Anti-diabetic properties of *Synsepalum dulcificum* and its potential inclusion in functional yogurt

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Abstract. There has been an enormous interest in the development of alternative medicines for type-2 diabetes, specifically screening for phytochemicals with the ability to delay or prevent glucose absorption. The goals of the present study were to provide *in vitro* evidence for potential inhibition of α-amylase and α-glucosidase enzymes, followed by inclusion of the extracts of *Synsepalum dulcificum* in yogurt to enhance the therapeutic properties of the yogurt. The screening results of seed, leaf and pulp of *S. dulcificum* showed that pulp extracts contained significantly (P<0.05) higher anti-diabetic activities than the other plant parts. More interestingly, *S. dulcificum* pulp also has stronger anti-diabetic properties than the standard drug, acarbose and hence it was chosen to be incorporated into yogurt. *S. dulcificum* yogurt had higher (P<0.05) anti-diabetic activities than the plain yogurt throughout the storage period with the highest α-glucosidase and α-amylase inhibitory activities were shown on day 7 of storage. Therefore, *S. dulcificum* pulp can be developed as functional factor with anti-diabetic activities in food application.

1. Introduction

In this century, it is estimated at least 346 million of people worldwide suffer from type-2 diabetes mellitus due to pancreatic β-cell dysfunction and/or increased resistance to insulin with impaired glucose tolerance [1]. An effective strategy for type-2 diabetes management is the inhibition of α-amylase and α-glucosidase. α-amylase is an enzyme found in the pancreatic juice and saliva functions to break down large insoluble starch molecules into absorbable molecules [2]. In the meantime, α-glucosidase in the mucosal brush border of the small intestine catalyzes the end step of digestion of starch and disaccharides that are abundant in human diet [3]. Inhibitors of both α-amylase and α-glucosidase will act to delay the breaking down of carbohydrates in the small intestine and diminish the postprandial blood glucose excursion [4]. Phenolic compounds are known to interact with proteins and can inhibit enzymatic activity. To date, many plant and herbal extracts have been reported to efficiently inhibit the enzymatic activity of α-amylase and α-glucosidase [4, 5].
Synsepalum dulcificum also known as miracle fruit or miracle berry is an indigenous tropical plant growing in West Africa. The plant can be harvested at least twice a year making the yield stable [6]. Miracle fruit is about an inch in length with a bright red colour and known for its ability in converting sour tasting foods to sweet taste. Glycoprotein known as miraculin found in the pulp of this fruit is the compound responsible for this unique taste modifying function [6, 7]. The binding of miraculin to the receptor cells of the tongue suppresses the response of a sour taste in the central nervous system. The effect would last until the miraculin is diluted and eliminated by saliva. The taste modification function gives a great potential for this fruit to be exploited in the food industry especially as an alternative sweetener or additive to mask the undesirable sour taste in food products such as yogurt [8]. Moreover, miracle fruit’s pulp and seed contained high nutrient contents which can be used for dietary supplement in food [9]. Stem of miracle fruit also contained antioxidant and antityrosinase effects which can be potential applications in food supplementation and medical cosmetology [10]. Hence, it is interesting for miracle fruit to be formulated into functional foods such as yogurt to improve the nutritional and therapeutic properties [11].

To date, very little is known about the health benefits of S. dulcificum plant extract. The ability of this plant to inhibit the enzymatic activity of α-amylase and α-glucosidase when incorporated into food product also has yet to be studied and explored. Keeping in view the potential of anti-diabetic properties of S. dulcificum plant extracts [12], the present study is endeavoured to develop functional yogurts fortified with S. dulcificum extract. In this regard, the inclusion of the plant extracts is expected to enhance the nutritional values of the yogurt and as such functional food that contained components which are able to inhibit α-amylase and α-glucosidase could be developed.

2. Materials and methods
2.1. Plant materials
The pulp, seed and leaf of S. dulcificum were obtained from local farm located in Selangor. They were thoroughly washed with tap water to remove unwanted materials. Then, the seeds were separated from the pulp of S. dulcificum using a knife, and the pulp and seed were freeze dried for 48 hours by Martin Christ Epsilon 1-80 freeze dryer. The leaf samples were dried in an oven at 50°C for 24 hours. All the samples were then grinded using a household blender into fine powder and kept in labelled zip lock plastics in a chiller (4°C) until usage.

2.2. Starter culture
Yogurt starter culture was purchased from Chris Hansen (Denmark) in the form of freeze-dried containing a mixture of bacteria strains (YC-380): Streptococcus thermophilus and Lactobacillus delbrueckii subs. Bulgaricus in the ratio of 1:1. Briefly, the freeze-dried yogurt starter was inoculated in fresh pasteurized cow milk in a ratio of 1:100 (wv⁻¹). The mixture was then incubated at 42°C overnight without shaking. The yogurt formed was kept at 4°C and used as a starter culture within 1 week.

2.3. Water extraction of S. dulcificum plant
Water extraction of plant samples was done following a method by Amirdivani [5] with some modifications. The fine powders of pulp, seed and leaf were added in distilled water in a ratio of 1:10 (wv⁻¹). Then, the solutions were incubated in a water bath (50°C) for 16 hours, followed by filtration with Whatman’s filter paper No. 5. The filtrate was harvested and centrifuged (5810R, Eppendorf, Germany) at 6000 g for 10 minutes. The pellet was discarded while the supernatant was stored in 4°C and used within 3 days.

2.4. Preparation of yogurt
Plain and S. dulcificum yogurts were prepared on the same day. S. dulcificum pulp extract (10 mL) was added into pre-warmed pasteurized cow milk (80 mL) followed by the addition of starter culture (10 g). Meanwhile, plain yogurt was prepared essentially in the same manner as the S. dulcificum yogurts.
with the exception that distilled water (10 mL) was used instead of the pulp extract. Yogurts were fermented (42°C) until its pH reduced to 4.5 followed by refrigeration (4°C) up to 21 days.

2.5. Preparation of yogurt extracts
Yogurt extracts were prepared following a method reported by Amirdivani [5] with some modifications. Aliquots of each 10 g yogurt sample was homogenized (by vortex) with 2.5 mL sterile distilled water. The pH of the yogurt was acidified to 4.0 with 0.1 M hydrochloric acid (HCl). The yogurts were then heated in a water bath (45°C) for 10 minutes before centrifuged (MiniSpin, Eppendorf, Germany) at 7500 g, 4°C for 10 minutes. The supernatants were harvested and pH was adjusted to neutral (pH 7) with an addition of 0.1 M sodium hydroxide (NaOH). The yogurt water extracts were re-centrifuged under the same condition and the supernatants were harvested and kept in -20°C prior to further analysis.

2.6. α-amylase inhibitory activity
Following a method described by Akoro et al., [13] with some modifications, 250 µL enzyme solution (1 U/mL of pancreatic alpha-amylase enzyme (E.C:3.2.1.1; Sigma, USA) dissolved in 0.02 M sodium phosphate buffer with 0.006 M sodium chloride) was added to 250 µL sample (plant extract/ control) and incubated for 10 minutes at 37°C. Then, 250 µL of 1% soluble starch (potato starch) was added and incubated again for 10 minutes at 37°C. The reaction was terminated by adding 500 µL of DNSA (3,5-dinitrosalicylic acid) reagent and then boiled in the water bath at boiling point for 5 minutes. The solution was cooled and diluted with 5 mL of water and absorbance reading was taken at 540 nm. To eliminate the absorbance produced by plant extract, an appropriate extract control without the present of enzyme was also included. The results were expressed as percentage of inhibition of extracts against concentration, calculated according to the Equation 1:

\[
% \text{inhibition} = \left( \frac{\text{Absorbance of control} - \text{Absorbance of extract}}{\text{Absorbance of control}} \right) \times 100
\]

2.7. α-glucosidase inhibition assay
The effect of S. dulcificum extracts on α-glucosidase activity was determined according to the method described by Kazeem et al., [4] with some modifications, using α-glucosidase from Saccharomyces cerevisiae. The substrate solution p-nitrophenyl glucopyranoside (pNPG) (3.0 mM) was prepared in 20 mM phosphate buffer, pH 6.9. 100 µL of α-glucosidase (1.0 U/mL) was pre-incubated with 50 µL of the extracts for 10 minutes. Then 50 µL of 3.0 mM pNPG dissolved in 20 mM phosphate buffer (pH 6.9) was added to start the reaction. The reaction mixture was incubated at 37°C for 20 minutes and stopped by adding 2 mL of 0.1 M Na2CO3. The α-glucosidase activity was determined by measuring the yellow coloured para-nitrophenol released from pNPG at 405 nm. The results (% inhibition) were expressed as percentage of the blank (control) as in Equation 1.

2.8. Statistical analysis
All experiments were performed on at least three different occasions (n=3). All data is expressed as mean value ± standard deviation. Data analyses and the graphs were analyses and plotted on Graph Pad prism 6. The statistical significance was determined at p value < 0.05. Statistical analysis was done using the Analysis of variance (ANOVA); P<0.05 was considered significant.

3. Results and discussion
3.1. Screening of anti-diabetic properties in S. dulcificum plant parts
3.1.1. α-amylase inhibitory activity
α-amylase catalyzes the hydrolysis of 1,4-glycosidic linkages of starch, glycogen, and various oligosaccharides into simpler sugars which can be readily available for the intestinal absorption [14]. Therefore, inhibition of this enzyme in the digestive tract of human is considered to be effective in
controlling diabetes by decreasing the absorption of glucose from starch [15]. In this study, porcine pancreatic α-amylase (PPA) was used since PPA is closely related to human α-amylase [16]. In vitro α-amylase inhibitory studies demonstrated that all the S. dulcificum extracts possessed α-amylase inhibitory activity (Table 1).

The evaluation of α-amylase inhibitory activity of acarbose (an anti-diabetic drug used to treat diabetes mellitus type 2) was also done to assess the validity of the assay used. The results revealed that a dose-dependent response (P<0.05) since increasing concentration of plant extracts increased the inhibitory activity (Table 1). At the lowest concentration of extracts (1.25 mg/mL), the pulp extract exhibited the highest inhibitory activity of 20.73% which was higher than that of acarbose (13.4%). However, at the highest concentration (20 mg/mL), the highest inhibition was observed in acarbose (78.35%) with leaf extract having the lowest α-amylase inhibitory activity of 31.96%. Nevertheless, the overall result showed that the S. dulcificum pulp extract is the most effective for the inhibitory of α-amylase activity since the pulp was having the lowest IC₅₀ value of 9.66 mg/mL when compared to acarbose and the other two extracts. This result indicated that the S. dulcificum pulp is a strong α-amylase inhibitor that could help to suppress the hydrolysis of starch leading to reduction in postprandial glucose level and hence reducing the complication of diabetes mellitus. The presence of α-amylase inhibitor in S. dulcificum pulp may be due to the presence of potential α-amylase inhibitors such alkaloids, flavonoids, terpenoids or glycosides [17]. To our knowledge till date, the present study is the first to report on α-amylase inhibitory activity in S. dulcificum.

**Table 1. α-amylase inhibitory activity of S. dulcificum plant parts and synthetic drug, acarbose**

| Concentration (mg/mL) | % Inhibition by seed | % Inhibition by leaf | % Inhibition by acarbose | % Inhibition by pulp |
|-----------------------|----------------------|----------------------|--------------------------|----------------------|
| 1.25                  | 18.56±(0.01)         | 4.12±(0.00)          | 13.4±(0.00)              | 20.73±(0.01)         |
| 2.50                  | 20.62±(0.03)         | 18.56±(0.01)         | 28.87±(0.02)             | 34.14±(0.02)         |
| 5.00                  | 20.62±(0.01)         | 19.59±(0.01)         | 41.24±(0.01)             | 39.02±(0.02)         |
| 10.00                 | 22.68±(0.02)         | 21.65±(0.01)         | 47.42±(0.01)             | 59.76±(0.01)         |
| 20.00                 | 35.05±(0.00)         | 31.96±(0.06)         | 78.35±(0.02)             | 71.95±(0.02)         |
| IC₅₀ value (mg/mL)    | 39.37                | 35.09                | 10.4                     | 9.66                 |

Data was expressed as mean ± standard deviation of triplicate determinations.

IC₅₀: Concentration of inhibitor to inhibit 50% of the enzyme activity under the assayed conditions.

Means in the same column expressed with different superscript letters are significantly different at (P<0.05)

3.1.2. α-glucosidase inhibitory activity

Glucosidase is the key enzyme involved in hydrolysis of carbohydrate and inhibitors of this enzyme may be exploited as therapeutic approaches for controlling postprandial hyperglycemia [18]. Phytochemicals from plants such as phenolic compounds, saponins, flavonoids, glycosides and alkaloids have been reported to play an important role in modulating glucosidase activity and can therefore contribute to the management of postprandial hyperglycemia [19].

From the result obtained, a similar trend as α-amylase inhibitory activity result was observed in which increasing the concentration of plant extracts resulted in increasing of α-glucosidase inhibitory activity (Table 2). At the highest concentration of 0.5 mg/mL, pulp extract showed a maximum and effective inhibition of nearly 98.94 % whereas acarbose (control), showed a lower inhibition of 40.10%, under similar assay condition. The IC₅₀ of S. dulcificum pulp, seed and leaf were 0.08, 0.23, 0.30 mg/mL, respectively, which were evidently less than that for acarbose (0.70 mg/mL). The lower value of IC₅₀ in S. dulcificum extracts showed that all the extracts are effective and strong inhibitors of α-glucosidase when compared to the synthetic drug, acarbose.

When comparing the results of α-amylase IC₅₀ values to α-glucosidase, all the S. dulcificum extracts showed higher α-glucosidase inhibitory activity than α-amylase. According to Mogela et al.,.S. dulcificum [20] natural inhibitors from plants are mostly reported to have a lower inhibitory effect against α-amylase but stronger inhibitory activity against α-glucosidase. Anuya and Abhay, [21] also found
similar results whereby the aqueous extract of *Ocimum sanctum* Linn showed lower α-amylase inhibitory activity and stronger α-glucosidase inhibitory activity than the ethanol extract.

Overall, due to the strong inhibitory properties, *S. dulcificum* pulp has great potential for use in functional food application and thus pulp extract was selected for the subsequent experiments of the yogurt preparation. Additionally, the strong α-glucosidase inhibition and mild inhibition of α-amylase makes the extract a good source for management of type-2 diabetes with potentially minimum side effects than that currently observed with several drugs being used for management of non-insulin dependent diabetes mellitus. For example, the use of acarbose is reported to be associated with gastrointestinal side effects caused by the excessive inhibition of pancreatic α-amylase, resulting in the abnormal bacterial fermentation of undigested carbohydrates in the large intestine [22].

### Table 2. α-glucosidase inhibitory activity of *S. dulcificum* plant parts and synthetic drug, acarbose

| Concentration (mg/mL) | % Inhibition by acarbose | % Inhibition by leaf | % Inhibition by seed | % Inhibition by pulp |
|-----------------------|--------------------------|----------------------|----------------------|----------------------|
| 0.03                  | 10.35±(0.05)             | 11.20±(0.02)         | 17.90±(0.01)         | 20.92±(0.01)         |
| 0.06                  | 11.04±(0.02)             | 23.10±(0.01)         | 26.57±(0.05)         | 48.49±(0.03)         |
| 0.13                  | 15.37±(0.01)             | 31.60±(0.05)         | 35.34±(0.01)         | 68.73±(0.02)         |
| 0.25                  | 16.80±(0.01)             | 45.90±(0.03)         | 54.89±(0.02)         | 92.34±(0.01)         |
| 0.50                  | 40.10±(0.02)             | 72.80±(0.07)         | 86.63±(0.04)         | 98.94±(0.06)         |
| IC_{50} value (mg/mL) | 0.70                     | 0.30                 | 0.23                 | 0.08                 |

Data was expressed as mean ± standard deviation of triplicate determinations.

IC_{50}: Concentration of inhibitor to inhibit 50% of the enzyme activity under the assayed conditions.

a,b,c,d,e Means values in the same column expressed with different superscript letters are significantly different at (P< 0.05).

### 3.2 Formulation of functional yogurt

#### 3.2.1. α-amylase inhibitory activity

In this study, the α-amylase inhibitory activities increased significantly (P< 0.05) for both types of yogurts after the fermentation step, up to day 7 (Figure 1). At day 7, which is the highest α-amylase inhibitory activities, high inhibition of 69.23% was observed in *S. dulcificum* yogurt when compared to plain yogurt (18.46%). According to Ni *et al.*, (2018), the increase of α-amylase inhibitory activities in yogurts supplemented with salal berry (*Gaultheria shallon*) and blackcurrant (*Ribes nigrum*) pomace during cold storage were attributed to the viability of lactic acid bacteria. The proteolytic activity of lactic acid bacteria [23] contributed to the release of peptides with moderate α-amylase inhibitory activity.

Refrigerated storage also has profound effect on yogurts inhibition of α-amylase activity. This was demonstrated by a decrease in α-amylase inhibition (P < 0.05) about 26% during storage from day 7 to day 21. The present study showed that the inclusion of *S. dulcificum* pulp extract in yogurt could increase the therapeutically values of the yogurt via higher α-amylase inhibitory activity during storage compared to plain yogurt.

#### 3.2.2. α-glucosidase inhibitory activity

The α-glucosidase inhibitory activity was significantly (P< 0.05) affected by both types of yogurt and storage duration as shown in Figure 2. Yogurt that supplemented with *S. dulcificum* pulp had higher activity than the plain yogurt with the highest α-glucosidase inhibitory activity was observed on day 1. High α-glucosidase inhibitory activity of yogurt fortified with *S. dulcificum* pulp indicated that the addition of pulp extract enhanced the inhibitory activity. This could be mainly due to the presence of phytochemical compounds in the pulp extract such as anthocyanin, flavonoids and phenolic compounds [6]. Anthocyanin is sub-type of organic compound of flavonoid family and a member of large group of compounds [24]. *S. dulcificum* was previously reported to have 11.04 mg/100 g FW of total anthocyanin content, that possibly responsible for the red color of the fruit [6].
However, when compared to the screening result, there was high reduction of inhibitory activity of this enzyme when fortified into yogurt. This could be due to the low content of flavonol compounds in the yogurt. Flavonol compounds, such as quercetins and its derivatives have particularly high affinity towards the α-glucosidase enzyme in vitro [25]. Additionally, the decreased of the activity as observed after day 7 might be due to the anthocyanin stability in the S. dulcificum pulp extract. Arueya and Akomolafe [26], stated that anthocyanin are highly unstable molecules in food matrix and their stability is strongly affected by pH, solvents, temperature, oxygen, light, enzymes, anthocyanin concentration, structure and other accompanying substances. Their studies also found similar results where there was a decrease in α-glucosidase inhibitory activity of yogurt fortified with Allium sativum from 15.2% to 12.8% during 21 days of cold storage. Likewise, the α-glucosidase inhibitory activity of probiotic yogurt supplemented with Roselle extract was decreased during 15 days of cold storage [27].

On the other hand, for plain yogurt, the inhibitory activity observed was most probably due to the presence of bioactive peptides produced through proteolysis by LAB [28]. During fermentation, protein content in the milk is degraded by LAB into peptides. Several bioactive peptides have been found to be able to inhibit the activity of α-glucosidase enzyme [29]. Moreover, probiotic strain also has been reported to have an anti-diabetic effect. For instance, Lactobacilli probiotic has the potency to be anti-diabetic probiotic due to its ability to act as α-glucosidase inhibitor [30].

**Figure 1.** α-amylase inhibitory activity of yogurt fortified with S. dulcificum pulp extract (■) and plain yogurt (●) during cold storage at 4°C. The error bars represent the standard deviations about the mean (n=3).

**Figure 2.** α-glucosidase inhibitory activity of yogurt fortified with S. dulcificum pulp extract (■) and plain yogurt (●) during cold storage at 4°C. The error bars represent the standard deviations about the mean (n=3).

### 4. Conclusion

Overall, the data reported in this study suggesting that S. dulcificum pulp extract contained high α-amylase and α-glucosidase inhibitory activities. S. dulcificum pulp showed stronger α-amylase (IC$_{50}$ 9.66) and α-glucosidase (IC$_{50}$ 0.08) inhibitory activities compared to standard drug, acarbose, seed and leaf. In the view of these traits, the consumption of S. dulcificum pulp could be of great value in prevention of oxidative stress related diseases (such as atherosclerosis, coronary heart diseases and cancer) and may also be useful in the management of diabetes. The inclusion of S. dulcificum pulp extract into yogurt has the potential to be a new functional food-based product with high nutritional appeal and beneficial properties. However, from this preliminary study it was found that the α-glucosidase inhibitory activity showed a gradual decrement when the extract was formulated into yogurt, thus it is interesting to identify the compounds that are responsible on affecting the interactions between food matrix and α-glucosidase enzyme.
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