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In Vitro Antidiabetic Activity of "Green Tea" Soursop Leaves Brew Through α-Glucosidase Inhibition

Hardoko*[1], Yuniwaty Halim[2], StevellaVerena Wijoyo[3]

[1]Full-time Lecturer, Faculty of Fisheries and Marine Science, University of Brawijaya, Malang, East Java - Indonesia and Part-time Lecturer, Department of Food Technology, Universitas Pelita Harapan, Tangerang, Banten Province- Indonesia;

[2,3]Food Technology Department, Unoversitas Pelita Harapan, Tangerang, Banten Province - Indonesia

Abstract: Soursop leaves contain phytochemical compounds that can inhibit α-glucosidase enzyme activity; thus, they show potentiality as an antidiabetic agent. This research was aimed to observe the effect of brewing time and temperature towards inhibition of α-glucosidase enzyme activity and also to observe the physical, chemical and organoleptic characteristics of the brew. Initially, soursop leaves brew was prepared using green tea preparation method. Then, soursop leaves were brewed at 80°C, 90°C and 100°C for 15, 30 and 45 minutes. Results showed soursop leaves brew contained tannin, flavonoids and triterpenoid. The best time and temperature combination, which had the highest inhibition towards α-glucosidase enzyme activity (showed by the lowest IC_{50} value), was 100°C and 30 minutes of brewing with IC_{50} value of 396.70 ppm, phenolic content of 205.37 mg GAE/L, tannin content of 100.33 mg CE/L and flavonoids content of 99.97 mg QE/L. The brew was categorized as non toxic with LC_{50} value of 3801.89 µg/ml. The brew had reddish yellow colour and soursop leaves specific aroma, which was slightly not preferred by the panelists.
Keywords: "green tea" soursop leaves brew, antidiabetic, α-glycosidase inhibition, brewing temperature, brewing time.

Introduction

Soursop is a part of *Annoceae* family. This plant grows at tropical and subtropical area. All parts of soursop tree have a function as natural medicines, including its bark, leaves, roots and seeds. The bark, leaves, roots and seeds of soursop tree can be used as sedative, antispasmodic, hypoglycemic and hypotension agent[1].

Soursop (*Anonna muricata*) leaves are potential to be used as an antihypertension, antispasmodic, sedative, hypoglycemic, anticancer, emetic, and vermifuge agent[2]. Chemical compounds in soursop leaves consist of alkaloids, essential oils and acetogenins[3].

Diabetes mellitus is a chronic disease, in which the blood glucose level is high. Diabetes is usually treated with insulin and antidiabetic medicines, such as sulfonylurea, bigua-nides, α-glucosidase inhibitors and glinides. In developing countries, these medicines are not easily obtained and the price of these medicines is also relatively expensive[4]. Since soursop leaves has hypoglycemic potential[2], traditional or natural diabetes treatment can be done using soursop leaves.

Moreover, it has been reported that the wáter extract from soursop leaves played role in decreasing oxidative stress in pancreatic cells by streptozotocin on diabetic rats[5]. Besides, it has also been reported that supplementation of soursop leaves brew on patients with diabetes mellitus type II could decrease blood glucose

level[6]. Soursop leaves brew consumption of about 200 mL/day for 3 days consecutively decreased the blood glucose level for about 40.45 mg/dL.

Based on the fact that soursop leaves are able to act as hypoglycemic agent, it is possible to apply them in form of beverages, such as green tea product. In green tea making, there are withering, fixation, curling and

drying process. These processes are aimed for non-gallic catechin formation and removal of fresh leaves aroma, also for oxidation and fermentation prevention; resulting in better bioactivity[7]. Besides, it has also been reported that green tea was 2.5 times better antioxidant agent than black tea. Green tea also has different characteristics, in terms of colour, taste and sensation, compared to black tea[8].

Other factors that influence tea beverages are brewing time and temperature. Brewing time and temperature influence caffeine and catechin solubility, therefore they will influence the effect and organoleptic profiles of tea brew[9]. Moreover, green tea is already known for its effect as antidiabetic agent, because of its epigallocatechin gallate content, which is able to decrease the oxidative stress in pancreatic cells[10].

Based on these reasons, it is required to study the antidiabetic activity and characteristics of soursop leaves brew that is processed with green tea preparation method. The antidiabetic activity of soursop leaves will be observed *in vitro* through inhibition of α-glucosidase enzyme.

Materials and Methods

Materials

Material used was "green tea" soursop leaves processed from fresh soursop leaves (*Annona muricata*), which came from Perumahan Melati Mas, BSD, Tangerang, Indonesia. Supporting materials for the research were α-glucosidase enzyme from *Saccharomyces cerevisiae* (G5003 Sigma), bovine serum albumin (Merck), p-nitrophenyl α-D-glucopyranoside (N1377 Sigma), dimethyl sulphoxide (Merck), sodium carbonate (Merck), HCl (Merck), NaOH (Merck), $NaNO_2$ (Merck), $AlCl_3$ (Merck) and *Folin Ciocalteau* reagent (Merck).

Preparation of "Green Tea" Soursop Leaves

Soursop leaves, which have been processed according to green tea preparation, later will be called as "green tea" soursop leaves. Soursop leaves was prepared based on green tea making process[11]. At the beginning, soursop leaves were sorted to discard defect leaves and contaminants. The sorted leaves were then washed with water, drained and withered at room temperature for 16 hours by spreading them on nets. The leaves were roasted using oven with temperature of 90 ^oC for 8 minutes and then manually curled for 20 minutes. After that, the leaves were size-reduced until reached 3-4 mm length and dried in cabinet dryer with temperature of 70 ^oC until the moisture content reached about 3-5%. Thus, "green tea" soursop leaves were obtained[12,13].

Brewing of "Green Tea" Soursop Leaves

Brewing of "green tea" soursop leaves was performed at temperature of 80^oC (A1), 90^oC (A2), 100^oC (A3) for each 15 (B1), 30 (B2) and 45 (B3) minutes. Brewing was done by dissolving 2.3 gram of "green tea" soursop leaves in 200 ml water[14], using waterbath.

Determination of α-glucosidase Enzyme Inhibition on "Green Tea" Soursop Leaves Brew[15]

Inhibition test was done first by making a mixture of solution for reaction, which consists of 250 µL of 20 mM p-nitrophenyl- α- D glucopyranose, 490 µL of 100 mM phosphate buffer and 10 µL of "green tea" soursop leaves brew sample in DMSO solution (S) or DMSO as a blank (C). Each mixture was incubated at 37^oC for 5 minute, and then 250 µL of enzyme solution was added into positive control and sample. Moreover, 250 µL of phosphate buffer (pH 7) was added into negative control and blank. The mixture was then incubated at 37^oC for 15 minutes. Inhibition reaction was stopped by addition of 1000 µL of Na_2CO_3 and the absorbance for each mixture was measured at 400 nm. The concentrations of "green tea" soursop leaves sample were 6.25 ppm, 12.5 ppm, 25 ppm and 50 ppm. % inhibition was calculated using a formula:

% inhibition = (control absorbance – sample absorbance)/ control absorbance x 100%

Sample concentration and %inhibition were plotted into linear regression equation: Y= a + bx. IC_{50} value (inhibition activity), defined as sample concentration that can inhibit 50% of enzyme activity, was calculated.

Determination of Total Phenolic Content[16]

Determination of total phenolic content was done using Folin Ciocalteau method. A 0.1 ml solution of sample was put into reaction tube and 0.1 ml of Folin Ciocalteau reagents was added. The mixture was homogenized using vortex and added with 2% Na_2CO_3. Solution mixture was then incubated in a dark room for 30 minutes. The absorbance of solution was measured at 750 nm. The absorbance results were plotted in standard curve equation which was made using gallic acid with concentration of 100, 150, 200 and 250 ppm. The measurement results were calculated as mg gallic acid equivalent (GAE)/ L of sample or ppm.

Determination of Total Flavonoids[17]

A 2 ml of "green tea" soursop leaves brew sample or standard solution was put into reaction tube and mixed with 2 ml of 2% $AlCl_3$ which has been dissolved in methanol, homogenized using vortex and measured for its absorbance at 425 nm. Sample absorbance was plotted into standard curve equation, which was prepared using quercetin with concentration of 25, 50, 75 and 100 ppm. The measurement results were calculated as mg quercetin equivalent (QE)/ L of sample or ppm.

Determination of Condensed Tannin[18]

A 0.5 ml of "green tea" soursop leaves brew sample or standard solution was added with 2 ml of 4% vanillin in MeOH. The solution was homogenized using vortex and added with 1 ml of concentrated HCl. The solution was then incubated at room temperature for 20 minutes and was measured for its absorbance at 500 nm. Sample absorbance was plotted into standard curve equation, which was prepared using catechin with concentration of 25, 50, 75 and 100 ppm. The measurement results were calculated as mg catechin equivalent (QE)/ L of sample or ppm.

Brine Shrimp Lethality Test[19,20]

This method used *Artemia salina* Leach larva as a testing animal. At the beginning, *A. salina* was hatched in artificial seawater (38 gram of table salt in 1000 ml of water) under 20 Watt TL lighting. After 38 hours, larva will hatch to become nauplii instar III/IV and ready to use as a testing animal. *A. salina* larva was put into vial which has already been filled with extract of sample solution with certain concentration. All vials were incubated at room temperature for 24 hours. An observation was performed after incubation by counting the number of dead *A. salina* at each concentration. LC_{50} value was calculated in µg/mL or ppm using a table of probit value.

Results and Discussion

Phytochemical Compound in "Green Tea" Soursop Leaves Brew

Tabel 1. Phytochemical compound in fresh soursop leaves brew and "green tea" soursop leaves brew

Phytochemical compound	Brew	
	Fresh soursop leaves	"Green tea" soursop leaves
Tannin	+	+
Flavonoids	+	+
Alkaloid	-	-
Steroid	-	-
Triterpenoid	+	+

Notes: + = detected
- = undetected

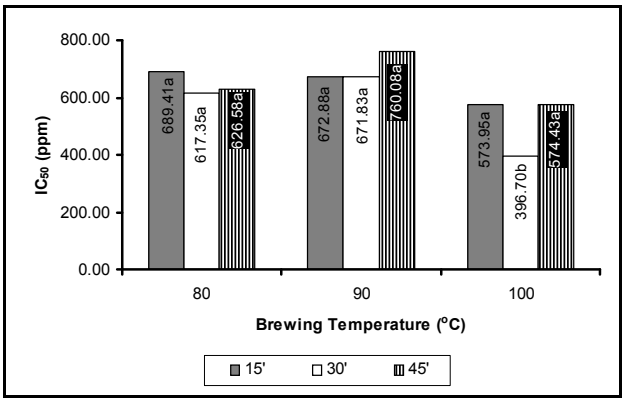
Table 1 show that soursop leaves brew contains condensed tannin, flavonoids and triterpenoid. This result was in accordance with a previous research[21], which reported that soursop leaves thin extract with water solvent, contained condensed tannin and flavonoids. It was also reported that condensed tannin could give antidiabetic effect through inhibition of α-glucosidase enzyme[22]. Flavonoids could also give a specific and

strong inhibition towards α-glucosidase enzyme[23]. Terpenoids in soursop leaves was also found in soursop leaves methanol extract[24]. Moreover, it was explained that triterpenoids compound could inhibit α-glucosidase enzyme[25]. Tannin, flavonoids and triterpenoids content in soursop leaves make them potential as an antidiabetic functional beverage or medicine.

Inhibition Activity of α-glucosidase Enzyme and Phytochemical Compound Content

α-glucosidase is an enzyme which plays role in starch and glycogen (in animal tissue) metabolisms. In the other words, α-glucosidase enzyme is involved in carbohydrate digestion into glucose[26]. α-glucosidase from *Saccharomyces cerevisiae* contains α-1,4-glucosidase (maltase) dan oligo-1,6-glucosidase (isomaltase), which are able to hydrolyze α-1,4 and α-1,6 bonds in starch or glycogen[27]. Thus, inhibition of α-glucosidase enzyme activity could decrease its ability to hydrolyze carbohydrate. This is often considered as hypoglycemic activity or antidiabetic activity.

ANOVA results show that inhibition of α-glucosidase enzyme by soursop leaves brew was influenced by brewing temperature ($p<0.05$), but was not influenced by brewing time ($p>0.05$). Further statistical results of inhibition data, in form of IC_{50} value, can be observed at Figure 1.



Notes: different notation shows significant difference at α=0.05
Figure 1. IC50 value of "green tea" soursop leaves at various brewing time and temperature

From Figure 1, it can be observed that brewing temperature at 100°C for 30 minutes resulted in the lowest IC_{50} value. It is related to a statement[28] that the higher the temperature, the more efficient the extraction process. The heat used can increase cell wall permeability, increase the solubility and diffusion of extracted phytochemical compounds. The extracted phytochemical compounds are suspected to be involved in enzyme inhibition. However, increasing the brewing time to 45 minutes did not increase the enzyme inhibition activity. It was suspected that there were bioactive phytochemical compounds which are destructed by heat in longer brewing time.

Compared to *Nicotiana tabacum* leaves extract[29], which has IC_{50} value of 5300ppm, "green tea" soursop leaves brew had a better inhibition activity towards α-glucosidase. Nevertheless, compared to acarbose medicine[30] which has IC_{50} value of 130 ppm, "green tea" soursop leaves brew had a lower inhibition activity towards α-glucosidase.

Inhibition activity towards α-glucosidase by "green tea" soursop leaves brew was related to phytochemical compound content, including tannin and flavonoids, as well as triterpenoid, as shown at Table 2. Table 2 shows that as the brewing time and temperature increase, there was also an increase in phytochemical content level.

Table 2. Phenolic, tannin and flavonoids content in "green tea" soursop leaves brew

Treatment	Total Phenolic (mg GAE/L)	Tannin (mg CE/L)	Total Flavonoids (mgQE/L)
80°C, 15'	135.28[a]	5.67[a]	50.44[a]
80°C, 30'	164.45[b]	61.67[cd]	56.40[a]
80°C, 45'	191.03[bc]	53.00[bc]	70.20[b]
90°C, 15'	122.70[a]	39.00[b]	64.48[ab]

90°C, 30'	170.28[b]	89.67[ef]	79.29[b]
90°C, 45'	223.53[c]	73.67[de]	86.43[c]
100°C, 15'	185.20[b]	110.33[f]	73.20[b]
100°C, 30'	205.37[c]	100.33[f]	99.97[d]
100°C, 45'	242.03[d]	94.33[f]	102.62[d]

Notes: different notation at the same column shows significant difference at α = 0.05

There is a correlation between inhibition activity towards α-glucosidase and tannin, flavonoids and triterpenoid content[22, 23, 25]. Condensed tannin can give antidiabetic activity through inhibition towards α-glucosidase enzyme[22], and it was also reported that tannin could delay the glucose absorption in human intestine[31]. Besides, it was also stated that flavonoids could give a strong and specific inhibition towards α-glucosidase[23]. Inhibition mechanism towards α-glucosidase is done through hydroxylation bonding and substitution at β ring. This results in delay of carbohydrate hydrolysis, glucose absorption and inhibition of carbohydrate metabolism into glucose[32].

Physical and Organoleptic Characteristics of "Green tea" Soursop Leaves Brew

ANOVA results show that brewing time of 15, 30 and 45 minutes did not influence the physical characteristics and organoleptic (hedonic and scaling) score at "green tea" soursop leaves brew ($p>0.05$). On the other hand, brewing temperature only influenced lightness, taste and aroma intensity (organoleptic scaling) of brew, as can be observed at Table 3.

Table 3. The influence of brewing temperature on physical and organoleptic characteristics of "green tea" soursop leaves brew

Treatment	Colour		Hedonic score		Scaling	
	(°Hue)	Lightness (L*)	Taste	Aroma	Aroma	Taste
80°C, 15'	90.1	53.5[a]	3.6[a]	4.0a	4.1[a]	4.5[a]
80°C, 30'	89.2	53.1[a]	3.7[a]	4.1a	4.6[a]	4.7[a]
80°C, 45'	88.9	52.1[a]	3.6[a]	4.1a	5.2[ab]	5.0[ab]
90°C, 15'	88.7	52.1[a]	3.6[a]	4.0a	4.8[a]	5.0[ab]
90°C, 30'	88.5	52.1[a]	3.6[a]	4.0a	5.1[ab]	5.1[ab]
90°C, 45'	87.7	51.5[a]	3.7[a]	4.2a	5.7[b]	5.1[ab]
100°C, 15'	85.1	51.2[a]	3.9[a]	4.3a	5.3[ab]	4.7[a]
100°C, 30'	84.4	50.5[ab]	3.5[a]	4.0a	5.6[b]	5.1[ab]
100°C, 45'	82.7	49.4[b]	3.5[a]	4.2a	6.3[b]	5.3[b]

Notes: different notation at the same column shows significant difference at α = 0.05
Hedonic score : 1-7= dislike extremely - like extremely
Aroma scaling: 0 - 10 = soursop leaves aroma very weak – soursop leaves aroma very strong
Taste scaling: 0 - 10 = very not astringent – very astringent

Colour and lightness results of "green tea" soursop leaves at Table 2 show the similar colour range, which was 54-90°Hue, defined as yellow-red. However, the lightness (L*) decreased as the brewing temperature increased. According to previous research[33], the colour change in *Camellia sinensis* brew into a darker colour is caused by degradation of chlorophyll compound by chlorophyllase enzyme. This condition can occur at higher brewing temperature. This result was supported by another research which reported that there was an significant increase in turbidity in water extracted chamomile (*Matricaria chamomilla* L.) at brewing temperature of 90°C and 100°C[34].

It was also reported that ethanol extract of *Camellia sinensis* green tea gave L* value of (76.92±0.02)[35]. "Green tea" soursop leaves brew had L* value of 49.5-53.5. It shows that ethanol extract of green tea is brighter than "green tea" soursop leaves brew.

Hedonic test results were at interval of 3-4, which means the preference was between slightly dislike and neutral. This shows that the panelists slightly dislike the taste and aroma of "green tea" soursop leaves brew. This is possibly caused by several plant compounds that are not slightly preferred, such as phenolic

compound, saponins and triterpenoid. These compounds cause a specific taste and aroma on each leaves types[36,37].

Taste and aroma organoleptic scaling was performed to determine the aroma and taste intensity of "green tea" soursop leaves brew. A lower preference on "green tea" soursop leaves brew is possibly related to the intensity of specific soursop leaves aroma which is detectable by the panelists (scaling score of 4.1-6.3). As the brewing time and temperature increased, the aroma intensity also increased. Moreover, the astringency taste tend to be undetectable (scaling score of 4.5-5.3), therefore it did not influence the preference and acceptance of panelists. The higher aroma intensity is related to the higher amount of extracted phytochemical compounds (Table 2). Besides, the increase in aroma intensity could be related to the presence of triterpenoid in "green tea" soursop leaves brew. This is possibly related to a statement that triterpenoid plays role in specific tea aroma[37]. The higher the brewing temperature, specific aroma of green tea will be more intense[38].

Another report stated that soursop leaves contain acetogenin compounds which are considered as toxic[39], but "green tea" soursop leaves brew had LC_{50} value 3801.89 µg/mL, so it can be categorized as non toxic. LC50 value higher than 1000 µg/mL means that a compound is categorized as non toxic[40]. As a comparison, LC50 value of ethanol extract of soursop leaves[41] is 2.74 µg/mL.

5.1 Conclusion

"Green tea" soursop leaves brew contained phytochemical compounds, i.e. tannin, flavonoids and triterpenoid, which are potential to inhibit α-glucosidase activity.

The best inhibition of α-glucosidase activity was from soursop leaves which were brewed at 100^{o}C for 30 minutes, with IC50 value of 396.70 ppm, phenolic content of 205.37 mg GAE/L, tannin content of 100.33 CE/L and flavonoids content of 99.97 mgQE/L. These results showed that soursop leaves brew have a potential as an antidiabetic functional drink.

Moreover, "green tea" soursop leaves brew was non-toxic, had reddish yellow colour and was still slightly not preferred by panelists because of its specific soursop leaves aroma.

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