), semi-micro balance (AND Model GR-200, Japan), weighing balance (AND Model GF-300, Japan), vortex (IKA Model V 1 S000, Selangor, Malaysia), pH meter (Sartorius Model PB-10, Kuala Lumpur, Malaysia), centrifuge (Hettich Model Rotofix 32, Selangor, Malaysia), water bath incubator (Memmert Model WNB 14, Selangor, Malaysia), sonicator (Hwashin Technology Co. Model Powersonic 405, Seoul, Korea), chiller, and fume hood (ESCO Model EFH-5A1, Singapore).

3.4 Preparation of Samples

Fresh samples of chocolate milk were prepared during each time of the experiments. Two brands of 85% dark chocolate (Lindt Excellence Dark 85% Cocoa; Belgian Dark 85% Chocolate) were homogenized and melted using a hot plate stirrer (Heidolph, Germany). Fresh commercial whole milk, soy milk, and almond milk were taken out from the chiller and mixed with the melted chocolate respectively to obtain different samples of chocolate milk. Dark chocolate was heated on the hot plate stirrer until it melted, without a fixed temperature or duration. Dark chocolate (50 g) was either added with 100 ml of drinking water, whole milk, soy milk or almond milk in a ratio of 1:2 (Serafini et al., 2003; Roura et al. 2007). In addition, 150 ml of whole milk, soy milk, and almond milk were prepared as samples as well in order to determine their existing polyphenols content and antioxidant activity for references.

There were seven (7) samples in total in this study. Dark chocolate was mixed with drinking water in sample (i) and has been used as control for the experiments; it contained the same ratio of dark chocolate to fluid as in other samples of chocolate milk, hence the concentration of dark chocolate was similar in all chocolate milk samples. The seven experimental samples were listed as below.

i. Dark chocolate + Drinking water (DC) (1:2)

ii. Dark chocolate + Whole milk (DCWM) (1:2)

iii. Whole milk (WM)

iv. Dark chocolate + Soy milk (DCSM) (1:2)

v. Soy milk (SM)

vi. Dark chocolate + Almond Milk (DCAM) (1:2)

vii. Almond Milk (AM)

3.5 Determination of Total Polyphenols Content

3.5.1 Folin-Ciocalteu (F-C) Assay

Folin-Ciocalteu (F-C) method was applied in this study to determine the total polyphenols content in different milk and chocolate milk samples (Singleton & Rossi, 1965; Ramirez-Aristizabal et al., 2017). Firstly, 0.48 ml of 1% fresh liquid sample was added with 4.26 ml of 10% Folin-Ciocalteu (F-C) reagent and 4.26 ml of 7.5% sodium carbonate (Na_2CO_3) solution in a screw cap test tube. The role of Na_2CO_3 solution was to provide and to adjust basic conditions in the mixture for the reaction to occur. The mixture was kept in the dark at room temperature for 2 hours; and the absorbance was then measured at 758 nm by using a UV-visible spectrophotometer (Secomam Prim Light, France). The presence of antioxidants caused the molybdenum in F-C reagent to receive electrons from the sample and undergo reduction, thus forming blue colour complexes.

In this experiment, gallic acid with concentrations ranging from 0.03 to 0.21 mg/ ml were prepared and used as standard. The reactions were carried out by using the same procedures as chocolate milk samples. The absorbance of gallic acid was plotted against their concentrations to form a standard calibration curve, in order to be used to quantify the total polyphenols content in each sample. Final results were expressed in mg of gallic acid equivalent (GAE) per milliliter (ml) of fresh liquid sample (mg GAE/ ml sample) after being multiplied with dilution factor. All analyses were carried out in triplicate in order to minimize the standard deviations of the results.

3.6 Determination of Antioxidant Activity

3.6.1 2, 2-Diphenyl-1-Picrylhydrazyl (DPPH) Assay

DPPH radical scavenging assay was used to determine the antioxidant activity in different samples (Brand-Williams et al., 1995; Ghasemzadeh-Mohammadi et al., 2017; Gangwar et al., 2014). Firstly, 0.1 mM of ethanolic DPPH solution was prepared. The sample could react well with DPPH radicals in the ethanol solution. Next, 0.2 ml of freshly prepared chocolate milk was added into 6.0 ml of the DPPH solution prepared. The violet DPPH solution was reduced to light yellow in the presence of antioxidants. Control solution was prepared as well by substituting the sample with distilled water. The mixture was swirled thoroughly using a vortex (IKA Model V 1 S000, Selangor, Malaysia), and then was left at room temperature in the dark.

After 30 minutes, the sample was centrifuged at 4000 rpm for 10 minutes (Hettich Model Rotofix 32, Selangor, Malaysia). Then, 2 ml of supernatant was taken out and mixed with 4 ml of absolute ethanol. The absorbance was measured at 517 nm against ethanol using a UV-visible spectrophotometer (Secomam Prim Light, France). The DPPH radical scavenging ability (%) of each sample was calculated based on the following formula, whereby A_{cont} referred to absorbance of control solution and A_{sample} was absorbance of the sample. This experiment was carried out in triplicate as well in order to obtain more accurate results.

$$\mathrm{SC}\,\% = \frac{A_{cont} - A_{samp}}{A_{cont}} \times 100$$

<u>3.6.2 Ferric Reducing Antioxidant Power (FRAP) Assay</u>

Besides, ferric reducing antioxidant power (FRAP) assay was also applied in this study to study the antioxidant activity in different samples (Benzie & Strain, 1999; Xiao et al., 2020). Firstly, FRAP reagent was prepared by adding 100 ml of 300 mM acetic acid buffer with pH 3.6 measured using a pH meter (Sartorius Model PB-10, Kuala Lumpur, Malaysia), 10 ml of 10 mM tripyridyltriazine (TPTZ) solution in 40 mM of hydrochloric acid (HCl), and 10 ml of 20 mM of ferric chloride ($FeCl_3$) solution, in a 10:1:1 ratio. The prepared FRAP reagent was incubated at 37 ℃ in an incubator (Memmert Model WNB 14, Selangor, Malaysia), and has to be used up within one to two hours soon after preparation.

To carry out the experiment, 0.125 ml of 1% freshly prepared chocolate milk sample was added with 4.5 ml of FRAP reagent, and then was mixed and shaken thoroughly using a vortex (IKA Model V 1 S000, Selangor, Malaysia). The mixture was incubated in the dark at 37 ℃ for 15 minutes. An intense blue colour could be observed if there was presence of antioxidants in the sample, due to the reduction of ferric tripyridyltriazine (Fe_3-TPTZ) complex to ferrous tripyridyltriazine (Fe_2-TPTZ).

The absorbance of mixture was measured at a wavelength of 593 nm using a UV-Visible spectrophotometer (Secomam Prim Light, France). Trolox was used as standard in this experiment; it was sonicated (Hwashin Technology Co. Model Powersonic 405, Seoul, Korea) and was prepared in different concentrations from 31.25 to 4000 μmol/ ml. A standard curve was plotted based on the absorbance and concentrations of trolox solutions to estimate the antioxidant capacity of each sample.

The final results were expressed in μmol of Trolox equivalent (TE) per liter (L) of fresh liquid sample (μmol TE/ L) after considering the dilution factor. The analyses were carried out in triplicate as well in order to obtain results with higher accuracy and to minimize the standard deviations of the results.

3.7 Data Analysis

IBM SPSS Statistics 25 was used to perform all statistical analyses. Results obtained were expressed in mean ± standard deviation. One-way analysis of variance (ANOVA) with Tukey's post-hoc test was used to compare the mean values in total polyphenols content and antioxidant capacity among different samples. Besides, Pearson's correlation test was used to determine the correlations between the total polyphenols content in different chocolate milk samples and their antioxidant activities. Results were statistically significant at $p < 0.05$.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Total Polyphenols Content

4.1.1 Folin-Ciocalteu (F-C) Assay

The total polyphenols content of different chocolate milk and milk samples had been estimated by using Folin-Ciocalteu (F-C) method, and results were shown in Table 4.1 in descending order. The samples were represented by different abbreviations, including DC (dark chocolate with drinking water), DCWM (dark chocolate whole milk), WM (whole milk), DCSM (dark chocolate soy milk), SM (soy milk), DCAM (dark chocolate almond milk), and AM (almond milk). Gallic acid standard was used to plot the standard curve with linear equation y = 2.2997x + 0.0120 (R^2 = 0.9970). The equation was used to determine the total polyphenols in unit of milligram of gallic acid equivalent per milliliter of each sample (mg GAE/ ml of sample).

Table 4.1 Total polyphenols content of different samples

Samples	**Total Polyphenols Content (mg GAE/ ml sample)**
DCWM	15.68 ± 0.17^{a}
DCAM	13.16 ± 0.09^{b}
DCSM	11.48 ± 0.06^{c}
DC	8.06 ± 0.11^{d}
AM	2.12 ± 0.11^{e}
WM	0.93 ± 0.13^{f}
SM	0.70 ± 0.15^{f}

Results in the table are mean values of triplicates ± standard deviation. Means with different superscript letters were significantly different (*p* < 0.05, ANOVA, Tukey's post hoc). DCWM = dark chocolate with whole milk; DCAM = dark chocolate with almond milk; DCSM = dark chocolate with soy milk; DC = dark chocolate; AM = almond milk; WM = whole milk; SM = soy milk.

The homogenized dark chocolate from two brands which were involved in this study contains 85% of cocoa solids respectively. The total polyphenols content estimated was 8.06 ± 0.11 mg GAE/ ml of dark chocolate in drinking water. Dark chocolate was mixed with drinking water (1:2) in this study to imitate the ratio of dark chocolate to fluid as in other chocolate milk samples and to act as a control for the experiment. The mean value obtained was approximately two-fold compared to a previous study with a reported mean of 3.64 ± 0.02 mg GAE/ g of dark chocolate containing 50% of cocoa solids (Kemsawasd et al., 2016). This was due to a huge difference in the cocoa solids percentage (up to 35%) between dark chocolates used in both studies. This has further proven that the higher the percentage of cocoa solids, the higher the total polyphenols content in the chocolate.

Furthermore, most of the previous studies had defatted chocolates before conducting the experiments while this study did not; this was to determine the antioxidants in chocolate and chocolate milk without eliminating any components which could potentially affect the antioxidants content and activity, hence a more accurate finding could be disseminated to the consumers or public in terms of the amount of antioxidants they could obtain from a particular beverage. Studies had showed that lipid or fat could enhance antioxidants (Otemuyiwa et al., 2017; Ackar et al., 2013; Lesser et al., 2004); hence the total polyphenols content of dark chocolate in this study was higher due to the reason of not being defatted. In fact, this statement was supported by a research by Ibrić and Ćavar (2014) which compared the phenolic content in both defatted and non-defatted samples from the same chocolate with 43% cacao, and the results obtained were 0.070 ± 0.005 mg GAE/ g and 0.135 ± 0.006 mg GAE/ g of chocolate respectively. Hence, the influence of lipids on the antioxidants has been certified, in which the defatted chocolate contains only approximately half of the polyphenols compared to its non-defatted counterpart.

However, the polyphenols content of dark chocolates in this study was on the other hand lower compared to a few other studies, such as 16.17 mg GAE/ g of dark chocolate with at least 46% cacao (Zujko & Witkowska, 2014) and 8.4 mg GAE/ g of bakers' chocolate with percentage of cocoa solids not mentioned (Waterhouse et al., 1996, as cited in Belšcak et al., 2009). This could be due to the dark chocolate (DC) sample in present study had been diluted with drinking water. Also, other studies used chocolate extract instead of using fresh sample of chocolate hence the values obtained were higher. Besides, the process of fermentation, roasting, storage, ripeness level, post-harvest conditions, and geographical origins of the cocoa beans used to manufacture the chocolate could also influence the antioxidants content (Wollgast & Anklam, 2000).

Based on Table 4.1, the total polyphenols content in dark chocolate (8.06 $\pm$ 0.11 mg GAE/ ml) was significantly enhanced after adding soy milk (11.48 $\pm$ 0.06 mg GAE/ ml) (p < 0.001), almond milk (13.16 $\pm$ 0.09 mg GAE/ ml) (p < 0.001), and whole milk (15.68 $\pm$ 0.17 mg GAE/ ml) (p < 0.001), in ascending order. This was in line with a study conducted by Otemuyiwa et al. (2017) which reported that the *in-vitro* phenolic availability of chocolate was significantly enhanced by the addition of milk when being conducted through the Folin-Ciocalteu method. However, the results were contradicted with most of other studies which demonstrated that milk could either reduce (Gallo et al., 2013; Neilson et al., 2010) or possess no significant effect (Keogh et al., 2007; Roura et al., 2007; Schramm et al, 2003) on the antioxidants content in chocolate. No plant-based chocolate milk had been studied previously. The types of milk involved were not mentioned clearly as well and hence cow's milk was assumed in the previous studies.

Besides, three types of milk involved in this study had been tested blankly as well to be used as references. Results showed that almond milk has the highest content of polyphenols, which is 2.12 ± 0.11 mg GAE/ ml of fresh commercially available almond milk, as compared to whole milk (0.93 ± 0.13 mg GAE/ ml) and soy milk (0.70 ± 0.15 mg GAE/ ml). This was due to the rich content of antioxidant vitamins A and E in almond milk (Yigit, A. A., 2019). In this study, soy milk was found to contain the least antioxidants although soy products are generally rich in isoflavones, a kind of polyphenols as well (Paul et al., 2019). According to one-way ANOVA, the total polyphenols content in all samples were significantly different from each other ($p < 0.05$), except whole milk and soy milk ($p = 0.534$).

After incorporating dark chocolate in milk, DCWM (dark chocolate whole milk) has the highest polyphenols content (15.68 ± 0.17 mg GAE/ ml) when compared to other types of chocolate milk, despite whole milk containing less polyphenols than almond milk. This might be due to the higher lipid or fat content in whole milk that exists naturally in animal-based milk. Lipid could enhance antioxidants as discussed earlier, as lipid could store fat-soluble antioxidants especially vitamin A and vitamin E, which could then directly increasing the antioxidants in DCWM after complementing with antioxidants in dark chocolate (Ajmal et al., 2019; Otemuyiwa et al., 2017; Rizvi et al., 2014; Ackar et al., 2013; Lesser et al., 2004). Moreover, whole milk has a higher content of protein than soy milk and almond milk. Protein was found to be reactive towards Folin-Ciocalteu reagent as this reagent was initially developed to determine tryptophan, a non-phenolic aromatic amino acid; hence the presence of protein in the sample might enhance the reactions and reduce the F-C reagent as well (Everette et al., 2010; Folin & Ciocalteu, 1927, as cited in Schendel, R. R., 2019). Overall, the results demonstrated that milk could enhance the total polyphenols content in dark chocolate.

4.2 Antioxidant Activity

4.2.1 DPPH Radical Scavenging Activity

DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay has been used to determine the antioxidant activity of different samples in this study. The synthetic radical DPPH was reduced from purple to yellow in the presence of antioxidants. Results were expressed in percentage in descending order as shown in Table 4.2. A higher percentage indicates a stronger ability of the sample to scavenge free radicals.

Table 4.2 DPPH radical scavenging activity of different samples

Samples	DPPH radical scavenging activity *M* (%) ± *SD*
DCAM	94.9 ± 1.13 [a]
AM	92.8 ± 1.54 [a]

DCWM	90.0 ± 0.75 [a]
DCSM	82.6 ± 1.54 [b]
DC	64.0 ± 2.39 [c]
WM	25.8 ± 1.02 [d]
SM	24.7 ± 2.09 [d]

Results in the table are mean values of triplicates in percentage ± standard deviation. Means with different superscript letters were significantly different ($p < 0.05$, ANOVA, Tukey's post hoc). DCAM = dark chocolate with almond milk; AM = almond milk; DCWM = dark chocolate with whole milk; DCSM = dark chocolate with soy milk; DC = dark chocolate; WM = whole milk; SM = soy milk.

Based on Table 4.2, the DPPH scavenging activity of dark chocolate was 64.0 ± 2.39%. When being introduced as chocolate milk, the scavenging ability percentage was statistically increased to 90.0 ± 0.75% ($p < 0.001$), 82.6 ± 1.54% ($p < 0.001$), and 94.9 ± 1.13% ($p < 0.001$) upon the addition of whole milk, soy milk, and almond milk respectively. DCAM (dark chocolate almond milk) has the highest free radical scavenging ability, but it has no significant difference with that of DCWM (dark chocolate whole milk) ($p = 0.09$) and AM (almond milk) ($p = 0.851$). If being compared among milk, the fresh commercially available almond milk used in this study has the highest DPPH scavenging percentage of 92.8 ± 1.54%, followed by whole milk (25.8 ± 1.02%) and soy milk (24.7 ± 2.09%) in descending order. This sequence of scavenging ability was in line with that among DCAM, DCWM, and

DCSM. This showed that the existing antioxidant activity of milk alone might possess a positive impact on the antioxidant capacity in their chocolate milk counterparts.

However, several previous studies had reported a significant decline in the antioxidant activity in chocolate after being incorporated with milk (Gallo et al., 2013; Neilson et al., 2010; Tabernero et al., 2006; Serafini et al., 2003). This was due to the formation of secondary complexes from the molecular interaction between chocolate flavonoids and milk protein; more specifically, proteomic analysis carried out in a previous study had reported that catechin and epicatechin from dark chocolate interacted and formed covalent bindings with whey protein β-lactoglobulin and non-covalent bindings with casein protein in milk, thus reducing or inhibiting the *in-vitro* bio-accessibility and absorption of polyphenols (Gallo et al., 2003; Serafini et al., 2003). Besides, protein in milk could also increase the stomach pH, then increasing ionization which could also negatively affect the polyphenols absorption in an indirect way (Chen et al., 2001, as cited in Otemuyiwa et al., 2017).

Otemuyiwa et al. (2017) also highlighted the strong affinity of polyphenols towards protein in their study; however, their results showed increased antioxidants availability as the volume of milk added into chocolate and cocoa drinks increased. On the other hand, a few other studies reported a neutral effect of adding milk on antioxidant activity in dark chocolate (Keogh et al., 2007; Roura et al. 2007; Schramm et al, 2003; Richelle et al., 2001). In this study, milk did not impair the antioxidants in dark chocolate, but enhanced its antioxidant activity. This might be due to the lacking of whey protein β-Lactoglobulin and casein protein in soy milk and almond milk, thus resulting in less milk protein-polyphenols interactions. On the other hand, higher lipid content in whole milk has helped to secure its antioxidant activity since lipids could store fat-soluble antioxidants (Ajmal et al., 2019; Rizvi et al., 2014).

4.2.2 Ferric Reducing Antioxidant Power (FRAP)

Ferric reducing antioxidant power (FRAP) assay had been applied in this study in order to determine the antioxidant activities of different samples, and the results were shown in Table 4.3 in descending order. Trolox was used as standard to plot a calibration curve with linear equation y = 0.0005x + 0.0883 (R^2 = 0.9734). The equation was then used to estimate the FRAP value in the unit of micro molar of trolox equivalent per liter of each sample (μmol TE/ L sample).

Table 4.3 FRAP values of different samples

Samples	FRAP (μmol TE/ L sample)
DCAM	325.67 ± 14.91 ^{a}
DCWM	133.67 ± 1.55 ^{b}
DCSM	98.61 ± 2.23 ^{c}
DC	95.94 ± 2.94 ^{c}

AM	24.81 ± 0.19 [d]
WM	22.41 ± 0.25 [d]
SM	20.34 ± 0.49 [d]

Results in the table are mean values of triplicates ± standard deviation. Means with different superscript letters were significantly different ($p < 0.05$, ANOVA, Tukey's post hoc). DCAM = dark chocolate with almond milk; DCWM = dark chocolate with whole milk; DCSM = dark chocolate with soy milk; DC = dark chocolate; AM = almond milk; WM = whole milk; SM = soy milk.

Based on Table 4.3, the estimated FRAP value of dark chocolate was 95.94 ± 2.94 µmol TE/ L, whereas the mean value obtained in another study was 104 ± 4 µmol TE/ g of 50% dark chocolate (Kemsawasd et al., 2016). According to Table 4.3, the antioxidant activity of dark chocolate (DC) was significantly enhanced after being added with whole milk and almond milk, with FRAP values of 133.67 ± 1.55 µmol TE/ L of DCWM ($p < 0.001$) and 325.67 ± 14.91 µmol TE/ L of DCAM ($p < 0.001$) respectively. There was an increase in the FRAP value of dark chocolate after being added with soy milk as well, which was 98.61 ± 2.23 µmol TE/ L of DCSM, but the difference was not statistically significant with dark chocolate (95.94 ± 2.94 µmol TE/ L of DC) alone ($p = 0.999$).

Similar to the results obtained from DPPH scavenging assay, almond milk has the highest antioxidant activity among three types of milk used in this study, with FRAP value of 24.81 ± 0.19 µmol TE/ L of AM, followed by whole milk and soy milk in descending order, with respective FRAP values of 22.41 ± 0.25 µmol TE/ L of WM and 20.34 ± 0.49 µmol TE/ L of SM. This trend was consistent with the FRAP values of DCAM, DCWM, and DCSM as well; hence further proven that the existing antioxidant activity in milk could complement that of dark chocolate to boost the antioxidant activity when they appear as chocolate milk. However, there was no significant difference between the FRAP values of WM, SM, and AM ($p > 0.05$).

Overall, the results obtained from the FRAP assay were consistent with that from the DPPH assay, where both experiments similarly showed the highest antioxidant activity in DCAM, followed by DCWM, DCSM, and DC among the chocolate milk samples. Both studies also demonstrated a similar finding with a previous study conducted by Otemuyiwa et al. (2017) that milk could enhance antioxidant activity in dark chocolate; whereby the higher the antioxidant activity of a milk, the higher the antioxidant capacity or activity of its chocolate milk counterpart.

4.3 Correlation between Total Polyphenols Content and Antioxidant Activity

Pearson's correlation test had been carried out to determine the relationship between total polyphenols content and antioxidant activity of all samples in this experiment. The results were shown in Table 4.4.

According to Pearson's correlation test, there was a strong and positive correlation between total polyphenols content and DPPH radical scavenging activity

(r = 0.714, $p < 0.01$), while there was also a strong and positive correlation between total polyphenols content and FRAP values (r = 0.741, $p < 0.01$); both results were statistically significant. This indicates that the antioxidant activity increases as the total polyphenols content increases. Previous studies had also reported a positive correlation between the total polyphenols content and antioxidant capacity or free radicals scavenging ability of the dark chocolate and chocolate milk respectively (Nurhayati et al., 2019; Urbańska et al., 2020). In brief, the higher the total polyphenols content, the higher the DPPH free radical scavenging abilities and FRAP values of the samples.

Table 4.4 Pearson's correlation between total polyphenols content and antioxidant activity of the samples

	Pearson's Correlation (*r*)
Assays	**Total Polyphenols Content (F-C)**
DPPH Radical Scavenging Activity (DPPH)	0.714**
Ferric Reducing Antioxidant Power (FRAP)	0.741**

**Correlation was significant at $p < 0.01$.

CHAPTER 5

CONCLUSION, LIMITATIONS AND FUTURE RECOMMENDATIONS

5.1 Conclusion

This study was conducted to investigate the effects of adding animal-based milk and plant-based milk on the total polyphenols content and antioxidant activity in dark chocolate. Three assays with seven samples respectively had been carried out. Whole milk represented animal-based milk while soy milk and almond milk represented plant-based milk. The total polyphenols content of different milk and chocolate milk were studied through Folin-Ciocalteu (F-C) method; while their antioxidant activities were determined through 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay and ferric reducing antioxidant power (FRAP) assay.

Based on the findings, DC (dark chocolate with drinking water) contains 8.06 ±

0.11 mg GAE/ ml DC of polyphenols, 64.0 ± 2.39% DPPH radical scavenging ability, and with FRAP value of 95.94 ± 2.94 µmol TE/ L of DC. The values obtained were higher than several studies since dark chocolate used in this study contained a higher percentage of cocoa solids (85%) and were not defatted prior to the experiment. However, the values were on the other hand lower than a few other studies due to the dilution of dark chocolate with drinking water, as well as the usage of fresh samples in this study instead of using sample extracts as in most of the previous studies.

Regarding chocolate milk, WM (whole milk) significantly boosted the total polyphenols content of DCWM (dark chocolate whole milk) to 15.68 ± 0.17 mg GAE/ ml DCWM ($p < 0.001$); DPPH scavenging ability to 90.0 ± 0.75% ($p < 0.001$), and FRAP value to 133.67 ± 1.55 µmol TE/ L of DCWM ($p < 0.001$). A combination of DC with AM (almond milk) also significantly enhanced the antioxidants in DCAM (dark chocolate almond milk), with polyphenols content of 13.16 ± 0.09 mg GAE/ ml DCAM ($p < 0.001$); 94.9 ± 1.13% ($p < 0.001$) DPPH scavenging ability, and FRAP value 325.67 ± 14.91 µmol TE/ L DCAM ($p < 0.001$). SM (soy milk) increased the antioxidants in DCSM (dark chocolate soy milk) as well, and had showed significant results in total polyphenols content (11.48 ± 0.06 mg GAE/ ml DCSM) ($p < 0.001$) and DPPH radical scavenging activity (82.6 ± 1.54%) ($p < 0.001$); however, the addition of SM to DC did not exhibit a significant result in the FRAP value of DCSM (98.61 ± 2.23 µmol TE/ L of DCSM) ($p = 0.999$). Besides, among milk, almond milk has the most antioxidant content and capacity, followed by whole milk and soy milk.

In addition, total polyphenol contents of chocolate milk samples were found to have statistically strong and positive correlations with their DPPH radical scavenging activities ($r = 0.714$, $p < 0.01$) and FRAP values ($r = 0.741$, $p < 0.01$). This indicates that polyphenols could contribute to a higher antioxidant capacity of chocolate milk.

In conclusion, whole milk, soy milk and almond milk significantly enhance antioxidants in dark chocolate; hence the public are recommended to consume chocolate milk than consuming dark chocolate or milk alone, as this is a more effective way to obtain greater health benefits from this functional food. In fact, consumers could opt for both animal-based milk and plant-based milk based on their personal needs and preferences since both types of milk possess positive results on antioxidants in chocolate with 85% cocoa solids. It is worth knowing that almond milk is the most recommended milk to consume as it contains the highest antioxidant content and activity compared to whole milk and soy milk. Besides, the existing antioxidants in milk could complement that of dark chocolate, thus boosting the antioxidant content and capacity in their chocolate milk counterparts. Also, the higher the total polyphenols content, the higher the antioxidant activity of the chocolate milk.

5.2 Limitations and Future Recommendations

This study could provide baseline data or references for future research concerning the effects of adding milk, especially soy milk and almond milk on the antioxidants in dark chocolate, since no previous study had involved plant-based milk regarding this topic.

However, preparing fresh samples during each time of experiments might lead to less accurate results as small deviations in the weight of dark chocolate used might occur despite the given ratio. Also, mankind errors, surrounding temperature, and duration to heat and melt the chocolate might affect the results as well. Samples were not prepared at a monitored and constant temperature in this study; while chocolate milk melted for a longer duration on the hot plate stirrer or melted at a higher temperature might exhibit less polyphenols as heat could destroy the antioxidants.

Also, milk was not deproteinized in this study in order not to eliminate any component which could potentially influence the antioxidants in dark chocolate milk. Protein might have affected the results of total polyphenols content in the samples since protein was reactive towards Folin-Ciocalteu reagent and could potentially reduce it. Besides, protein in the samples formed precipitates after reacting with the ethanolic DPPH solution. It was because protein could precipitate in alcohol-based solvents such as ethanol, which had been used to dilute and prepared the DPPH solution in this study. Hence, modification of the method has been applied. The reacting mixtures were centrifuged; the supernatants were then taken out and used to measure the absorbance.

Future studies need to consider carefully whether to use fresh samples or sample extracts based on their study objectives, since both methods have their respective advantages and disadvantages. Besides, future researchers are encouraged to conduct related studies *in-vivo* in order to obtain results with higher reliability and higher accuracy. Last but not least, since convenient sampling has been applied in this study, future researchers are recommended to use probability sampling methods to obtain more valid yet representative samples of chocolate milk.

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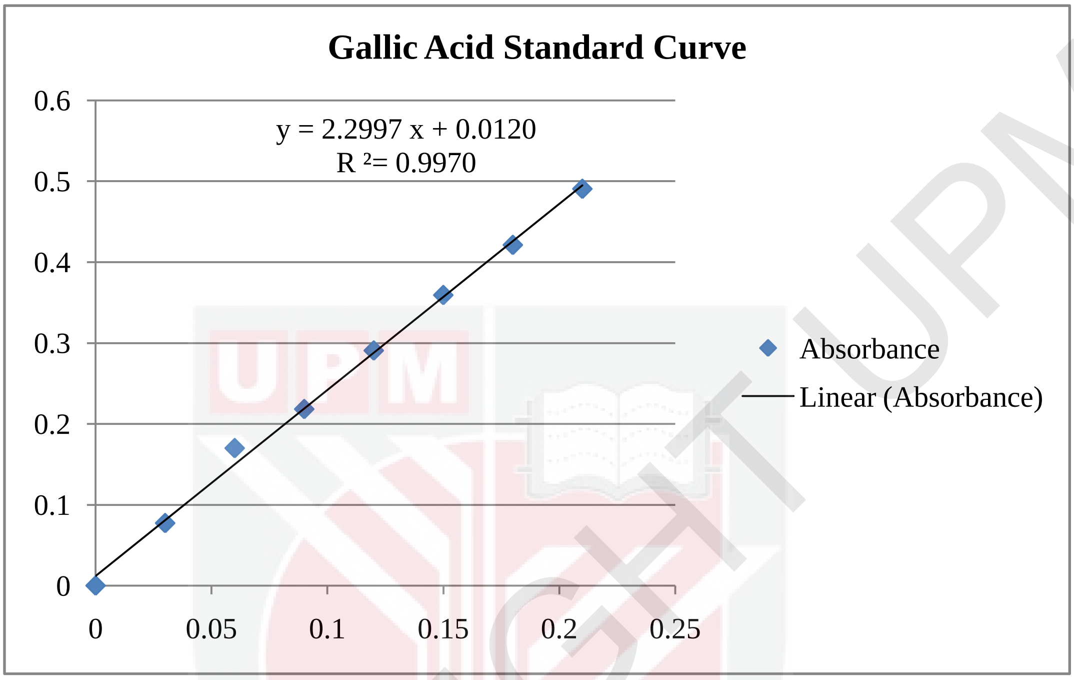
APPENDICES

Appendix A. Gallic acid standard curve for Folin-Ciocalteu assay



Appendix B. Trolox standard curve for FRAP assay