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Clitoria ternatea L. Flower Extract Inhibits α-amylase During in Vitro Starch Digestion

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Abstract: This study aimed to investigate the inhibitory effect of *Clitoria ternatea* flower against α-amylase during simulated *in vitro* wheat starch digestion. The dark-blue tropical flower is used as a food colorant but its ability to modulate starch digestion has not been tested before. The aqueous extract of the flower containing anthocyanins was a competitive inhibitor against α-amylase with an IC_{50} value (concentration of inhibitor required to reduce the enzyme activity by half) and inhibition constant, K_i, of 0.91 mg/mL and 0.75 mg/mL, respectively. Subjecting the extract to pasteurisation (72°C for 15 s) and boiling (for 30 min) it significantly (P<0.05) decreased the anthocyanin content as determined by a pH-shift method, although the light absorbance profile of the extract remained virtually unchanged, suggesting that the equilibrium mixture of anthocyanin species was unaffected. The thermal degradation of the anthocyanins explained the partial loss of inhibition activity of the extract, as indicated by the decrease in Michaelis-Menten constant, K_m, from 14.8 mg/mL in the systems with unheated extract to 11.3 and 6.1 mg/mL in pasteurised and boiled extracts, respectively. The thermal treatments, however, did not change the type (competitive) of inhibition. The results from this work demonstrated the potential of *C. ternatea* flower extract in inhibiting α-amylase during starch digestion, which might lead to development of functional food/drink for controlling postprandial blood glucose level.

Keywords: Alpha-amylase, anthocyanins, *Clitoria ternatea*, inhibition, starch digestion.

Introduction

The rising prevalence of chronic diseases related to excessive caloric intake is a critical public health concern in many developed and developing countries, with almost a fourfold increase in the number of adults living with diabetes since 1980 (WHO, 2016). The sharp increase is largely due to the rise in type 2 diabetes which represents 90% of the diabetes diagnoses (WHO, 2016). This chronic condition is a metabolic disorder that, if untreated, can lead to high blood glucose levels known as hyperglycaemia, as well as other medical complications including heart attack, stroke, renal failure, blindness and neuropathy (Liebl et al., 2002). However, treating diabetes and the associated complications is expensive –it has been estimated that the direct annual cost of diabetes to the entire world is more than US$827 billion (Seuring et al., 2015). Lowering a postprandial hyperglycemic response offers an effective alternative way of preventing the development of type 2 diabetes (UKPDS, 1998) and this can be achieved by modulating starch digestion using natural enzyme inhibitors.

Research has shown that anthocyanin-containing extracts of certain fruits,roots, grains and legumes such as Maqui berries (Rojo et al., 2012), black currant (Castro-Acosta et al., 2016), black carrot (Türkyilmaz et al, 2012) and blue maize (Camelo-Méndez et al., 2016) inhibit α-amylase and/or glucosidase during *in vitro* starch digestion to varying extents. Furthermore, *in vivo* studies have showed that feeding diabetic mice with purple corn containing a high level of anthocyanin cyanidin-3-glucoside resulted in reduced hyperglycaemia, hyperinsulinemia and hyperleptinemia, demonstrating the potential of anthocyanins to be used in functional

foods to naturally prevent early onset of obesity and diabetes (Tsuda et al., 2013). It is widely known that anthocyanins provide multiple health benefits including antioxidative (Li et al., 2017) and anti-inflammatory properties (Szymanowska et al., 2015), and research has shown that they possess anti-diabetic effect as well. Anthocyanins are phenolic compounds found in plants which are collectively known as flavonoids. These secondary plant metabolites are wide spectrum colour pigments varying from blue to red or pink to orange (Swer et al., 2016). Structurally, anthocyanins are glycosides of polyhydroxy- and polymethoxy- derivatives of flavylium salts (Kong et al., 2003). It appears that the degree of enzyme inhibition relies on the composition of anthocyanins, which is plant species- or sub-species-dependent. For example, a particular group of anthocyanins, so-called diacylated anthocyanins, in Glen Ample raspberries were found to effectively inhibit glucosidase (Mc Doughall et al., 2005), while proanthocyanidins, another group of anthocyanins from faba bean showed inhibition effects on digestive enzyme (Yuste et al., 1992).

This work was initiated to assess the inhibitory activity of *Clitoria ternatea* flower against α-amylase during *in vitro* starch digestion. *C. ternatea*, commonly known as butterfly pea, belongs to the Fabaceae family and is herbaceous, slender and tall with climbing vines. The tropical plant blooms in around 6 weeks with bluish purple flowers and a yellow centre. The anthocyanins in the petals of the flower are exceptionally stable in weakly acidic or neutral aqueous solution (Goto & Kondo, 1991) and for this reason the flowers are traditionally used as food colorants in Southeast Asia (Mohamad et al., 2011). Delphinidin glucoside is the main anthocyanin responsible for the distinctive blue colour of the flower (Terahara et al., 1996). A total of 15 species of anthocyanins, so-called ternatins, have been isolated from the flower. Their common chemical structures have been characterised as malonylated delphinidin 3,3',5'-triglucosides (Terahara et al, 1990; 1996), having 3',5'-side chains with alternating D-glucose and *p*-coumaric acid units and delphinidin 3-*O*-(2'-*O*-α-rhamnosyl-6'-*O*-malonyl-β-glucoside (Kazuma et al. 2003). While many parts of *C. ternatea* plant have been used to treat various diseases as in the folklore medicine (Gupta et al., 2010), little is known about the potential of anthocyanins of the flower in modulating starch digestion.

In this study, an aqueous extract of *C. ternatea* flower was prepared and its ability to slow down α-amylase activity was tested in simulated starch digestion. Since heating can cause degradation and polymerization of anthocyanins and potentially the loss of enzyme inhibitory activity, the anthocyanin-rich extract was also subjected to pasteurisation and boiling/cooking to assess its thermal stability. The process described here provides an understanding of the inhibition behaviour of *C. ternatea* extract against α-amylase.

Materials and Methods

Materials

Sundried *C. ternatea* flowers were purchased from a local market place in Penang, Malaysia. In the laboratory, the flowers were dried in an oven at 45°C and stored in an air tight container and kept in a dark cool place until required. Lyophilised α-amylase from *Bacillus* sp.(Type II-A, ≥1,500 units/mg protein) and wheat starch were obtained from Sigma-Aldrich (Dorset, UK).All of the other chemicals used were also purchased from Sigma-Aldrich (Dorset, UK) and were of analytical grade.

Preparation of C. Ternatea Extract

Approximately 10 g of dry *C. ternatea* flower petals were soaked in 100 mL of distilled water overnight at room temperature. The solution was then filtered through doubled layer of cheese cloth and freeze-dried in Edwards Micro Modulyo freeze-dryer (Bristol, UK).The lyophilised extract was ground into powder and a stock solution of 97.5 mg/mL in sodium phosphate buffer (SPB, 0.1 M, pH 6.9) was prepared. The anthocyanin-rich stock solution was kept at –20°C until use.

Determination of Anthocyanin Content

The pH shift method adapted from Ribereau-Gayon and Stone street (1965) was used to determine the total monomeric anthocyanin content of the extract. Briefly, aliquots of the *C. ternatea* extract were separately dissolved in sodium phosphate-citric acid buffer (0.1 M, pH 4.5) and 2% hydrochloric acid solution (pH 1) in the presence of 7.5% ethanol with a predetermined dilution factor. The absorbance of the samples in these two solutions was measured using a Genesys UV-Vis spectrophotometer (Model 6, Thermo Fisher Scientific, Waltham, USA) at 520 and 700 nm using the respective buffer solutions as the blank. The monomeric anthocyanin pigment concentration, expressed as delphinidin-3-glucoside equivalent, was determined as follows:

$$\text{Anthocyanin pigment concentration, } mg/L = \frac{A \times MW \times DF \times 1000}{\varepsilon x l} \quad [1]$$

where $A = (A_{520nm} - A_{700nm})_{pH1.0} - (A_{520nm} - A_{700nm})_{pH4.5}$, and MW is the molecular weight of delphinidin-3-glucoside, 518.5 g/mol; DF is the dilution factor; ε is the molar extinction coefficient of delphinidin-3-glucoside, 29000 L/mol cm; and *l* is the path length of 1 cm.

Starch Digestion Experiments

The inhibitory effect of the extract against α-amylase during *in vitro* starch digestion as described by Sui and co-workers (2016) was investigated as a function of *C. ternatea* or wheat starch concentration. Briefly, aliquots of the extract stock solution were diluted to final concentrations of 1.3, 2.6, 3.9 and 5.2 mg/mL in Eppendorf tubes by SPB (0.1 M, pH 6.9) in the presence of 40 mg/mL calcium chloride. Exactly 20µL of α-amylase solution was added to each Eppendorf tube and incubated at 37°C for 15 min to allow the enzyme interaction with the extract. The digestion was started by adding 60 µL of wheat starch solution (12.5 mg/mL), which was gelatinised before hand by boiling an appropriate amount of starch in SPB for 30 min, and continued incubating the mixture at 37°C for another 5 min. The concentrations of both α-amylase and starch in the mixture were fixed at 1 mg/mL, and the total volume of the reaction mixture was 750 µL. The extent of starch digestion was analysed by measuring the concentration of glucose liberated. To this end, 100 µL of 3,5-dinitrosalicylic (DNS) acid reagent was added to the mixture at the end of the 5-min digestion, followed by heating in boiling water for 10 min to develop the colour. The reaction was then stopped by cooling the mixture in ice bath for 20 min and the absorbance of the sample was read at 540 nm using a UV-Vis spectrophotometer (Genesys Model 6, Thermo Fisher Scientific, Waltham, USA).To compensate the interference of anthocyanin's colour in the spectrophotometric measurement, respective blanks were prepared in the same way with different extract concentrations but replacing the starch solution with SPB. For control, a sample was prepared similarly without the presence of *C. ternatea* extract. Another set of experiments was carried out with varying wheat starch concentrations of 0.5, 1.0,1.5, 2.0 and 2.5 mg/mL while the extract concentration was set at 1.3 mg/mL. The concentration of liberated glucose was determined from a calibration curve (R^2=0.9982) plotted using glucose solutions with concentrations ranged from 0.01 to 0.25 mg/mL.

Effects of Thermal Treatments on the Extract

Aliquots of *C. ternatea* stock solution were separately heated at 72°C for 15 s and 100°C for 30 min to mimic industrial pasteurisation and cooking processes, respectively. The samples were diluted appropriately and absorbance spectra was obtained by scanning the samples at pH 6.9 from 350 to 750 nm using a UV-Vis spectrophotometer and the anthocyanin content was determined as described above. To investigate the thermal effect on the inhibitory activity of *C.ternatea* extract against α-amylase, a set of digestion experiments were conducted with varying wheat starch concentrations and the glucose concentration in the samples was determined using the DNS method as mentioned above.

Statistical Analysis

All experiments were triplicated and, where applicable, the results were analysed with ANOVA test at the 95% significant level (P<0.05) using SPSS (version 22.0, SPSS Inc., Chicago, IL, USA).

Results and Discussion

Type and Inhibition Parameters

Figure 1 shows the effect of *C. ternatea* extract on the extent of wheat starch digestion by α-amylase. The presence of extract (1.3 mg/mL) in the reaction mixture significantly ($P<0.05$) decreased the glucose liberated from 0.197 mg/mL in the control to 0.044 mg/mL after 5 min of digestion, resulting in 78% enzyme inhibition. The extract inhibited α-amylase activity in a dosage-dependent manner – the addition of more extract resulted in a higher inhibition of α-amylase as shown by further decrease in liberated glucose; about 90% of inhibition was achieved when the extract concentration was increased to 5.2 mg/mL (Figure 1).

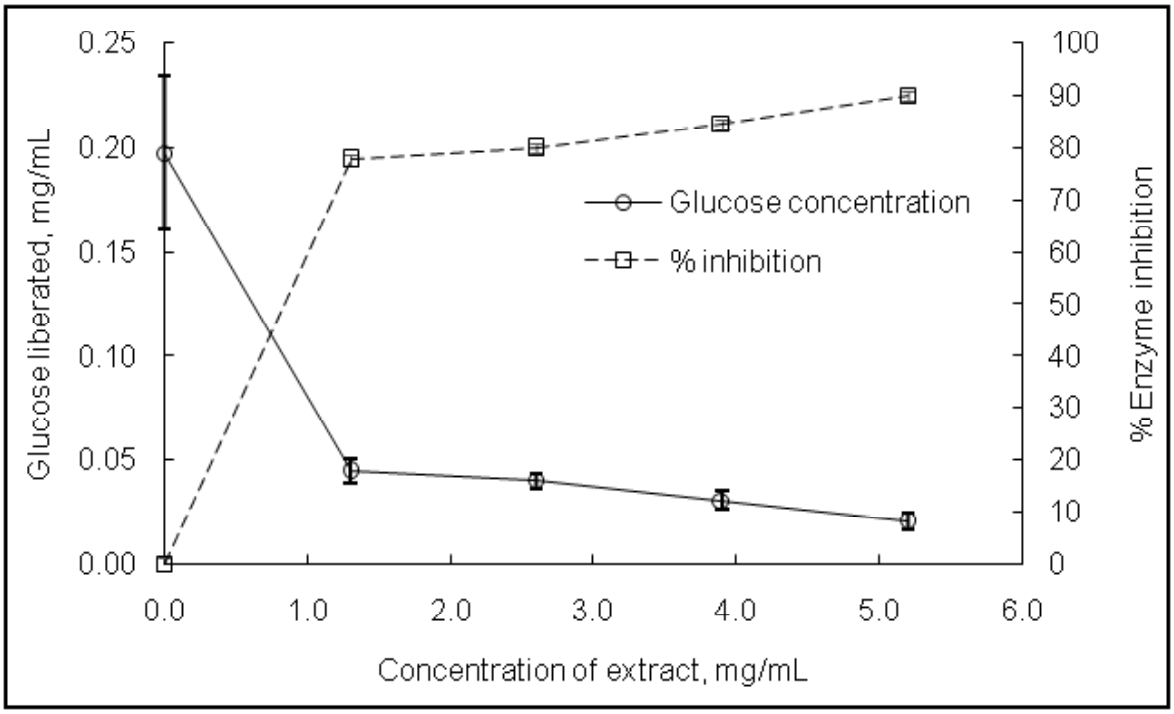

Fig1. *Effect of C. ternatea extract on the inhibition of α-amylase activity and concentration of liberated glucose.*

To determine the inhibition type of the extract against α-amylase, double-reciprocal Line weaver-Burk graphs were plotted and the results are shown in Figure 2. Both *C. ternatea* and control systems had similar *y*-intercepts but different slope and *x*-intercepts, characteristic of competitive inhibition. Anthocyanins such as cyanidin-3-glucoside and cyanidin-3,5-glucoside and peonidin-3-glucoside have also been reported to be competitive inhibitors against α-amylase (Sui et al., 2016). Competitive inhibition is relative to the amount of inhibitor bound in the active site of the enzyme and is consequently proportional to inhibitor concentration. As the inhibitor reversibly binds, the substrate can compete with the inhibitor in high concentrations, so at higher substrate concentration there is a less inhibitory effect. Therefore, a competitive inhibitor does not alter the maximal velocity (V_{max}) of the catalytic reaction of an enzyme. The V_{max} of the digestion in the systems in this study was similar but the enzyme activity decreased in the presence of *C. ternatea* extract, as indicated by the increase in Michaelis-Menten constant, K_m, from 1.73 mg/mL in the system without the extract to 14.84 mg/mL in the presence of the extract (Table 1). Competitive inhibitors increase the K_m value of an enzyme and higher concentrations of substrate would be required to achieve the half-maximal velocity (Pelley, 2012). The results suggest that *C. ternatea* extract could occupy the active site of α-amylase and competitively inhibit the enzyme.

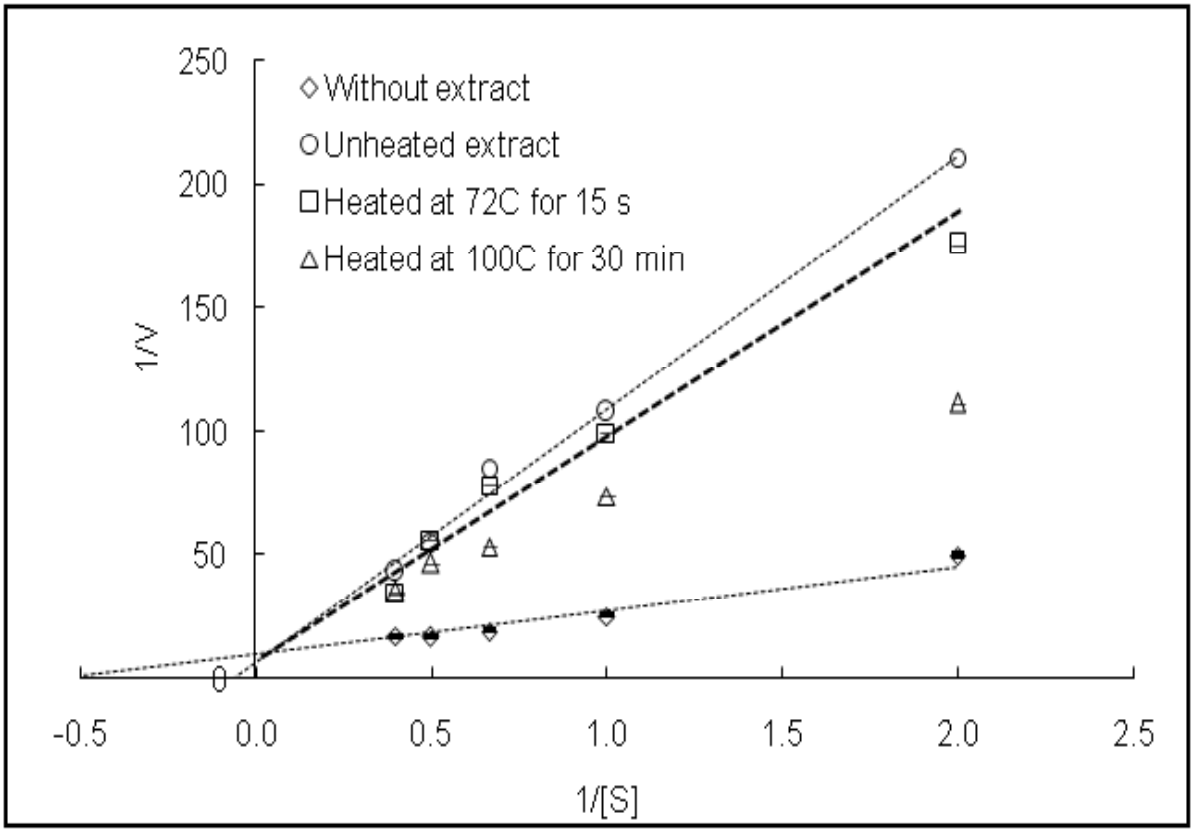

Fig2. *Lineweaver-Burk plots of wheat starch digestionby α-amylase with and without the presence of unheated, pasteurised and boiled C. ternatea extract.*

Table1. *The K_m and V_{max} values and the type of inhibition of the in vitro wheat digestion in the presence of C. ternatea extracts*

System	K_m	V_{max}	Type of inhibition
Unheated extract	14.8363	0.1449	Competitive
Extract heated at 72°C for 15 s	11.2897	0.0978	Competitive
Extract heated at 100°C for 30 min	6.0580	0.1455	Competitive
Control (without extract)	1.7338	0.1215	Not applicable

The effectiveness of *C. ternatea* extract as α-amylase inhibitor was evaluated by determining its half maximal inhibitory concentration, IC_{50} which is the amount of inhibitor required to reduce the catalytic activity of an enzyme by half. The IC_{50} can be estimated graphically by plotting the substrate concentration divided by rate of the reaction, [S]/V, on the y axis, as a function of the extract concentration as shown in Figure 3. The intercept of the extrapolated line on the *x*-axis (inhibitor concentration) indicates the $-IC_{50}$ (Cortés, et al., 2001). The curve plotted from the experiment results (Figure 3) yielded a correlation coefficient, R^2, of 0.9362 and the IC_{50} calculated from the regression equation of the plot was 0.91 mg/mL. The inhibition activity of the extract was also assessed by determining the inhibition constant, K_i. The K_i is inversely related to the binding affinity of the extract towards α-amylase; the smaller the value, the smaller the amount of inhibitor is required to inhibit the enzyme. In this study, the K_i was calculated based on the Michaelis-Menten model for competitive inhibition as below:

$$\frac{1}{V} = \frac{K_m}{V_{max}\ [S]}\left(1 + \frac{[CT]}{K_i}\right) + \frac{1}{V_{max}} \quad [2]$$

where V is the initial reaction rate, [S] is the substrate concentration and [CT] is the extract concentration, which gave a K_i of 0.75g/mL. The value was slightly higher than expected because, for a competitive inhibition, the K_i should be about half that of the IC_{50} (Bachmann & Lewis, 2005). Nevertheless, inhibition parameters determined here have shown the potential of the extract as α-amylase inhibitor.

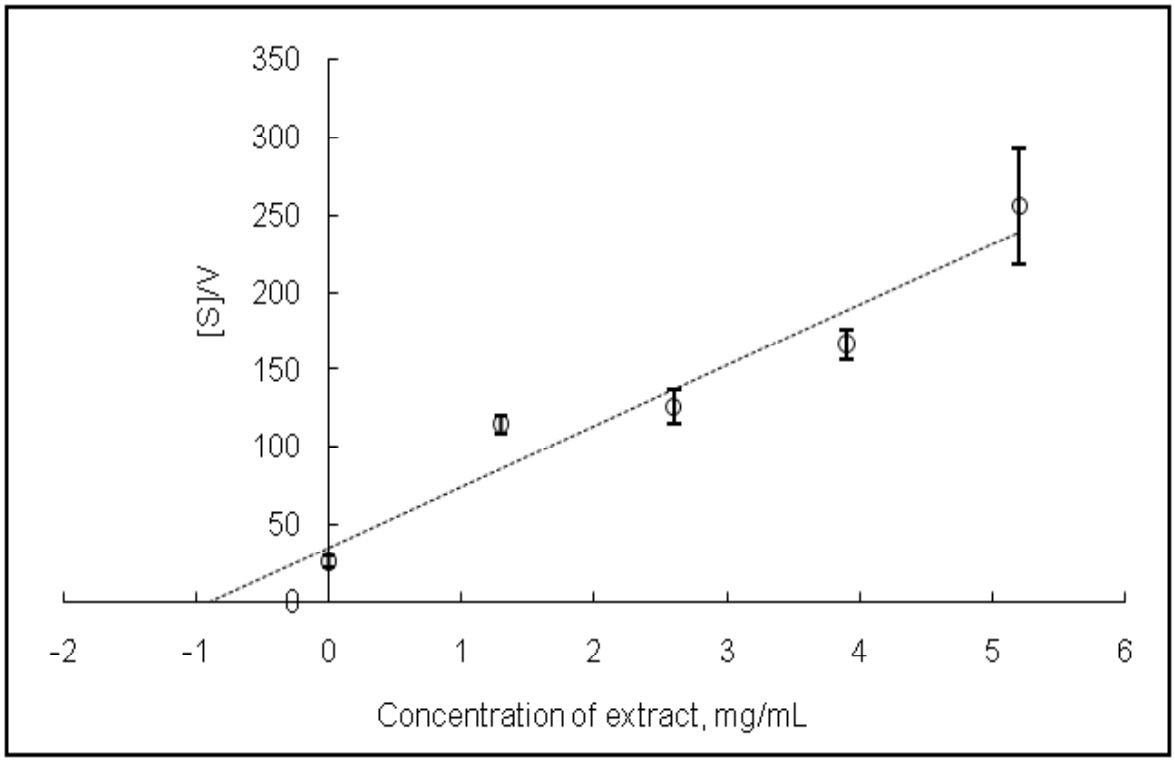

Fig3. *Graphical determination of IC_{50} of C. ternatea for inhibition of α-amylase.*

It is speculated that the anthocyanins (or ternatins) in the flower extract are responsible for the inhibition of the α-amylase. Research has shown that anthocyanins from different plant sources inhibit α-amylase to varying extent, and that the inhibition activity depends on the type of glycoside that is linked to the aglycon, rather than the aglycon skeleton of the anthocyanins. Akkarachiyasit et al. (2010) reported that cyanidin-3-glucoside was the most potent inhibitor against porcine pancreatic α-amylase with an IC_{50} of 0.3 mM. The ternatins of *C. ternatea* have a melonylated delphidinin structure with much more complex triglucosides. However, it is not possible to compare their inhibition activity with that of cyanidin-3-glycoside in this study since a crude extract has been used. Molecular docking studies on porcine pancreatic α-amylase suggest that anthocyanins form 7-8 hydrogen bonds with the enzyme and the inhibition is most likely implemented through binding to the side of glutamic acid-233 residue at the catalytic site of the enzyme (Sui et al., 2016). Although the exact amino acid residues at the catalytic site of *Bacillus* sp. α-amylase may be different to those of porcine pancreatic α-amylase, it is still possible that a similar mechanism of inhibition occurred in this work in which the glucosides of ternatins bind to the amino acid residues at the active site of the enzyme through hydrogen bonding which leads to inhibition. It is also likely that the 15 species of delphinidin-based ternatins exhibit varying inhibition activity owing to their different glucoside moieties thus affecting their polarity and chemical structure (Akkarachiyasit et al., 2011, Sui et al., 2016).

Influence of Thermal Treatments

To be successfully used as an ingredient in functional foods, *C. ternatea* extract must be able to withstand common food processing temperatures without affecting their ability to inhibit the enzyme activity. Therefore, the effect of pasteurisation and 30-min boiling on the stability of *C. ternatea* anthocyanins and their amylase inhibition properties were investigated. Figure 4 compares the absorbance spectra of the thermally treated with untreated extracts both measured at neutral pH. All samples had a very similar spectra profile with maximal absorbance at 574 and 619 nm and a shoulder at 540 nm. The same spectral characteristics were reported by Lee and co-workers (2011). It is thought that the visible spectrum is attributed to the equilibrium mixtures of red flavylium cations appeared at the shoulder at 543 nm and the two tautomers of blue quinonoidal bases at 574 and 619 nm (Lee at al., 2011). The spectra suggested that equilibrium mixture of anthocyanin species in the extract was virtually unaffected by the thermal treatments. However, the results of pH shift experiments showed that the thermal treatments reduced the anthocyanin content. The total anthocyanin content significantly (P<0.05) decreased from 3987±252 mg/L in the unheated extract solution to 3607±94and 1193±13 mg/L in the pasteurised and boiled samples, corresponding to 9.5% and 70.1% loss, respectively. Although *C. ternatea* anthocyanins were reported to be relatively thermally stable in comparison to other anthocyanins (Lee at al., 2011)probably due to the intermolecular co-pigmentation of the anthocyanins (Bloor, 1997; Eiro & Heinonen, 2002; Lee et al., 2011), the results of this work show that it is still a challenge to keep the anthocyanins stable during heat treatment especially at boiling temperature. Increasing temperature can induce the loss of glycosyl moieties of anthocyanins through the hydrolysis of glycosyl bond (Adams, 1973). Heat treatments are also known to cause polymerisation of monomeric anthocyanins which leads to the development of browning (Somers & Evans, 1986)as seen in thermally processed fruit or vegetable juices (Türkyilmaz et al., 2012).

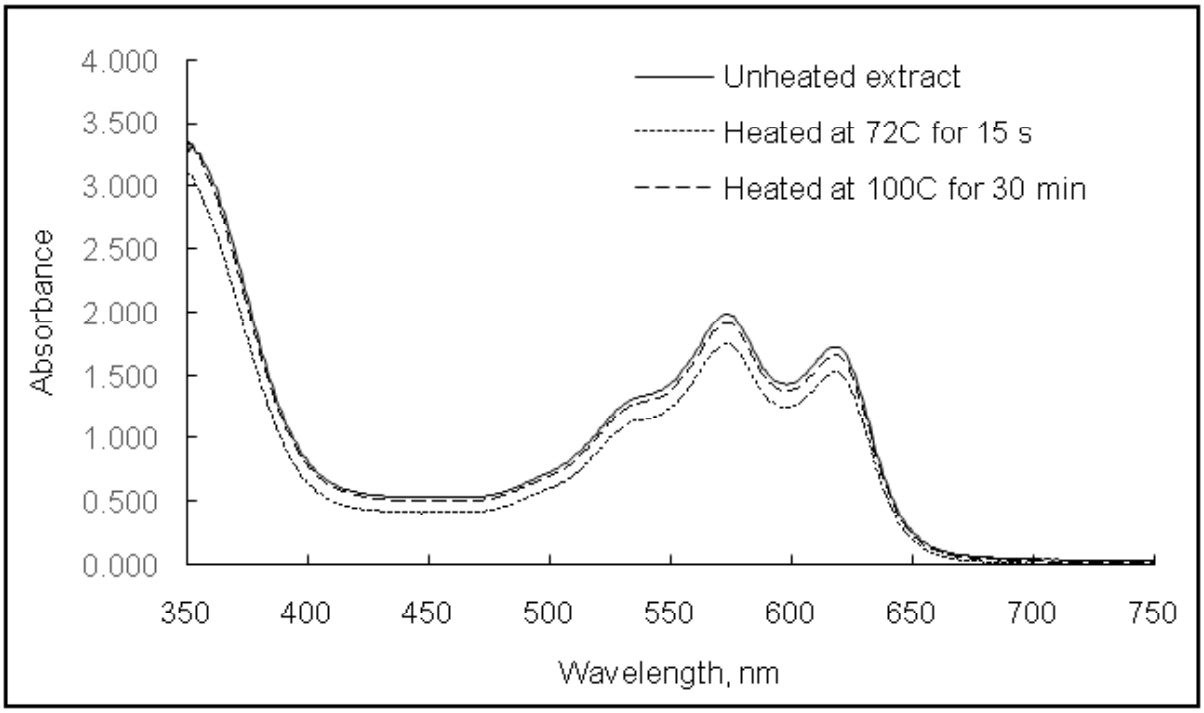

Fig4. *Spectrophotometric absorbance profiles of unheated, pasteurised and boiled C. ternatea extracts.*

The thermal treatments of the *C. ternatea* extract dampened its amylase inhibitory properties to different degrees as shown in the Line weaver-Burk plots in Figure 3. The inhibition activity decreased with the extent of heating – the 30-min boiling caused a larger loss in inhibition activity of the extract than the milder pasteurisation. For

example, unheated extract (1.3 mg/mL) inhibited amylase by as much as 78% but the boiled extract reduced the enzyme activity to 65%; whilst pasteurisation at 72°C for 15s to 74%, a small decrease compared to that of the unheated extract. Similarly, thermal treatments decreased the K_m values of α-amylase from 14.84 mg/mL in the system with unheated extract to 11.29 and 6.06mg/mL in pasteurised and boiled extracts, respectively (Table 1), indicating that the effectiveness of the extract as amylase inhibitor decreases with the extent of the heating. On the other hand, the V_{max} values were relatively constant in all systems (Table 1& Figure 3). The results showed that the thermal treatments did not change the type of amylase inhibition by the extract but remained as competitive inhibition.

Conclusion

The current work demonstrates the potential of the aqueous extract of *C. ternatea* flower in inhibiting α-amylase during *in vitro* starch digestion. It is thought that the anthocyanins or ternatins in the extract were responsible for the competitive inhibition of the enzyme. Mild thermal treatments reduced the anthocyanin activity to certain extent in the extract whilst more severe heat treatment caused a further loss. The next step of this work is to confirm the inhibition activity of *C. ternatea* using a purer extract and individual ternatins with α-amylase and glucosidases relevant to the physiological conditions of human.

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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