The Role of *Psidium Guajava* Leaves as A Functional Agent for Anti-Diabetic Therapeutics

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ABSTRACT

Background: *Psidium guajava* leaves have traditionally been used for the prevention and treatment of various diseases such as rheumatism, diarrhea, diabetes mellitus, and cough. The pharmacological research *in vitro* as well as *in vivo* has discovered numerous health beneficial effects of *P. guajava* leaves. **Aims and Objective**: To evaluate the *in vitro* antidiabetic activity of *Psidium guajava* leaves. **Methods**: α-amylase inhibitory assay was examined via dinitrosalicylic acid reaction. Glucose uptake was investigated using the LO-2 cell model. DPPH and $ABTS^+$ scavenging assay were performed by spectrophotometry. Nitric oxide production was measured by the Griess reaction. Cell viability was conducted by MTT assay. **Results**: It was found that PGL extract considerably inhibited enzyme α-amylase activity up to (65 ± 6.2)% at the concentration of 200 µg/ml. Furthermore, the glucose adsorption efficiency of PGL was revealed at (3.1 ± 0.28) mM glucose/g extract. On the other hand, the extract significantly enhanced glucose uptake up to (186 ± 9.1)% in human liver LO-2 cells. Notably, PGL extract was effective in scavenging DPPH and $ABTS^+$ radicals up to (77.2 ± 3.7)% and (82.5 ± 5.6)%, respectively, and reducing NO production up to (31.1 ± 4.6)% from RAW264.7 cells without any cytotoxic effects. **Conclusion**: These biological activities of *P. guajava* leaves indicate its important role in the management of diabetes.

Key Words: α-amylase; *Psidium guajava*; glucose uptake; antioxidant; antidiabetes.

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INTRODUCTION

Type 2 diabetes is a very prevalent disease, causing a substantial increase in premature mortality, comorbidity, and increased healthcare costs metabolic disorder [1-3]. Indeed, insulin deficiency causes carbohydrate metabolism disorders resulting in chronic hyperglycemia in the diabetic patient. Moreover, diabetes is associated with severe long-term complications, such as retinopathy, nephropathy, neuropathy, myocardial infarction, cerebral embolism, and blood vessel damages [4-6]. Luckily, diabetes can be managed via proper diet, exercise and pharmacologic interventions [7, 8]. Especially, various herbal plants have been considered as apart of currently available therapeutics for diabetes treatment [9-12]. They have been used as traditionally around the world due to their effectiveness, fewer side effects, and relatively low cost [13]. Therefore, the scientific pieces of evidence regarding the hypoglycemic effect of herbal plants need to be investigated for the further development of natural antidiabetic products.

Psidium guajava L., commonly called as guava, is a small plant belonging to the Myrtaceae family. Guava is widely grown in tropical countries and traditionally used due to its food and nutrition values. Especially, *P. guajava* leaves have long been used in folk medicine in several countries, mainly as anti-gastrointestinal disturbance remedies such as diarrhea, peristaltic reflex, and gastroenteritis. Moreover, it has been used for the treatment of female-related diseases like dysmenorrhoea, uterine bleeding, and premature labor [14]. Up to now, various bioactive compounds have been found from *P. guajava* leaves, especially polyphenols. Simultaneously, numerous health beneficial effects of *P. guajava* leaves were also evidenced including hepatoprotective, antioxidant, anti-inflammatory, antispasmodic, anticancer, antimicrobial, and anti-hyperglycemic activities. Notably, two important flavonoids including quercetin and guaijaverin from *P.*

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guajava leaves was indicated by their important pharmaceutical properties [15]. Recently, *P. guajava* leaves have got much attention from researchers and consumers due to a decrease in hyperglycemia. So far, the hypoglycemic effect of *P. guajava* leaves was determined *in vivo* models [16, 17]. In the present study, the *in vitro* antidiabetic activity of *P. guajava* leaves was further investigated.

MATERIALS AND METHODS

Materials

The *Physalis angulate* leaves were collected from Tay Ninh province, Vietnam. Ethanol was obtained from Xilong (China). α-amylase (A4582) and other reagents were obtained from Sigma–Aldrich (St. Louis, MO, USA).

Extraction

The powder of *P. angulate* leaves was extracted by the following conditions: ethanol 98%, time (4 h), ratio (1/8, w/v), and temperature (60 ^{0}C). The ethanol extract was kept at a low temperature for further investigation.

The α-amylase inhibitory assay

The α-amylase inhibitory assay was conducted as reported by Bhutkar and Bhise [18]. Briefly, 1ml of extract or Acarbose was pre-incubated with 1 ml of α-amylase (A4582, diluted for 10.000 in 20 mM sodium phosphate buffer, pH 6.9) for 30 min. Afterward, 1 ml of starch solution (1% w/v) was added and incubated for 10 min at 37°C. Subsequently, 1 ml of DNS reagent was added and heated for 5 min. The control (C) was absent from the extract while the blank (B) is absent from the amylase enzyme. The absorbance was measured at 540 nm using Genova Nano (Jenway, UK). The inhibition was determined by the following equation:

Inhibition (%) = $[(OD_C - OD_B) – (OD_{sample} - OD_B)]/(OD_C - OD_B)*100\%$

The efficiency of glucose adsorption

The efficiency of glucose adsorption was conducted as reported by Ou et al. [19]. Briefly, 25 ml of a mixture containing extract (1%, w/v) and glucose solution (10, 50, or 100 mM) was incubated for 6 h at 37 °C. The mixture was then centrifuged for 20 min at 4,000g and the glucose content in the supernatant was determined.

Glucose adsorption efficiency = [(G1 – G2)*Volume of solution]/Weight of sample

Where:

- G1 = glucose concentration in the mixture without extract
- G2 = glucose concentration in the mixture with extract

Glucose uptake capacity

The glucose uptake capacity was determined as reported by van de Venter et al. [20]. In brief, LO-2 cells ($1x10^4$ cells/ml) were treated with the extractor metformin for 48 h. The supernatant was then replaced by a 50 µl incubation buffer containing 8 mM of glucose and further maintained for 3 h at 37 °C. After incubation, the concentration of glucose in the culture medium was determined by using Contour™ Plus Meter (Ascensia Diabetes Care, Switzerland). Glucose uptake capacity was determined as the following equation:

Glucose uptake capacity (%) = [(8 – T)/(8 – C)]*100

Where:
- C = the group without extract treatment
- T = the group with extract treatment

The free radical scavenging ability

The free radical scavenging ability of extract was conducted by the 1,1-diphenyl-2-picryl-hydroxyl (DPPH) and 2,2-Azinobis-3-Ethyl benzothiazoline-6-sulfonic acid (ABTS) assays as reported by Vo et al. [21]. The free radical scavenging ability was determined as the following equation:

Free radical scavenging ability (%) = $[(OD_{control} – OD_{sample})/OD_{control}]$ x 100%

Where:

- $OD_{control}$ = absorbance of the group without extract treatment
- OD_{sample} = absorbance of the group with extract treatment

Nitric oxide (NO) production assay

The investigation of NO production from the cultured cells was conducted via Griess reaction as shown by Vo et al. [22]. The level of NO was determined as the following equation:

NO production (%) = (T – B)/(C – B) × 100

Where, B is absent from extract and LPS, C is present with LPS without extract, while T is present both LPS and extract.

Cell viability assay

The viability levels of the cells were determined by MTT assay. In brief, the cells ($1x10^5$ cells/ml) were treated with extract for 24 h. The medium was replaced by MTT solution (1 mg/ml) for 4 h before adding 100 µl of DMSO.

The absorbance was determined at 540 nm using a microplate reader. The percentage of the cell viability was calculated as compared to the blank group (without extract treatment).

Statistical analysis

Data were analyzed using Pair sample T-test of statistical package for the social sciences (SPSS). The statistical differences among groups were considered significant at $p < 0.05$.

RESULTS

The α-amylase inhibitory activity of PGL extract

To evaluate the inhibitory capability of *P. guajava* leaves (PGL) on α-amylase activity, the extract was incubated with α-amylase before adding a starch solution. The inhibition was indicated by measuring the formation of reducing glucose in this reaction mixture. The result showed that PGL extract exhibited significant inhibition on enzyme α-amylase activity in a concentration-dependent manner (Fig. 1). The inhibitory effect was shown up to (65 ± 6.2)% at the concentration of 200 µg/ml. Meanwhile, acarbose exhibited inhibition up to (59 ± 5.3)% at 100 µg/ml.

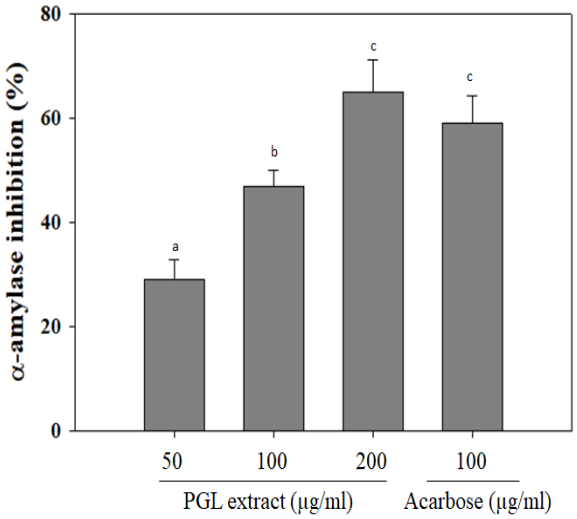

Fig. 1. The inhibitory activity of *P. guajava* leaves (PGL) extract on enzyme α-amylase. Each test was independently repeated in three experiments. Different letters a–c show difference among groups significantly ($p < 0.05$).

The efficiency of glucose adsorption of PGL extract

Glucose adsorption efficiency of PGL extract was examined by using a mixture containing the extract and the indicated glucose concentration. In this assay, PGL extract (1%, w/v) exhibited glucose adsorption efficiency at various doses of glucose (Fig. 2). The level of glucose bound increased with increased glucose dose. The Fig. 2 showed adsorption efficiency of PGL up to (0.9 ± 0.11), (2.4 ± 0.29), and (3.1 ± 0.28) mM glucose/g extract at glucose dose of 10, 50, or 100 mM, respectively.

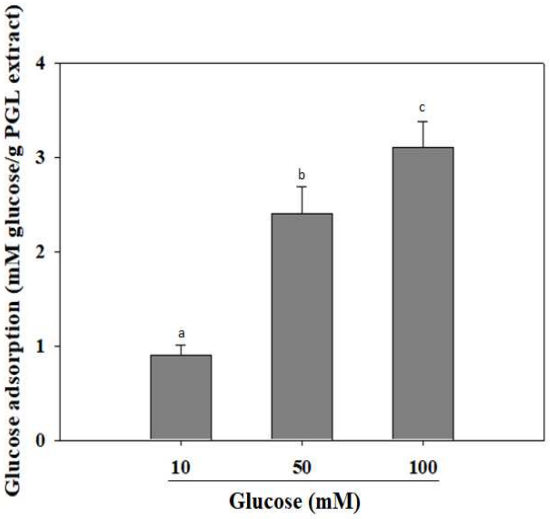

Fig. 2. The glucose adsorption efficiency of *P. guajava* leaves (PGL) extract. Each test was independently repeated in three experiments. Different letters a–c show difference among groups significantly ($p < 0.05$).

Glucose uptake ability of PGL extract

To investigate whether PGL can stimulate glucose uptake, hepatic LO-2 cells were pre-treated with extract before incubated with the indicated concentration of glucose. The stimulatory capacity of PGL extract on glucose uptake into LO-2 cells was shown in Fig. 3. PGL extract enhanced glucose uptake into LO-2 cells in a dose-dependent manner as compared to the control group (without extract treatment). The glucose uptake level was shown up to (186 ± 9.1)% at 200 µg/ml of PGL treatment. Meanwhile, metformin stimulated glucose uptake up to (200 ± 11.3)% at the concentration of 20 µg/ml.

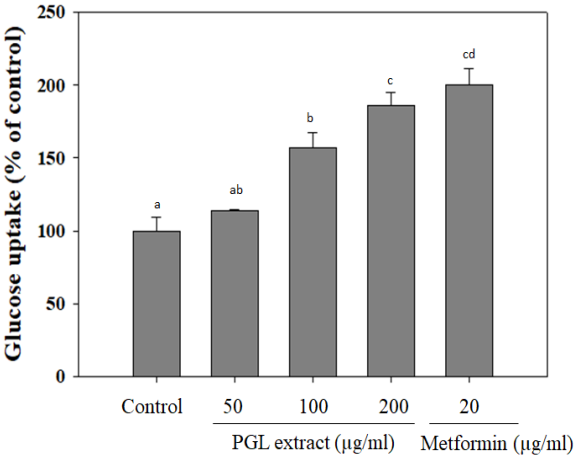

Fig. 3. The glucose uptake ability of *P. guajava* leaves (PGL) extract on in LO-2 cells. Each test was independently repeated in three experiments. Different letters a–d show difference among groups significantly ($p < 0.05$).

Free radical scavenging activity of PGL extract

To determine the antioxidant activity of PGL extract, its scavenging ability on DPPH and $ABTS^+$ radicals was examined. As shown in Fig. 4, the scavenging capacity of PGL extract on DPPH and $ABTS^+$ radicals was shown up to (77.2 ± 3.7)% and (82.5 ± 5.6)% at the concentration of 200 µg/ml, respectively. Meanwhile, vitamin C was effective in scavenging (81.8 ± 4.9)% DPPH and (98 ± 3.8)% $ABTS^+$ radicals at the concentration of 20 µg/ml.

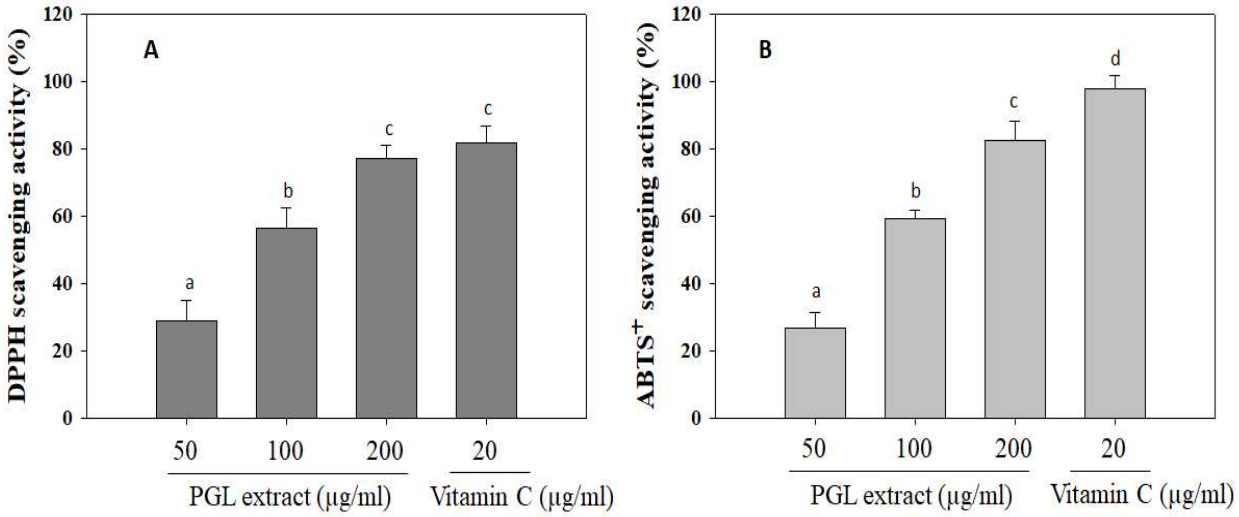

Fig. 4. The free radical scavenging ability of *P. guajava* leaves (PGL) extract. Each test was independently repeated in three experiments. Different letters a–d show difference among groups significantly ($p < 0.05$).

Inhibitory effect of PGL extract on nitric oxide (NO) production

In this study, the suppressive effect of PGL extract on LPS-induced NO production from RAW 264.7 macrophage cells was examined. Fig. 5 showed that PGL extract significantly decreased NO production from the cultured cells. The NO production levels were reduced to (77.3 ± 6.8)%, (43.4 ± 5.9)%, and (31.1 ± 4.6)% at the concentration of 50, 100, and 200 µg/ml, respectively. Meanwhile, ibuprofen reduced NO production to (22 ± 6.3)% at the concentration of 100 µg/ml.

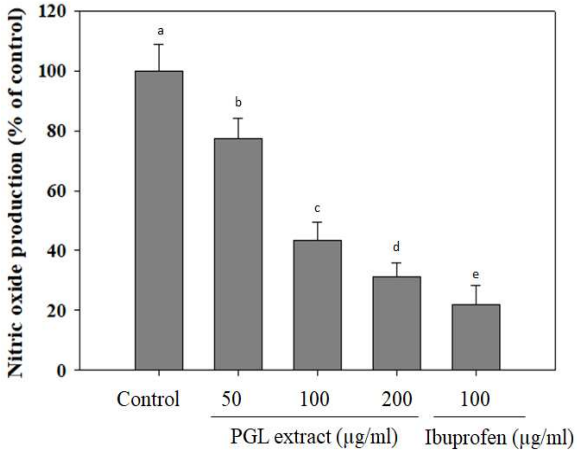

Fig. 5. The inhibitory activity of *P. guajava* leaves (PGL) extract on NO production from LPS-stimulated RAW 264.7 cells. Each test was independently repeated in three experiments. Different letters a–e show difference among groups significantly ($p < 0.05$).

The effect of PGL extract on cell viability

In the present study, the cytotoxicity effect of PGL extract was investigated on RAW 264.7 macrophage cells and hepatic LO-2 cells. Fig. 6 showed that the cell viability levels in the presence of PGL extract were identified in a range of (88 – 96) % for LO-2 cells and (92 – 99)% for RAW 264.7 cells as compared with the blank (no addition of extract). These results indicated that PGL extract has no significant cytotoxic effect on hepatic cells and macrophage cells in a range concentration of (50 - 200 µg/ml).

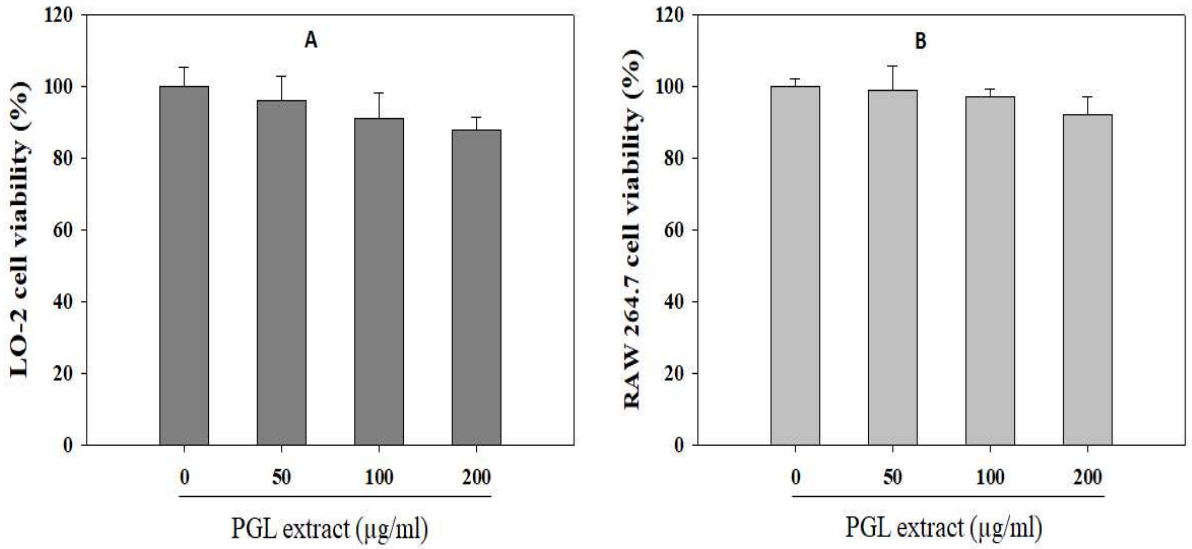

Fig. 6. The cytotoxic effect of *P. guajava* leaves (PGL) extracts on LO-2 cells (A) and RAW 264.7 cells (B). Each test was independently repeated in three experiments.

DISCUSSION

It is literature that the α-amylase is secreted by the pancreas and salivary gland responsible for the hydrolysis of complex carbohydrates to oligosaccharides and disaccharides in the intestinal mucosa. The enzyme α-amylase contributes to the increase in postprandial glucose concentration in diabetic patients [23]. Thus, the inhibition of α-amylase activity can decrease carbohydrate digestion and reduce postprandial hyperglycemia [24]. The current therapeutics for α-amylase inhibition exhibit several side effects such as diarrhea and flatulence [25]. Therefore, alternative medicine with a low side effect for the management of diabetes is necessary. *P. guajava* leaves have long been used as a folk medicine for the management of diabetes. Indeed, PGL extract was determined to be effective in the inhibition of α-amylase activity in this study. Especially, PGL extract possessed stronger inhibition than *Momordica charantia* ($IC_{50} = 0.267 \pm 0.024$ mg/ml) on α-amylase activity [26]. It has evidenced that mice treated with specific α-amylase inhibitors slow the breakdown of sucrose and starch [27]. Hence, the inhibitory activity of PGL extract on α-amylase partly contributes to the reduction of hyperglycemia in type 2 diabetes.

Glucose adsorption property of certain natural products is useful in the prevention of carbohydrate absorption after food intake. In particular, some natural products such as bitter melon, banana, ginseng, *Gymnema Sylvestre*, fenugreek, and *Coptis Chinensis* were indicated as glucose adsorption agents and exhibited hypoglycemic effect in type 2 diabetes [28]. Moreover, PGL extract was also found as a potential adsorbent of the glucose. It was found that grain of cereals and millets possessed glucose adsorption capacity at (0.49 ± 0.02) – (1.07 ± 0.02) mM glucose/g fiber [29]. As a result, the capacity of glucose adsorption of PGL extract was significantly higher than that of these insoluble fibers. Hence, PGL extract was suggested to be able to decrease the transport across of glucose in the intestinal lumen and reduce the postprandial hyperglycemia.

It is well-known that insulin enhances glucose uptake into its target tissues. Conversely, insulin resistance or deficiency causes a decrease in glucose uptake and an increase in endogenic hepatic glucose production in diabetic patients [30]. Thus, nutraceuticals that can modulate glucose uptake represent a promising dietary tool for the management of hyperglycemia [31]. Notably, PGL extract significantly stimulated the glucose uptake into the hepatic LO-2 cells. This action of PGL extract could be suggested to be related to the recruitment of glucose transporters from a large intracellular pool to the plasma membrane of the cells, and thus stimulating glucose metabolism in its target tissues [32].

Oxidative stress is known as an imbalance between the accumulation and production of free radicals in the body. It has been associated with the apoptosis of pancreatic beta-cells and insulin resistance [33]. Moreover, the role of oxidative stress has evidenced in the pathogenesis and development of complications from diabetes including retinopathy, nephropathy, neuropathy, and accelerated coronary artery disease [34]. Meanwhile, antioxidant acts via scavenging free radicals or increasing the antioxidant defense enzyme capabilities. Therefore, it can stop oxidative stress and related diabetic complications [35]. Interestingly, PGL extract was found to possess strong antioxidant via scavenging DPPH and $ABTS^+$ radicals. *P. guajava* leaves have been reported to contain various phenolic compounds that may contribute to its high antioxidant activity. As a result, the antioxidant capacity of

PGL extract partly leads to a delay in the development of diabetes and its complications.

Nitric oxide (NO) has been reported to exhibit different regulations depending on its concentration [36]. The high levels of NO may be cytotoxic for beta-cells via inhibiting insulin secretion, disrupting electron transport, and inducing lipid peroxidation and apoptosis [37]. Moreover, NO may cause the development of diabetes complications, both microvascular and cardiovascular [38]. In this study, NO production level from LPS-stimulated RAW 264.7 cells was decreased by the presence of PGL extract. Importantly, the suppression of PGL extract on NO production was higher than that of oolong tea (*Camellia sinensis*) [39] and Red Ginger (*Zingiber officinale*) [40]. The action of PGL extract on radical scavenging activity and NO production inhibition could contribute to the management of diabetes-related complications.

Herbal plants have played a vital role in treating different diseases since ancient times [41]. Besides the profound therapeutic advantages, some medicinal plants also possess potentially cytotoxic effects. Numerous studies have found that plant-derived medicines caused hepatic failure and even death followed on the ingestion of herbal medicine [42]. Thus, the knowledge related to the safety of these natural products is important, and thus their cytotoxic effects need to be determined to ensure relatively safe use. Especially, MTT assay has been known as a common *in vitro* model for the cytotoxicity test. It is based on the metabolic reduction of the soluble MTT salt, which reflects the normal function of mitochondria dehydrogenase activity and cell viability [43]. Especially, MTT assay has revealed that PGL extract did not cause any cytotoxicity on hepatic and macrophage cells at the tested concentrations. However, a further study due to the cytotoxic effect of PGL extract on an *in vivo* model is needed to achieve adequate knowledge regarding the safe use of this herbal plant.

Recently, *P. guajava* leaves have been found as a rich source of polyphenol compounds with various pharmaceutical properties. A chemical analysis of *P. guajava* leaves has identified various phenolic compounds such as quercetin, myricetins, catechin, garllic and ellagic acids, and their derivatives [44]. The positive effects of polyphenols have been evidenced in various *in vitro*, animal models, and some human trials. It has reported that polyphenols or foods and beverages rich in polyphenols were able to reduce postprandial and fasting hyperglycemia and stimulate the secretion of insulin [45]. The possible mechanisms have been determined via decreasing glucose absorption, suppressing carbohydrate digestion, enhancing insulin secretion, and activating glucose uptake [45]. Moreover, the high antioxidant and anti-inflammation of polyphenols have brought to their preventive effect against diabetes-related complications such as retinopathy, nephropathy, and neuropathy [46]. As a result, *P. guajava* leaves rich in polyphenols are also considered as potential agents that have the capacity in the prevention of diabetes and diabetes-related complications.

CONCLUSION

P. guajava leaves are well-known as an effective folk medicine for the treatment of various disorders such as rheumatism, diarrhea, diabetes mellitus, and cough. Recently, *P. guajava* leaves are developed as commercial products with health beneficial effect related to the modulation of blood glucose levels. In this study, the extract of *P. guajava* leaves has been evidenced as a potential antidiabetic agent due to suppressing α-amylase enzyme activity, possessing glucose adsorption and glucose uptake abilities, scavenging free radicals, and inhibiting NO production. Therefore, *P. guajava* leaves could be believed as a functional ingredient that may reduce hyperglycemia and prevent diabetes-related complications. However, further studies related to the safety and efficacy of *P. guajava* leaves are necessary.

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Conflicts of interest:
No conflict of interest associated with this work.

Author contributions:
TSV and DHN conceptualized the study and performed experiments. DHN analyzed data. TSV wrote the paper. All authors approved the final manuscript version.

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