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Research article

Antioxidant potentials and inhibitory activities against α-amylase and α-glucosidase, and glucose uptake activity in insulin-resistance HepG2 cells of some medicinal plants

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Abstract

Non-communicable diseases, especially diabetes mellitus, are a growing health problem worldwide. Although many synthetic drugs have been used to manage diabetic conditions, adverse side effects such as nausea or an upset stomach have been reported. Therefore, the use of medicinal plants for prevention and alleviation of diabetes has become attractive. This research investigated the antioxidant potential and inhibitory activity against α-amylase and α-glucosidase, and the glucose uptake activity in insulin-resistant HepG2 cells of some medicinal plants (*Aloe vera*, *Carthamus tinctorius*, *Cosmos sulphureus, Gymnanthemum extensum* and *Terminalia bellirica*). The ethanolic extracts of these plants were analyzed for total phenolic content and anti-oxidative properties. The results showed that *T. bellirica* extract had the highest total phenolic content (1,360.79 mg gallic acid equivalents/mL extract) followed by *C. sulphureus*, *C. tinctorius*, *G. extensum* and *A. vera* extracts, respectively. *T. bellirica* extract also had the highest anti-oxidative properties, followed by *C. sulphureus*, *C. tinctorius*, *A. vera* and *G. extensum* extracts, respectively. The inhibitory effects of ethanolic plant extracts against α-amylase and α-glucosidase were analyzed. *T. bellirica* extract had the highest efficacy to inhibit α-amylase and α-glucosidase activities compared to the other extracts. Additionally, *T. bellirica* extract at 0.001%, 0.01% and 0.1% enhanced the glucose uptake in insulin-resistant HepG2 cells by 35.5%, 37.9% and 21.0%, respectively, compared to the insulin-resistant HepG2 cells. Collectively, these results suggested that *T. bellirica* could be used as functional food for diabetes.

Introduction

Diabetes mellitus (DM) is a growing health problem worldwide and is a disease associated with abnormal carbohydrate metabolism (Pantidos et al., 2014). A prominent feature of diabetes is hyperglycemia, with the chronic high blood sugar level causing damage to several organs including the eyes, kidneys and heart, and to nerves and blood vessels (American Diabetes, 2010). Ninety percent of diabetic patients have been diagnosed with type 2 diabetes, which is a result of defects in insulin sensitivity. Although an early phase of clinical approaches to DM management involves exercise and diet intervention, most DM patients are recommended to start a treatment of diabetic drugs if they are unable to control their blood sugar levels (Manukumar et al., 2017).

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Several mechanisms underlying the anti-diabetic activities of some plants and dietary compounds have been reported, including an insulin sensitizing function (Chang et al., 2014), AMP-activated protein kinase activity (Yang et al., 2015), reduction of β-cell destruction and oxidative stress, regulation of glucose metabolism and lipid metabolism (Vinayagam and Xu, 2015) and inhibition of α-amylase and α-glucosidase (Lordan et al., 2013). Several pharmacological agents such as acarbose, metformin and thiazolidinediones have been approved and are widely used to manage hyperglycemia through inhibition of carbohydrate-metabolizing enzymes and modulation of glucose metabolism. However, many side effects have been reported such as flatulence, diarrhea, liver disorder, abdominal distention and meteorism (Farsi et al., 2011; Li et al., 2011). Therefore, natural or plant-derived compounds have become more attractive due to their reduced side effects, despite possible toxicity and contaminants.

A variety of natural compounds extracted from plants have been characterized and reported for their biological activities, and some have been suggested as alternative therapy (Van Duyn and Pivonka, 2000; Tang et al., 2017). Many plants have been consumed since ancient times and some have been used for diabetic treatment in traditional practices but information about their chemical profiles and underlying mechanisms is still lacking (Neamsuvan et al., 2015; Moradi et al., 2018; Phumthum and Balslev, 2018). The current study focused on five medicinal plants: *A. vera*, *C. tinctorius*, *C. sulphureus*, *G. extensum* and *T. bellirica*.

However, there is no scientific evidence to prove the anti-diabetic effects of these five medicinal plants. The present study aimed to investigate the anti-diabetic effects of these medicinal plants based on antioxidant activity and inhibitory effects against carbohydrate-metabolizing enzymes and modulatory effects on glucose uptake. The results will be used to encourage the use of these plants to manage and alleviate hyperglycemia.

Materials and Methods

Materials and chemicals

A. vera ('Wan Hang Chora Khe'), *C. tinctorius* ('Khumfoi' or safflower), *C. sulphureus* ('Dawkajay'), *G. extensum* ('Nanchaowei') and *T. bellirica* ('Samopipek'), as shown in Fig. 1 were purchased from local markets in Bangkok Thailand during October–November 2018. α-Amylase and α-glucosidase were purchased from Sigma Aldrich (USA). All other chemicals were analytical grade and obtained from reputable suppliers.

Sample preparation

Leaves of *A. vera* and *G. extensum*, petals and pollen of *C. tinctorius* and *C. sulphureus* and whole fully matured fruits of *T. bellirica* were used. *A. vera*, *C. sulphureus* and *G. extensum* were washed with tap water and drained. *C. tinctorius* and *T. bellirica* were purchased in sun-dried form. All samples were dried at 50 ± 3°C for 12 hr until the moisture content was less than 10%. All dried samples were ground using a mortar and pestle until the particle size was about 1–3 mm and kept in a desiccator for further analysis. The samples were extracted according to previously described methods with some modifications (Yang et al., 2014). The samples were extracted using maceration with 80% ethanol at a solid-to-solvent ratio of 1:10. The extraction was performed at room temperature (25 ± 3°C) by stirring with a glass stirring rod for 5 min every day for 7 d at 0900 hours to 1000 hours. After the maceration period, the soaked sample-solvent mixtures were passed through Whatman No.1 filter paper. The filtrate was concentrated using a rotary evaporator (Rotavapor® model R-205; Buchi, Switzerland) at 70 revolutions per minute and 50 ± 1°C until no condensation occurred. Extracts were kept in conical tubes at -20°C for further analysis.

Determination of total phenolic content

The total phenolic content of extracts was determined using Folin-Ciocalteu reagent (Sumczynski et al., 2015). Briefly, 0.03 mL of extract was mixed with 0.15 mL of 10% Folin-Ciocalteu reagent and equilibrated for 5 min. The mixture was neutralized with 0.3 mL of 7.5% Na_2CO_3 and distilled water was added to 1 mL. The solution was mixed and kept in the dark for 30 min. The absorbance of the mixture was determined at 765 nm using a spectrophotometer (CECIL 1011; Cecil Instruments Ltd, UK). Gallic acid was used as a reference standard and the results were expressed as milligrams of gallic acid equivalents (GAE) per milliliter of extract.

Determination of antioxidant activity

Antioxidant activity was determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method (Khanam et al., 2012). Briefly, 10 µL of extract was mixed with 990 µL of 0.1 mM freshly prepared DPPH. The solution was mixed and kept in the dark for 30 min at room

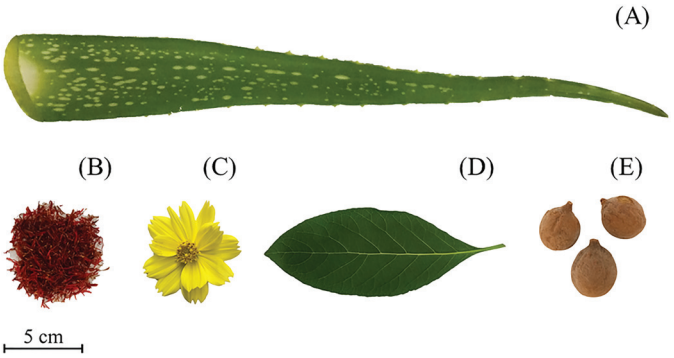


Fig. 1 Medicinal plants studied: (A) *Aloe vera*; (B) *Carthamus tinctorius*; (C) *Cosmos sulphureus*; (D) *Gymnanthemum extensum*; (E) *Terminalia bellirica*

temperature. The absorbance of the mixture was determined at 517 nm with a spectrophotometer (CECIL 1011; Cecil Instruments Ltd, UK). Trolox was used as a reference standard and the antioxidant activity was expressed as milligrams of Trolox equivalents per milliliter of extract.

α-Amylase inhibition assay

The inhibitory effects of the extracts against α-amylase were determined using a previously described method (Sae-tan and Kunpanya, 2017). The extracts were diluted with distilled water to get different concentrations (0.2%–100%). Briefly, 125 µL of the extracts at different concentrations were mixed with α-amylase from porcine pancreas (2 Unit/mL) at 37 ± 0.1°C for 5 min. Then 250 µL of 0.2% starch solution was added and incubated at 37°C for another 5 min. Then, 250 µL of dinitrosalicylic acid color reagent was added after the incubation. To stop the reaction, the mixture was boiled at 100°C, then the mixture was cooled on ice to room temperature. The absorbance of the mixture was determined at 540 nm using a microplate reader (Tecan Infinite 200 Pro, Switzerland). The control was analyzed in an identical manner, replacing the extract with deionized water. In the blank, the enzyme was replaced with deionized water. Acarbose was used as a positive control. The inhibitory activity of the extract was calculated as % inhibitory activity of extract relative to the control.

α-Glucosidase inhibition assay

The inhibitory effects of the extracts against α-glucosidase were determined using the previously described method (Sae-tan and Kunpanya, 2017). The extracts were diluted with distilled water to get different concentrations (0.2%–100%). Briefly, 20 µL of the extracts at different concentrations and 20 µL of α-amylase from *Saccharomyces cerevisiae* (1 Unit/mL) were mixed and pre-incubated in 460 µL of 0.1 M potassium phosphate buffer solution (pH 6.8) at 37C for 20 min. Then the reaction was initiated with the addition of 200 µL of 1 mM para-nitrophenyl-α-D-glucopyranoside. The reaction was processed at 37 ± 0.1°C for 15 min and terminated by the addition of 500 µL of 1 M Na_2CO_3. The absorbance of the mixture was determined at 405 nm with a microplate reader (Tecan Infinite 200 Pro, Switzerland). The control was analyzed in an identical manner replacing the extract with deionized water. For the blank, the enzyme was replaced with deionized water. Acarbose was used as a positive control. The inhibitory activity of the extract was calculated as % inhibitory activity of extract relative to the control.

Cell culture

The plant with the highest total phenolic content, antioxidant activity and inhibitory activity against α-amylase and α-glucosidase was selected to test in HepG2 cells. The HepG2 cells were purchased from the American Type Culture Collection (ATCC; USA). The HepG2 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 Units/mL of penicillin, 100 Units/mL of streptomycin and 1% of non-essential amino acid) at 37 ± 0.1°C in a humidified 5% CO_2 atmosphere (Zhang et al., 2018). The medium was changed every 2 d and subcultured once the cells reached 90% confluence.

Cytotoxicity assay

The plant with the highest total phenolic content, antioxidant activity and inhibitory activity against α-amylase and α-glucosidase was selected to test cytotoxicity in HepG2 cells. Cytotoxicity of extracts on HepG2 cells was assessed using 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetra-zolium bromide (MTT) assay (Zhang et al., 2018). The HepG2 cells were seeded at a density of 4×10^4 cells/well in 96-well plates. After 24 hr, the medium was changed and incubated with different concentrations of extracts for 24 hr. After incubation, 10 µL of 5 mg/mL of MTT was added into each well and incubated for 4 hr. Then, the MTT-containing medium was gently removed and replaced with 100 µL dimethyl sulfoxide to dissolve the formazan crystals. Then, the plate was gently shaken for 5 min to completely dissolve the crystals. Cell viability was observed by recording the absorbance at 570 nm using a microplate reader (TECAN, Infinite 200 Pro, Switzerland). The results were presented as percentages of the control values.

Cellular glucose uptake assay

The plant with the highest total phenolic content, antioxidant activity, α-amylase and α-glucosidase inhibitory activity was selected to evaluate cellular glucose uptake activity. Cellular glucose uptake was determined using the 2-deoxy-2-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]-d-glucose (2-NBDG) assay previously described (Zou et al., 2005). Briefly, HepG2 cells were seeded at a density of 5×10^4 cells/well in 96-well fluorescence plates. After 24 hr, the medium was changed and incubated with serum-free medium containing 1 µM insulin for 24 hr to induce insulin resistance. Then, the medium was replaced with serum-free medium containing different concentrations of the extract and incubated for another 24 hr. After 24 hr of incubation, the medium was replaced with 100 nM insulin in phosphate buffered saline (PBS) and incubated for 30 min. Then, 10 µL of 40 µM 2-NBDG was added and incubated for 30 min and the cells were washed three times with iced-cold PBS. The fluorescence intensity was immediately determined at an excitation wavelength of 460 nm and an emission wavelength of 528 nm. The percentage of relative 2-NBDG uptake was calculated using the Equation 1:

$$\%\ \text{relative}\ 2-\text{NBDG uptake} = (FI_{ext}/FI_{cont}) \times 100\% \quad (1)$$

where FI_{ext} is the fluorescence intensity of cells treated with extract and FI_{cont} is the fluorescence intensity of the control cells.

Statistical analysis

All data were expressed as mean ± SD and triplicate independent analyses were applied. SPSS version 23.0 (IBM Software; USA) was used to analyze statistical differences of data with one-way analysis of variance followed by the Duncan post-hoc test. Pearson correlation analysis was applied using the SPSS program. The GraphPad Prism 7 statistical package (GraphPad Software Inc.; USA) was used to determine values of the concentration of extract in parts per million (ppm) that inhibited 50% activity (IC_{50}). A value of $p < 0.05$ was considered statistically significant.

Results

Total phenolic content and antioxidant activity of plant extracts

The total phenolic contents of all plant extracts were significantly different (Table 1). *T. bellirica* extract contained the highest total phenolic content followed by *C. sulphureus, C. tinctorius, G. extensum* and *A. vera* extract (1360.79 mg gallic acid equivalents/mL extract, 296.42 mg gallic acid equivalents/mL extract, 172.04 mg gallic acid equivalents/mL extract, 33.50 mg gallic acid equivalents/mL extract and 20.00 mg gallic acid equivalents/mL extract, respectively). The antioxidant activities of plant extracts were also determined. The results showed that all extracts had significantly different antioxidant activity (Table 2). *T. bellirica* extract had the highest antioxidant activity (818.58 mg Trolox equivalents/mL extract) followed by *C. sulphureus, C. tinctorius, A. vera* and *G. extensum* extract (155.87 mg Trolox equivalents/mL extract, 94.55 mg Trolox equivalents/mL extract, 69.21 mg Trolox equivalents/mL extract and 58.96 mg Trolox equivalents/mL extract, respectively). However, IC_{50} is recommended for comparison of the current results with other studies.

Inhibition of α-amylase and α-glucosidase of plant extracts

The inhibitory effects of plant extracts against α-amylase and α-glucosidase were studied to investigate the possible mechanisms of plant extracts on delaying carbohydrate digestion. Isolated α-amylase from porcine and α-glucosidase from *S. cerevisiae* were used to investigate the inhibitory effects of each extract. The results showed that all extracts inhibited α-amylase and α-glucosidase in a dose-dependent manner. Specifically, *T. bellirica* extract had the highest efficiency to inhibit α-amylase with an IC_{50} value of 8.61×10^2 ppm followed by *C. sulphureus*, *C. tinctorius*, *G. extensum* and *A. vera*, respectively (Table 3). *T. bellirica* extract also had the highest efficiency to inhibit α-glucosidase with an IC_{50} value of 3.41×10^3 ppm followed by *C. tinctorius*, *C. sulphureus*, *G. extensum* and *A. vera*, respectively (Table 4).

Table 1 Total phenolic content of medicinal plant extracts

Extract	Total phenolic content (mg gallic acid equivalents/mL extract)
A. vera	20.00±0.003^e
C. tinctorius	172.04±0.001^c
C. sulphureus	296.42±0.004^b
G. extensum	33.50±0.002^d
T. bellirica	1360.79±0.004^a

Values are shown as mean ± SD from three replicated experiments and different lowercase superscripts in a column denote significantly ($p < 0.05$) different.

Table 2 Antioxidant activity of medicinal plant extracts

Extract	Antioxidant activity (mg Trolox equivalents/mL extract)
A. vera	69.21±1.27^d
C. tinctorius	94.55±0.59^c
C. sulphureus	155.87±3.92^b
G. extensum	58.96±3.53^e
T. bellirica	818.58±1.53^a

Values are shown as mean ± SD from three replicated experiments and different lowercase superscripts in a column denote significantly ($p < 0.05$) different.

Table 3 Inhibition of α-amylase of medicinal plant extracts

Extract	IC_{50} (ppm)
A. vera	3.06×10^8
C. tinctorius	2.16×10^4
C. sulphureus	1.93×10^4
G. extensum	1.38×10^5
T. bellirica	8.61×10^2
Acarbose	1.00×10^1

IC_{50} = concentration of extract in parts per million (ppm) that inhibits 50% activity of α-amylase

Table 4 Inhibition of α-glucosidase of medicinal plant extracts

Extract	IC_{50} (ppm)
A. vera	2.97×10^8
C. tinctorius	2.60×10^4
C. sulphureus	7.24×10^5
G. extensum	7.53×10^5
T. bellirica	3.41×10^3
Acarbose	7.18×10^2

IC_{50} = concentration of extract in parts per million (ppm) that inhibits 50% activity of α-glucosidase

Cytotoxicity of T. bellirica extract on HepG2 cells

The results of the total phenolic content and antioxidant activity of the five plant extracts showed that *T. bellirica* extract had the highest potential to delay carbohydrate digestion according to values for the total phenolic content, antioxidant activity and inhibitory effects

against α-amylase and α-glucosidase. Therefore *T. bellirica* extract was selected to further investigate its effect in cellular-based assay. Concentrations of *T. bellirica* extract in the range 5–1,000 ppm did not show cytotoxicity on HepG2 cells, while the higher concentrations showed the cytotocixity on HepG2 based on MTT assay (Fig. 2). The concentrations used were based on the objective of this study, which was to investigate the effect of the extract cytotoxicity and cellular glucose uptake activity disregarding to serving size. The effects of *T. bellirica* extract at concentrations in the range 10–1,000 ppm on cellular glucose uptake in insulin-resistant HepG2 cells were investigated.

Effects of T. bellirica extract on cellular glucose uptake in insulin-resistant HepG2 cells

Insulin-resistant HepG2 cells were developed, and they showed a significantly lower cellular glucose uptake compared to the control (Fig. 3). Pretreatment with *T. bellirica* extract significantly improved the cellular glucose uptake in insulin-resistant HepG2 cells (Fig. 3). Pretreatment with 10 ppm, 100 ppm and 1000 ppm of *T. bellirica* extract increased the cellular glucose uptake in insulin-resistant HepG2 cells by 35.5%, 37.9% and 21.0%, respectively (Fig. 3). However, the cellular glucose uptake ability of insulin-resistant HepG2 cells did not return to the normal level.

Discussion

Many medicinal plants have historically been acclaimed for their anti-diabetic activities; however, scientific reports about their underlying mechanisms are limited. The current study investigated the antioxidant potential and inhibitory activity against α-amylase and α-glucosidase, and glucose uptake activity in insulin-resistance HepG2 cells of five selected medicinal plants. Consumption of plants rich in phenolics and flavonoids have been linked with the reduction of risk for many chronic diseases including diabetes (Scalbert et al., 2005; Zhang et al., 2011). Phenolics and flavonoids in plants were hypothesized to play a key role in this effect due to free radical scavenging activity (Choi et al., 2002; Shahidi and Ambigaipalan, 2015). Increased reactive oxygen species and disturbed scavenging ability in subjects with a hyperglycemic condition were recognized as central events in developing diabetic complications (Rahimi et al., 2005). Therefore, antioxidants seem to be a key player in diabetes management. Five selected Thai medicinal plants were determined for total phenolic content and antioxidant activity. The positive correlation between total phenolic content and antioxidant activity (correlation coefficient = 0.99) in this study was consistent with previous study (Misbah et al., 2013) (data shown in Supplementary Fig. 1), which was consistent with Misbah et al. (2013). The current study showed that *T. bellirica* extract had the highest total phenolic content and antioxidant activity compared to the other extracts (Tables 1 and 2). The total phenolic content in *T. bellirica* extract was 1,360.79 mg GAE/mL extract, which was much higher than the total phenolic content of *T. bellirica* methanolic extract in the study by Chalise et al. (2010) of 327.89 mg GAE/mL extract. This difference may have been due to the method of extraction as the current study macerated the *T. bellirica* in ethanol for 7 d, while the previous study macerated *T. bellirica* for 1 d. The phenolic compounds found in the *T. bellirica* extract were predominantly in two forms: gallic acid and simple gallates (Pfundstein et al., 2010).

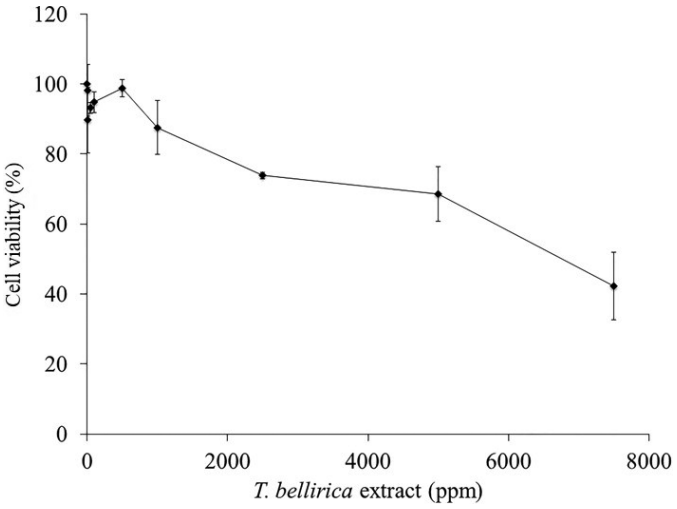


Fig. 2 Cytotoxicity of *Terminalia bellirica* extract on HepG2, where data are mean ± SD from three replicated experiments.

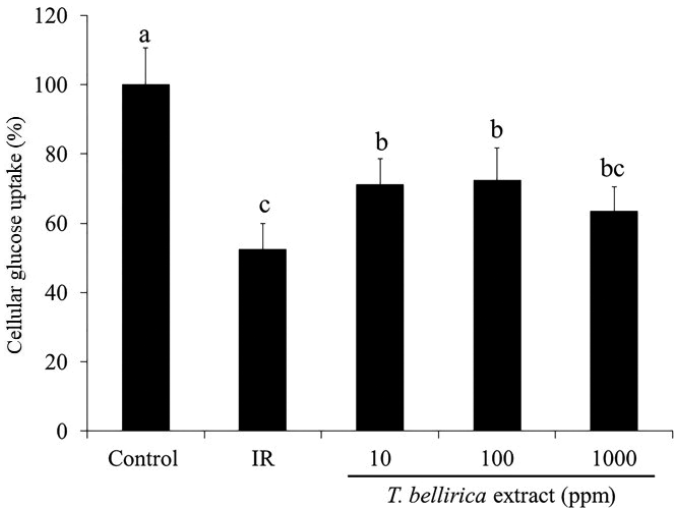
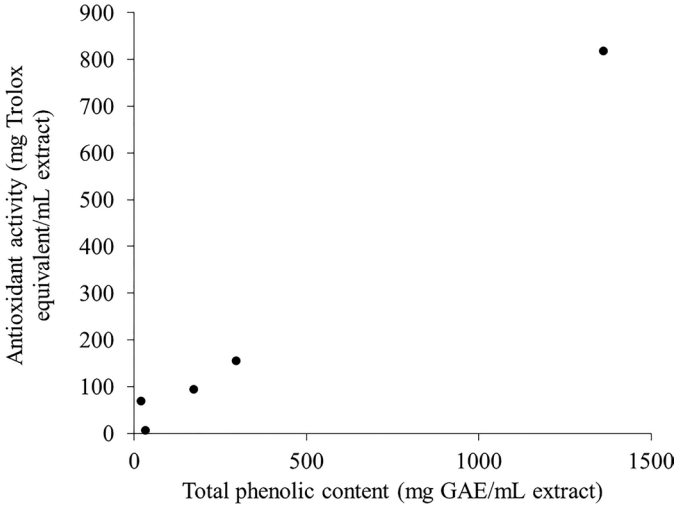


Fig. 3 Effects of *Terminalia bellirica* extract on cellular glucose uptake in insulin-resistant HepG2 cells, where IR = Insulin-resistant HepG2 cells, data are shown as mean ± SD from three replicated experiments and columns with different lowercase letters are significantly ($p < 0.05$) different.



Supplementary Fig. 1 Correlation between total phenolic content and antioxidant activity

The control of increased postprandial blood sugar is the key to diabetes management. One of the approaches in managing hyperglycemia is the use of carbohydrate metabolizing-enzymes inhibitors. α-Amylase and α-glucosidase are the key enzymes responsible for carbohydrate digestion, producing monosaccharides into the bloodstream as the inhibition of carbohydrate digestion results in the delay and reduction of increased postprandial blood glucose (Kwon et al., 2008; Tundis et al., 2010; Lordan et al., 2013). The medicinal plant extracts investigated in the current study had inhibitory effects against α-amylase and α-glucosidase with different levels of potency. The *T. bellirica* extract had the highest potency to inhibit α-amylase and α-glucosidase with IC_{50} values of 8.61×10^2 ppm and 3.41×10^3 ppm, respectively. The high potency of the *T. bellirica* extract may have been due to the phenolic content; however, the order of potency of the extracts inhibiting the enzymes was different from the order for the total phenolic contents and antioxidant activities. This suggested that other chemical constituents may also be responsible for the inhibitory effects of these plant extracts. The inhibitory effect against α-amylase and α-glucosidase of *T. bellirica* extract may contribute to its ability to lower the level of postprandial blood glucose.

One of the predictors for type 2 diabetes development is impaired insulin signaling, called insulin resistance. Insulin resistance becomes a therapeutic target once hyperglycemia occurs (Taylor, 2012). The effects of *T. bellirica* extract on cellular glucose uptake in insulin-resistant HepG2 cells were investigated in this study. Non-cytotoxic concentrations of *T. bellirica* extract increased the cellular glucose uptake of insulin-resistant HepG2 cells (Fig. 3) even though the effects were not dose-dependent. This suggested that *T. bellirica* extract modulated cellular glucose uptake of the insulin-resistant HepG2 cells. The current result was consistent with previous study showing that ethanolic *T. bellirica* extract enhanced insulin-stimulated glucose uptake in 3T3-L1 cells (Kasabri et al., 2009). The glucose uptake enhancement activity of *T. bellirica* extract may have been due to its phytochemicals. The most abundant polyphenolic compounds in *T. bellirica* are gallic acid and gallate esters (Pfundstein et al., 2010). Both gallic acid and gallates were also reported to have antioxidant activity (García-Pérez et al., 2019). Therefore, the antioxidant activity of *T. bellirica* in the current study may have been partly due to the gallic acid and gallates. Gallic acid has had preventive effects against insulin resistance via AMP-activated protein kinase activity (Doan et al., 2015). It was reported that elevated blood glucose increased oxidative stress, which contributes to diabetic complications (Yasunari et al., 1999). Previous studies evaluated the acute toxicity of methanolic *T. bellirica* extract and acetone *T. bellirica* extract in rats (Badoni et al., 2016; Jayesh et al., 2017). Their results showed that both methanolic and acetone extracts of *T. bellirica* up to a dose of 2,000 mg/kg body weight were safe with no toxic reaction or mortality. Therefore, *T. bellirica* extract may lower hyperglycemia via two possible mechanisms: 1) inhibition of α-amylase and α-glucosidase, resulting in delaying carbohydrate digestion and 2) reducing oxidative stress, resulting from hyperglycemia. However, the beneficial effects of *T. bellirica* on lowering hyperglycemia *in vivo* depend on its bioaccessibility and bioavailability. Based on the current results additional *in vitro* and *in vivo* studies are needed to further investigate the possible mechanism of *T. bellirica* extract. Collectively, *T. bellirica* should be encouraged for consumption as a functional food to manage and alleviate hyperglycemia. However, further studies are needed to show the effects of *T. bellirica* on the insulin-signaling pathway and its potential as a functional food.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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