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Antioxidant Capacity, Alpha Amylase Inhibition, and Calorie Value of Dark Chocolate Substituted with Honey Powder

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ABSTRACT

The antioxidant and antidiabetic properties of honey have led to studies exploring using honey powder as a sugar substitute in chocolate innovation for its health benefits. In this study, sugar was substituted with 70% honey powder to evaluate the effect on the antioxidant, alpha-amylase inhibition, and calorie value of dark chocolate. The honey powder was produced by adding 70% of either dextrose (honey/dextrose, H/D), maltodextrin (honey/maltodextrin, H/M), or sucrose (honey/sucrose, H/S) and vacuum dried at 40°C for 6 hr. The substitution of honey powder into chocolate at a 70% level was based on the preliminary study, which showed the most acceptable particle size value. The addition of honey powder into dark chocolate showed a significant increase ($p < 0.05$) in antioxidant capacity, assessed by 2,2-diphenyl-1-picrylhydrazyl and ferric reducing antioxidant power. Chocolate containing H/M showed the highest antioxidant capacity for both assays, followed by chocolate with H/D, H/S, and the control. Alpha amylase inhibition was also significantly higher ($p < 0.05$) for H/M chocolate relative to other samples. The calorie values of dark chocolate were not affected by the honey powder substitution, which remained at 600 kcal/100 g. Therefore, chocolate containing H/M showed the best properties due to its highest antioxidant capacities and alpha-amylase inhibition effect. Hence, it can be recommended for further application in chocolate.

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INTRODUCTION

Chocolates comprise approximately 70% sugar and cocoa in fine solid particles suspended in cocoa butter, which serves as the continuous fat phase (Afoakwa et

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al., 2007). Chocolates can be classified into various types, including dark, milk, and white chocolate, which differ in their composition of cocoa solids, cocoa butter, sugar, and milk fat content. Dark chocolate was reported to have more benefits compared to other chocolate varieties due to the high percentage of cocoa and higher phenolic antioxidant compounds (Montagna et al., 2019). Cocoa, from a cocoa tree fruit (*Theobroma cacao*), has been identified as one of the richest naturally occurring sources of antioxidants, higher than red wine, blueberries, and green tea (Shafi et al., 2018). Cocoa contains relatively high amounts of flavonoids, including catechin, epicatechin, and procyanidin (Övet, 2015). However, up to 50% of the sucrose in chocolate evokes health concerns, especially on the risk of diabetes and other cardiovascular diseases. Therefore, the impact of sucrose replacement was investigated using sugar alcohols (maltitol, isomalt, xylitol, lactitol, sorbitol, and mannitol), intense sweetener (acesulfame K, aspartame, sucralose, and steviosides), bulking agent (inulin, polydextrose, and maltodextrin) and natural sweetener (stevia, lucuma, yacon, dried carrot, acacia flowers, palm sugar, and coconut sugar) in several previous studies (Aguilar-Villa et al., 2020; Aidoo et al., 2013; A. M. E. Ali et al., 2021; Cikrikci et al., 2017; Furlán et al., 2017; Kusumadevi et al., 2021; Palazzo et al., 2011).

Honey has been shown to have a variety of beneficial impacts on human health, including antioxidant, antidiabetic, anti-cancer, antibacterial, anti-inflammatory, treatment of cardiovascular disorders, and wound-healing properties (Ahmed & Othman, 2013; Meo et al., 2017). In Malaysia, Tualang honey (*Apis dorsata*) is the most favourable as it has a darker brown colour, which correlates to its high phenolic content (Ahmed & Othman, 2013). Previous studies have reported that Tualang honey shows superior antioxidant and free radical scavenging activities compared to other local honey varieties like Gelam, Acacia, Pineapple, Borneo, and Kelulut, which is likely due to its elevated levels of phenolics and flavonoids (Khalil et al., 2011; Kishore et al., 2011; Mohamed et al., 2010; Moniruzzaman et al., 2013). Gallic, benzoic, syringic, p-coumaric, trans-cinnamic, and caffeic acids are phenolic compounds found in Tualang honey, along with catechin, naringenin, kaempferol, luteolin, and apigenin as flavonoids (Khalil et al., 2011). These compounds may act as antidiabetic agents and contribute to alpha-amylase inhibition activity in Tualang honey (H. Ali et al., 2020). Additionally, Tualang honey has an intermediate glycaemic index, according to a study on healthy individuals, suggesting a positive role in regulating blood glucose levels (Ahmed & Othman, 2013; Robert & Al-Safi, 2009). Furthermore, Tualang honey has the potential to reduce cardiovascular risk factors (Ahmed & Othman, 2013; Yaghoobi et al., 2008).

Honey powder is gaining popularity in the food industry due to its health and functional properties. It has been used as a sucrose replacer in bread (Ram, 2011;

Sathivel et al., 2013; Tong et al., 2010), cookies (Kılınç & Demir, 2017), and isotonic drinks (Tomczyk et al., 2020). Studies have shown that using honey powder in bread reduces the staling rate and increases the food's phenolic content (Kılınç & Demir, 2017; Sathivel et al., 2013). Replacing some sucrose in chocolate with honey powder can increase the polyphenol content of the chocolate due to the combination of cocoa and honey powder polyphenols. This synergistic effect can enhance the health properties of the chocolate, particularly its antioxidant and antidiabetic properties. This study compares the substitution effect of vacuum-dried honey powder on the antioxidant properties, alpha-amylase inhibition, and caloric value of chocolate using three different sugar carriers (dextrose, maltodextrin, and sucrose).

MATERIALS AND METHODS

Materials

Cocoa mass (54% fat content) and cocoa butter were purchased from Barry Callebaut (Banbury, United Kingdom), and soy lecithin from Eugene Chemical Sdn. Bhd. (Malaysia), and castor sugar (Kijang, Malaysia) from a local supermarket in Malaysia. Tualang honey was obtained from Hutan Hujan Tasik Kenyir, Kuala Terengganu, supplied by Koperasi Pemungut Madu Lebah Terengganu Berhad (Malaysia). The DE 10 maltodextrin was procured from Sim Supplies Sdn. Bhd., Malaysia and the dextrose was obtained from Eugene Chemical Sdn. Bhd., Malaysia. The solvents and reagents used in this study included hexane, acetone, acetic acid, sodium hydrogen phosphate, methanol, TPTZ (2,4,6-tripyridyl-s-triazine), ferric chloride hexahydrate, hydrochloric acid, and sodium chloride obtained from the EMSURE®, Merck (Germany), diethyl ether from Fisher Scientific (United Kingdom), dinitrosalicyclic acid (DNS) colour reagent from Santa Cruz Biotechnology Inc. (USA), trolox, 2,2-diphenyl-1-picrylhydrazyl (DPPH), amberlite XAD-2-resin with a pore size of 9 nm, and particle size of 0.2-0.8 µm, sodium acetate, and α-amylase were procured from Sigma Aldrich (USA).

Honey Powder Preparation

Honey powder containing either dextrose (H/D), maltodextrin (H/M), and sucrose (H/S) were used as a sucrose substitute. The proportions, time, and drying temperature were determined based on preliminary studies. Honey and carrier (dextrose/maltodextrin/sucrose) at 30:70 was homogenised for 15 min at 4,000 rpm with a homogeniser (Ultra-turrax® T25 Digital Disperser, IKA, China). Subsequently, 10 g of every sample was weighed and evenly spread onto a Petri dish before drying in a vacuum oven (Binder, USA) at 40°C under 25 in Hg for 6 hr. After being dried, the samples were pulverised into a powder using a mortar and pestle and stored in a desiccator until they were analysed.

Dark Chocolate Preparation

Dark chocolate samples with a total fat content of 37.7% were prepared according to the formulation (Table 1). The formulation

categorises dark chocolate as "semisweet" based on its cocoa component content, which falls between more than 38% and less than 72%, in accordance with the method outlined by Belščak-Cvitanović et al. (2012). In the formulation, sucrose was substituted with honey powder at a 70% level (w/w). The dark chocolate was prepared using a tabletop wet grinder (Lakshmi Platinum Plus, India) as the mixer. The drum and stones were pre-heated for 20 min with a hot air gun (Stanley Stel670, China) to ensure smooth grinding. This friction warmed the stones, breaking the cocoa mass faster (Masonis et al., 2017). Cocoa butter and cocoa mass were added to the wet grinder and mixed for 10 min before incorporating sugar, honey powder, and lecithin. A hot air gun was used throughout the mixing process to maintain a constant temperature of around 45°C for 30 min. Afterwards, the hot air gun was removed as the wet grinder generated sufficient heat to maintain a 42–52°C. The temperature was monitored using a food digital thermometer (Dr Yonimed®, India). The mixing period for each formulation was set to 12 hr. Extending the mixing period to 14 and 16 hr did not yield further particle size reduction for all any samples of dark chocolate, as observed using a grindometer. The molten dark chocolate was subsequently stored in an airtight container and placed in a freezer at -20°C for further analysis.

Table 1
Dark chocolate formulations

Ingredients	Percentage (g/100 g)	
	Control	Treated
Sugar	36.5	10.9
Honey powder	0	25.6
Cocoa mass	55	
Cocoa butter	8	
Lecithin	0.5	

Chocolate Defatting

The samples were defatted to remove lipids before analysing their antioxidant capacity and alpha-amylase inhibition. The chocolate sample weighing 20 g was subjected to three rounds of extraction using 100 ml of hexane each time. The resulting defatted cocoa solids were left to air-dry at room temperature for 24 hr to remove any residual organic solvent (Todorovic et al., 2015).

Sample Extraction for Antioxidant Capacity

The method for extracting antioxidants was followed by the procedure outlined by Todorovic et al. (2015). Firstly, approximately 1 g of defatted dark chocolate was weighed and placed in a screw-cap tube. Polyphenols were extracted by adding 5 ml of a solvent extraction solution, which consisted of a mixture of acetone, distilled water, and acetic acid in a ratio of 70:29.8:0.2 (v/v/v). The extraction was enhanced by vortexing the mixture for 1 min and then sonicated for 30 min in an ultrasonic bath (Power Sonic 410, Hwashin Technology, Korea). Afterwards, the mixture was centrifuged for 10 min at 2,516 × *g*. Afterwards, the supernatant was filtered through the Whatman No.1 filter paper, and the extraction step was repeated twice.

Determination of DPPH Radical Scavenging Assay

The assessment of antioxidant activity was carried out using DPPH assays, which gauged the electron donation ability of a substance towards a hydrogen atom. This ability was determined by monitoring the reduction reaction of DPPH, which caused a change in its colour from purple to yellow hue. The method used for the DPPH assays followed the protocol outlined by Brand-Williams et al. (1995) with minor adjustments. Initially, a fresh stock solution of 0.06 mM DPPH was prepared by dissolving 1.2 mg of DPPH powder in 50 ml of 70% methanol. The flask containing the DPPH stock solution was wrapped with aluminium foil and then stirred on a magnetic stirrer (HEB Heating Magnetic Stirrer, China) until the DPPH was fully dissolved in the methanol. The solution was then stored in a bottle covered with aluminium foil at room temperature.

Aliquoted samples or standard (200 µl) were mixed with 2.8 ml of the DPPH radical solution in a screw-cap test tube. After combining the samples or standards with the DPPH radical solution, the mixture was vigorously shaken and then incubated in a dark place by wrapping the test tube with aluminium foil for 1 hr at room temperature. The capacity of the compounds to scavenge free radicals was assessed by measuring the absorbance at 525 nm. The antioxidant activity of the sample was determined by constructing a calibration curve of the standard within the range of 0.2–0.7 mmol Trolox/ml. The outcomes were expressed as µM Trolox Equivalents (TE) per gram of the samples. The scavenging activity percentage was then calculated using Equation 1:

$$\% \text{ Inhibition} = \frac{A_{525\ control} - A_{525sample/standard}}{A_{525control}} \times 100\% \quad [1]$$

where,

$A_{525\ control}$ = Absorbance of control (a methanol solution)

$A_{525sample/standard}$ = Absorbance of the sample's extract or standard

Determination of Ferric Reducing Antioxidant Power Assay (FRAP)

This method was conducted based on the protocol established by Benzie and Strain (1996), with some minor alterations. The FRAP reagent was prepared freshly by mixing acetate buffer, 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ), and ferric chloride hexahydrate ($FeCl_3.6H_2O$) in a ratio of 10:1:1 (v/v/v). Approximately 25 ml of 300 mM acetate buffer solution was mixed with 2.5 ml of TPTZ solution (prepared by dissolving in 40 mM hydrochloric acid) before being added to 2.5 ml of 20 mM of ferric chloride hexahydrate. Then, the solution was warmed at 37°C using a water bath for 30 min. About 100 µl of extracted sample were mixed with 3,000 µl of FRAP reagent in a screw cap test tube and shaken vigorously. The absorbance of the reaction mixture was measured using a spectrophotometer (Varian Cary 50 Probe, Malaysia) at 593 nm after 40 min in dark conditions. The calibration curve determined the antioxidant activity within

the 0.1–0.8 mmol Trolox/ml range. The findings were then reported in µM Trolox Equivalents (TE)/g of the samples.

Sample Extraction for Alpha-Amylase Inhibition

Approximately 25 g of dark chocolate samples were dissolved in 250 ml of distilled water, and the pH of the resulting solution was adjusted to pH 2.0 by adding concentrated HCl. The sample solution was then passed through an Amberlite XAD-2 resin column. An additional 250 ml of water acidified with HCl to pH 2 was added to flush the sample solution into the column. The column was then washed with 300 ml of distilled water to eliminate sugars or other polar substances in the honey. A total of 250 ml of methanol was used to elute the phenolic chemicals from the sorbent. The methanol extracts were then evaporated under a vacuum in a rotary evaporator (Buchi Rotavapor R-300EL, Switzerland) at 40°C until most of the methanol was evaporated. The residue was diluted with 5 ml of distilled water and subjected to extraction three times, each with 5 ml of diethyl ether to eliminate non-flavonoid phenolic compounds. The ether extracts were combined, and the diethyl ether was eliminated by flushing the solution with nitrogen gas (Devarajan & Venugopal, 2012).

Determination of Alpha Amylase Inhibition

The procedure utilised for the inhibition assay of alpha-amylase was adapted from Devarajan and Venugopal (2012). The test tube was filled with 500 µl of 0.02 M sodium phosphate buffer (pH 6.9) containing 0.006 M sodium chloride and 0.5 mg/ml of alpha-amylase solution. Then, different sample concentrations (4, 8, 12, 16, and 20 µg/ml) in methanol were added and vigorously shaken. The alpha-amylase solution was incubated at 25°C for 10 min. After pre-incubation, 500 µl of 1% starch solution was added to each tube. Afterwards, the reaction mixtures were incubated at 25°C for 10 min. One millilitre of dinitrosalicylic acid (DNS) colour reagent was added to halt the reaction. The test tubes were then subjected to a boiling water bath at 100°C for 5 min, then cooled to room temperature. After adding 10 ml of distilled water, the reaction mixture was further diluted, and its absorbance was subsequently measured at 540 nm. The formula utilised to determine the calculation of enzyme inhibition is expressed in Equation 2:

$$\% \text{ Inhibition} = \frac{A_{540\ control} - A_{540extract}}{A_{540control}} \times 100\% \quad [2]$$

where,

$A_{540\ control}$ = Absorbance of control (a buffer solution)

$A_{540\ extract}$ = Absorbance of the sample's extract

Determination of Calorie Value

The caloric value measurement was carried out using a bomb calorimeter (Model-IKA® C 2000, China) following the outlined preparation of the sample and procedure in

the Operating Instruction Manual (2010). Approximately 1.0 ± 0.1 g of dark chocolate sample was weighed in an empty beaker using a digital balance. The sample was pressed into a pellet shape using a pellet press maker (IKA® Werke GmbH & Co, Germany). The sample was carefully removed from the pellet press maker and re-weighed before being placed in a crucible. The crucible was placed inside the decomposition vessel and transferred into the bomb calorimeter. After combustion, the vessel was automatically removed, and the analytical value of the energy content was recorded. The analysis was conducted in triplicate.

Statistical Analysis

The statistical analysis was carried out using Statistical Product and Service Solutions (SPSS) (version 23) (IBM Corp, USA). One-way analysis of variance (ANOVA) was utilised, and significance was determined using Tukey's post-hoc test. A significant difference was defined as a probability level of $p < 0.05$. Based on measurements made in triplicate, all data are provided as means ± standard deviations (SD).

RESULTS AND DISCUSSION

Antioxidant Capacity

The DPPH and FRAP of dark chocolate containing honey powder (H/D, H/M, and H/S) are shown in Figure 1. The substitution of sucrose with honey powder in dark chocolate has increased the antioxidant capacity of chocolate samples, demonstrating that honey powder has high antioxidant properties even though the amount of honey was only 30% in the honey powder. It proves that the low drying temperature (40°C) used during honey powder production helps preserve the antioxidant properties of the honey powder. Studies have shown that exposure to temperatures below 50°C can protect phenolic compounds in honey from degradation (Halim et al., 2021; Keke & Cinkmanis, 2021; Ramli et al., 2017). Previous studies also observed the same trend, where the addition of black mulberry, raspberry, and sea buckthorn to dark chocolate has resulted in higher antioxidant content, leading to increased DPPH and FRAP values compared to control dark chocolate (Godočiková et al., 2017; Todorovic et al., 2015).

Dark chocolate containing H/M honey powder exhibited the highest antioxidant capacity, followed by H/D, H/S, and control. Maltodextrin possesses excellent

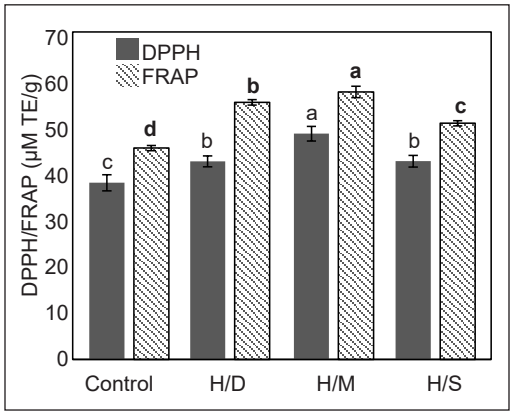

Figure 1. Antioxidant capacity in dark chocolate
Note. Control = Dark chocolate with no honey powder; H/D = Dark chocolate with honey/dextrose; H/M = Dark chocolate with honey/maltodextrin; H/S = Dark chocolate with honey/sucrose. Different letters indicate statistically significant differences ($p < 0.05$) between samples

encapsulating properties and is widely used as a drying agent due to its low viscosity, low bulk density, and high solubility in water and produce solutions that are monochromic in appearance (Buljeta et al., 2022; Hussain et al., 2018; Yang et al., 2022). Maltodextrin can effectively and stably encapsulate naturally occurring active compounds derived from plants, such as honey and cocoa (Jafari et al., 2008; Luna-Guevara et al., 2017). Studies have reported that adding maltodextrin to Mahkota Dewa fruit and pomegranate juice increased their antioxidant capacity and phenolic content compared to other encapsulating agents (Adetoro et al., 2020; Kathiman et al., 2020;). Maltodextrin has also been utilised to protect polyphenols in chokeberry, blackberry powder and acai (Ćujić-Nikolić et al., 2019; Ferrari et al., 2012; Tonon et al., 2010).

Alpha Amylase Inhibition

The inhibition percentage of dark chocolate extract against amylase enzyme is shown in Figure 2. Alpha amylase exhibited a concentration-dependent inhibitory action at 4, 8, 12, 16, and 20 extracted concentrations. The findings revealed a consistent trend for all samples, wherein the inhibition of alpha-amylase increased with an increase in concentration. The figure demonstrated that dark chocolate with honey powder had a significantly higher ($p < 0.05$) inhibition

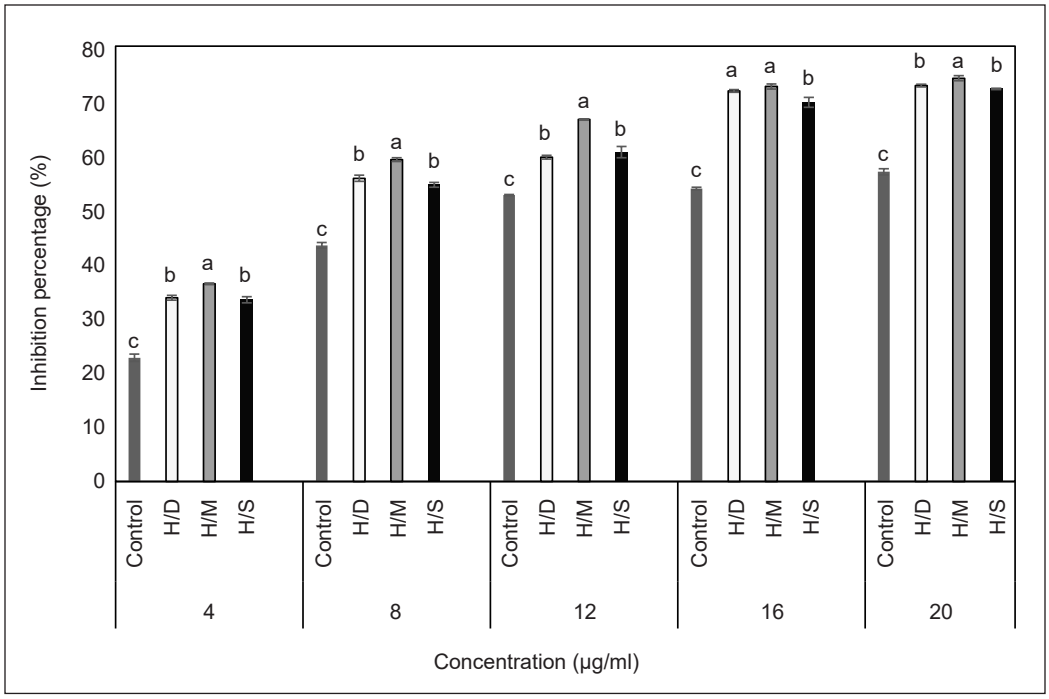

Figure 2. Alpha amylase inhibition in dark chocolate
Note. Control = Dark chocolate with no honey powder; H/D = Dark chocolate with honey/dextrose; H/M = Dark chocolate with honey/maltodextrin; H/S = Dark chocolate with honey/sucrose. Different letters indicate statistically significant differences ($p < 0.05$) between samples

percentage than the control. This finding indicates that combining cocoa polyphenols and honey may promote hypoglycemic effects due to their high antioxidant value. The high correlation between the inhibitory activity of enzymes and the antioxidant activity in the samples is attributed to polyphenols. Research has shown that honey has a specific antidiabetic role as it inhibits the enzyme alpha-amylase and treats glycemia by lowering blood glucose levels when consumed (Adefegha et al., 2018; H. Ali et al., 2020). Devarajan and Venugopal (2012) stated that flavonoid compounds in the honey extract are the source of antidiabetic behaviour. Additionally, another study suggested that luteolin, myrcetin, and quercetin in honey have potent inhibitory effects against the amylase enzyme (Tadera et al., 2006).

Flavonoids, including kaempferol, and phenolic acids, such as caffeic acid and *p*-coumaric acid, have been identified as the active ingredients in Tualang honey that contribute to the inhibition of alpha-amylase (Ahmed & Othman, 2013; Bharti et al., 2018; H. Ali et al., 2020). Chocolate is also a rich source of flavonoids, which are responsible for inhibiting alpha-amylase, as the samples demonstrate. Flavanols, such as catechin, epicatechin, and procyanidin, are flavonoids in cocoa that inhibit the alpha-amylase enzyme (Ramos et al., 2017; Yusuf et al., 2021). Furthermore, tannins, such as proanthocyanin and ellagitannin, in cocoa have also been reported to impact the inhibition of the amylase enzyme (Barrett et al., 2013). As a result, the combination of chocolate and honey extract can delay the digestion and absorption of starch by inhibiting alpha-amylase activity.

Dark chocolate containing H/M showed the highest inhibition effect compared to other dark chocolate samples. This study found a proportional relationship between alpha-amylase inhibition activity and antioxidant capacity, with dark chocolate containing H/M showing the highest DPPH and FRAP scavenging activities. Maltodextrin has been reported to efficiently preserve polyphenols and other bioactive compounds during drying, resulting in high antidiabetic activity demonstrated by encapsulated plant extract powder (Cian et al., 2019; Ćujić-Nikolić et al., 2019; Nguyen et al., 2022; Nurhayati et al., 2020; Tran et al., 2020).

Calorie Value

The caloric value of control dark chocolate and dark chocolate containing honey powder is shown in Figure 3. The figure shows no significant difference ($p > 0.05$) between all the samples. It represents that all dark chocolate has a calorie value of about 600 kcal/100 g. This value obtained was comparable to previous studies of dark chocolate, which ranged between 533 to 604 kcal/100 g (Ali et al., 2021).

The result demonstrates that the substitution of honey powder did not influence the caloric value of dark chocolate. According to the Malaysian Food Composition Database (1997), honey has a lower caloric value (313 kcal/100 g) than sucrose (398 kcal/100 g). However, as

the honey powder contained sugar carriers such as dextrose, maltodextrin, and sucrose at 70%, the value showed no significant difference ($p > 0.05$) to the control when the honey powder was incorporated into the chocolate. The caloric value of dextrose and maltodextrin was reported to be similar to sucrose (Lê et al., 2016; Tiefenbacher, 2017).

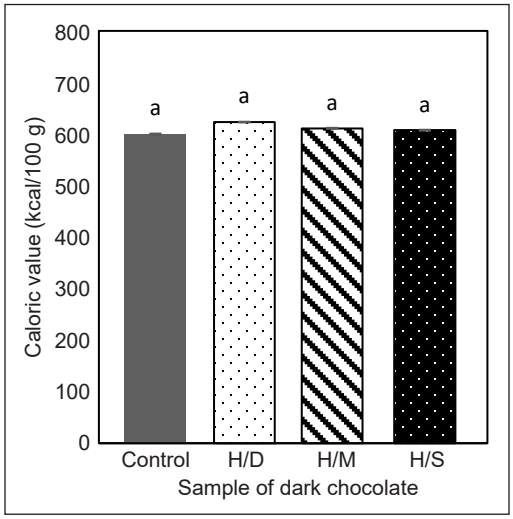

Figure 3. The caloric value of dark chocolate
Note. Control = Dark chocolate with no honey powder; H/D = Dark chocolate with honey/dextrose; H/M = Dark chocolate with honey/maltodextrin; H/S = Dark chocolate with honey/sucrose. Different letters indicate statistically significant differences ($p < 0.05$) between samples

CONCLUSION

The incorporation of honey powder into dark chocolate had been shown to increase its antioxidant activity and alpha-amylase inhibition, indicating that the phenolic content of the honey powder was preserved. Among the samples, dark chocolate containing H/M exhibited the highest antioxidant and alpha-amylase inhibition, demonstrating the effective preservation of phenolic content by maltodextrin. Therefore, chocolate containing H/M could be formulated as a functional food that may help decrease the risk of diabetes. However, to confirm the efficiency of the chocolate for human benefits, further studies on the bioavailability of polyphenol need to be carried out. An *in vivo* study on rats is suggested as the first stage for this confirmation.

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