In vitro anti-hyperglycemic, antioxidant activities and intestinal glucose uptake evaluation of *Endiandra kingiana* extracts

Mohamad Hafizi Abu Bakar, Pui Yee Lee, Mohamad Nurul Azmi, Nurul Syifa’ Lotfiamir, Mohamad Shamir Faris Mohamad, Nor Shahiqah Nor Shahril, Khairul Anuar Shariff, Harisun Ya’akob, Khalijah Awang, Marc Litaudon

**Abstract**

Optimal control of postprandial hyperglycemia is essentially important in the management of diabetes mellitus. The present investigation was undertaken to evaluate the in vitro anti-hyperglycemic, antioxidant properties and intestinal glucose uptake inhibition of *Endiandra kingiana* (E. kingiana) extracts. Previously, our group has identified and characterized the bioactive compounds of *E. kingiana* extracts, which had discovered several polyketides; endiandric acids and kingianins. Here, the inhibitory potential of bark-ethyl acetate (BEA), bark-methanol (BM), leaf-ethyl acetate (LEA), and leaf-methanol (LM) extracts of *E. kingiana* against carbohydrate-hydrolyzing enzymes with their mode of inhibitions were evaluated. Further, the antioxidant activities and inhibitory potential on glucose uptake in Caco-2 human intestinal cell monolayers were determined. Our finding showed that BEA extract exhibited the most potent inhibition activities against α-amylase (IC₅₀ = 2.32 μg/mL) and α-glucosidase (IC₅₀ = 1.83 μg/mL) by following inhibition mode of competitive and non-competitive manners, respectively. Meanwhile, BM extract exhibited notable antioxidant capabilities, as evidenced by strongest free radical scavenging (IC₅₀ = 1.18 μg/mL) and reducing power effect (118.53 mM Fe²⁺ equivalent/g extract) in couple with highest total phenolic contents (10.17 mg GAE/g extract) compared to other extracts. Mechanistically, both BEA and BM extracts of *E. kingiana* significantly inhibited glucose uptake in Caco-2 cell monolayers under sodium-dependent condition. Collectively, these findings suggest that BM and BEA extracts of *E. kingiana* exert in vitro anti-hyperglycemic and antioxidant properties, which can be further utilized as a potential candidate for treatment of hyperglycemia–induced oxidative stress conditions.

**Keywords:**
*Endiandra kingiana*, α-amylase, α-glucosidase, antioxidant, Caco-2 cell lines

**Article Info**

**A R T I C L E   I N F O**

**A B S T R A C T**

Optimal control of postprandial hyperglycemia is essentially important in the management of diabetes mellitus. The present investigation was undertaken to evaluate the in vitro anti-hyperglycemic, antioxidant properties and intestinal glucose uptake inhibition of *Endiandra kingiana* extracts. Previously, our group has identified and characterized the bioactive compounds of *E. kingiana* extracts, which had discovered several polyketides; endiandric acids and kingianins. Here, the inhibitory potential of bark-ethyl acetate (BEA), bark-methanol (BM), leaf-ethyl acetate (LEA), and leaf-methanol (LM) extracts of *E. kingiana* against carbohydrate-hydrolyzing enzymes with their mode of inhibitions were evaluated. Further, the antioxidant activities and inhibitory potential on glucose uptake in Caco-2 human intestinal cell monolayers were determined. Our finding showed that BEA extract exhibited the most potent inhibition activities against α-amylase (IC₅₀ = 2.32 μg/mL) and α-glucosidase (IC₅₀ = 1.83 μg/mL) by following inhibition mode of competitive and non-competitive manners, respectively. Meanwhile, BM extract exhibited notable antioxidant capabilities, as evidenced by strongest free radical scavenging (IC₅₀ = 1.18 μg/mL) and reducing power effect (118.53 mM Fe²⁺ equivalent/g extract) in couple with highest total phenolic contents (10.17 mg GAE/g extract) compared to other extracts. Mechanistically, both BEA and BM extracts of *E. kingiana* significantly inhibited glucose uptake in Caco-2 cell monolayers under sodium-dependent condition. Collectively, these findings suggest that BM and BEA extracts of *E. kingiana* exert in vitro anti-hyperglycemic and antioxidant properties, which can be further utilized as a potential candidate for treatment of hyperglycemia–induced oxidative stress conditions.

**1. Introduction**

Diabetes mellitus (DM) is a chronic metabolic disorder of nutrient metabolism characterized by chronic hyperglycemia due to insufficient insulin secretion by pancreas or defects in insulin action, termed as insulin resistance (Abu Bakar et al., 2015). In this regard, DM is strongly associated with development of oxidative stress in peripheral tissues via increased level of free radical including reactive oxygen species, leading to a redox imbalance, oxidative insults and mitochondrial dysfunction (Giacco and Brownlee, 2010; Hafizi Abu Bakar et al., 2015). This may lead to the progression of many metabolic diseases, including obesity, insulin resistance and various DM complications.

Notably, the application of glucose lowering therapies has been widely utilized in the DM treatment to decrease blood sugar level in the body after a meal (Hays et al., 2009; Mousavi et al., 2016). As such, the preventive and therapeutic strategies for hyperglycemia could be achieved by inhibition of carbohydrate-hydrolyzing enzymes including α-amylase (EC 3.2.1.1) and α-glucosidase (EC 3.2.1.20). Consequently, this may slow down the carbohydrate absorption and increase the carbohydrate digestion time in gastrointestinal tract, thereby decreasing...
postprandial hyperglycemia, thus preventing the onset of diabetes (Kazeem et al., 2013). Examples of these inhibitors are acarbose, miglitol and voglibose that have been approved for clinical use (Ali et al., 2006). These drugs exhibit several advantages for glycemic control over the short-term. Despite considerable benefits, their administrations are physiologically linked to undesirable side effects including flatulence, abdominal cramping and hypoglycemia which are mostly associated with incomplete carbohydrate absorption (Gulati et al., 2015; Hung et al., 2012).

Over the last decades, there is increasing interest in drug discovery from a natural origin for treatment of many chronic diseases. The need to search for new antidiabetic drugs from plant sources is of great interest considering a number of undesirable side effects exhibited by commercialized synthetic drugs (Jemain et al., 2011). As a result, plant-based drugs can be considered as one of the best possible alternatives for the therapeutic treatment of DM because of its cost-effectiveness compared to synthetic drugs and minimized side effects to human body (Jemain et al., 2011; Mousavi et al., 2016). Accordingly, there has been a rising interest in searching natural antioxidants that are abundantly present in numerous medicinal plants (Sylvie et al., 2014). Antioxidants is a substance which able to prevent the disease by neutralizing the damage caused by oxidative stress. Synthetic antioxidants are claimed to produce adverse side effects, therefore they have been replaced by natural antioxidants which can reduce the risk of disease progression (Sahoo et al., 2013). A number of antioxidant compounds have been isolated from medicinal plants and many studies showed that these compounds exhibit numerous attributive properties in attenuating various metabolic diseases such as obesity, insulin resistance, type 2 diabetes and cancer (Abu Bakar et al., 2018; Nunes et al., 2012).

The Caco-2 cell lines are widely utilized as cellular models to investigate the expression of glucose transporters and intestinal nutrient transport and absorption (Alzaid et al., 2013). The liberated glucose from carbohydrate digestions is mainly absorbed in the apical membrane of enterocytes mediated via specific glucose transporters; sodium-dependent glucose transporter 1 (SGLT1) and the facilitated-transporter glucose transporter 2 (GLUT2), depending the luminal glucose concentration (Pico and Martínez, 2019). As far as such disease is concerned, the expressions of these intestinal glucose transporters were found to be 3- to 4-fold higher in diabetes individuals than controls, leading to elevated postprandial glycemia (Ait- Omar et al., 2011; Corpe et al., 1996). Thus, therapeutic strategy aimed on the inhibition of intestinal glucose absorption may provide some protective targets for better glycemia after high-carbohydrate meals.

*Endiandra kingiana* (*E. kingiana*) belonging to the family Lauraceae, is a large sub-canopy tree distributed through Peninsular Malaysia and Borneo (Azmi et al., 2014). This family plant has been long utilized as sources of various classes of secondary metabolites and widely used in traditional medicines (Lenta et al., 2015). In particular, numerous reports have demonstrated that this plant family exhibits a number of biological properties including anti-inflammatory, antimalaria, anticancer, antimicrobial and enzymes inhibition activities (Zenta et al., 2015; Mollataghi et al., 2012). However, to the best of our knowledge, there has been no previous report being published on the attributable properties of *E. kingiana* extracts against hyperglycemia and oxidative stress. Therefore, in ongoing efforts aimed to further investigate these biological properties, the present study systematically evaluated the *in vitro* inhibitory effect of *E. kingiana* extracts against α-amylase and α-glucosidase activities with their mode of inhibitions. Given oxidative stress is strongly associated with DM, the *in vitro* antioxidant analyses in correlation to total phenolic content of *E. kingiana* extracts were further verified. The cytotoxicity of *E. kingiana* extracts was carried out using the cellular model of human small intestine, Caco-2 cell monolayers. Finally, the metabolic assessment of glucose uptake inhibition activity in Caco-2 cell monolayers by *E. kingiana* extracts was determined to investigate the glucose absorption into the small intestine.

## 2. Materials and methods

### 2.1. Materials

Solvents of analytical or HPLC grade were purchased from QReC, Malaysia and Merck, Germany. *Saccharomycyes cerevisiae* α-glucosidase (≥10 U/mg), pancreatic α-amylase (≥250 U/mg), and acarbose (BJ05882) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Other reagents were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA), unless otherwise stated.

### 2.2. Plant material

The barks and leaf of *E. kingiana* Gamble was collected at Reserved Forest Sg. Temau, Kuala Lipis, Pahang, Malaysia. This plant was identified by T. Leong Eng, a botanist at the University of Malaya (Burkill, 1966; Mabberley, 2017; Whitmore, 1983). A voucher specimen (KL-5243) has been deposited at the Herbarium of the Department of Chemistry, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia.

### 2.3. Extractions, identification and characterization of bioactive compounds

The air-dried barks and leaf of *E. kingiana* (200 g) were sliced, ground and extracted with ethyl acetate (3 × 0.5 L) and followed by methanol (3 × 0.5 L) for 72 h in tightly closed containers at room temperature using maceration (Organization et al., 2008). The crude extracts were filtered, and solvents were removed using rotary evaporator. Collectively, four *E. kingiana* crude extracts were produced for biological evaluation in this study, BEA: bark-ethyl acetate; BM: bark-methanol; LEA: leaf-ethyl acetate; LM: leaf-methanol. A series of study from our previous reports were done towards structural identification of bioactive compounds from *E. kingiana* extracts. Our group has previously identified, isolated and characterized several polyketides; (kingianic acids A-H) and kingianin analogues (kingianin O-Q) derived from bark of *E. kingiana* Gamble. Their structural elucidations and spectral data were previously determined by 1D and 2D NMR analysis in combination with HRESIMS experiments (Azmi et al., 2014, 2016; Leverrier et al., 2011).

### 2.4. Preliminary phytochemical screening

To facilitate the screening process of the biological properties of *E. kingiana* extracts, the qualitative phytochemical analysis was first determined to investigate the presence of secondary metabolites in the crude extracts. Accordingly, several phytochemical screening analyses including terpenoids, flavonoids, phenols and saponins were carried out following previous protocols. Aqueous extracts were prepared by dissolving 40 mg of crude extracts in 5 mL of distilled water and filtered.

#### 2.4.1. Test for terpenoids

**Salkowski test:** The screening for terpenoids in the extracts was determined with slight modifications (Ayoola et al., 2008). A total volume of two mL of chloroform was added to the tube containing 2 mL of aqueous extract and gently shaken. 2 mL of concentrated sulfuric acid was then added to the mixture. The presence of terpenoids was detected by the formation of reddish-brown color at the interface of the mixture.

#### 2.4.2. Test for flavonoids

**Alkaline reagent test:** Test for flavonoids was performed with some modifications (Sharma et al., 2017). Firstly, 2 mL of 2% sodium hydroxide solution were added to the tubes containing 2 mL of aqueous extract. Then, 2 mL of diluted acid was added to these tubes containing the samples. The formation of an intense yellow color was first observed in the test tube. Then, the solution was turned to colorless. The changes of color showed the presence of flavonoids.
2.4.3. Test for tannins

Lead acetate test: Test for tannins was performed according to Singh and Bag (2013) with some modifications. Firstly, 3 mL of aqueous extract was prepared and followed by the addition of few drops of 10% lead acetate solution. The presence of tannins was detected by the formation of precipitate in the test tube.

2.4.4. Test for saponins

Foam test: Test for saponins was determined according to Sumbul et al. (2012) with some modifications. Firstly, 50 mg of extract was weighted in a test tube and shaken vigorously with 20 mg of sodium bicarbonate and distilled water. Saponins were detected by the formation of honeycomb-like froth, which persist after 5 min.

2.5. Alpha-amylase inhibitory assay

The inhibition assay of α-amylase was conducted following previous procedures (Kazeem et al., 2013) with slight modifications. To prepare α-amylase solution, 0.05 g of enzyme was dissolved in 100 mL of phosphate buffer (20 mM, pH 6.9). The 3,5-dinitrosalicylic acid (DNS) reagent was prepared by weighing 5 g of DNS powder, 8 g of NaOH and 150 g of sodium potassium tartrate accordingly and dissolved homogeneously in 350 mL of distilled water by using magnetic stirrer. The DNS solution was then topped up by 500 mL of distilled water. All crude extracts of *E. kingiana* were weighted accordingly and diluted with equal volumes of dimethyl sulfoxide (DMSO) and distilled water. A volume of 250 μL of α-amylase solution diluted with starch was added into the set of tubes containing 250 μL of extract. After 10 min incubation at 25 °C in water bath, 0.5 mL of DNS reagent was added to the tubes to stop the reaction. Then, the tubes were placed in a boiling water bath for 5 min and cooled to room temperature. The mixture was diluted with 5 mL of distilled water and the absorbance of final product was measured at 540 nm by using MPR-96 microplate reader (Halo, Dynamica, Australia). The negative control was prepared using the same procedure with addition of buffer to replace the plant extracts. Acarbose was used as positive control. The following Eq. (1) was applied to calculate the α-amylase inhibitory activity:

\[
\text{Percentage of inhibition} \% = \left(1 - \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{extract}}}{\text{Abs}_{\text{control}}} \right) \times 100
\]

where Abscontrol denotes the absorbance value of control and Absextract represents the absorbance value of *E. kingiana* extracts. The concentration of *E. kingiana* extracts required to inhibit 50% of α-amylase activity (IC50) was determined from the graph of percentage activity inhibition versus extract concentrations.

2.6. Alpha-glucosidase inhibitory assay

The α-glucosidase inhibition assay was determined with slight modifications (Kazeem et al., 2013). To prepare 1.0 U/mL α-glucosidase solution, 3.827 g of enzyme was weighted and dissolved in 100 mL of phosphate buffer (20 mM, pH 6.9). All crude extracts of *E. kingiana* were weighted accordingly and diluted with equal volumes of DMSO and distilled water. Then, 100 μL of α-glucosidase solution was added into the set of tubes containing 50 μL of extracts. After 10 min preincubation at 37 °C in water bath, 50 μL of 4-nitrophenyl α-D-glucopyranoside (pNPG) (3.0 mM) was added as a substrate. Then, the mixture was incubated for 20 min. Further, 2 mL of 0.1 M sodium carbonate (Na2CO3) was added to the mixture to stop the reaction. The absorbance value of the released p-nitrophenol at 405 nm was measured. Acarbose solution was included as positive control. The following Eq. (2) was applied to calculate the α-glucosidase inhibitory activity:

\[
\text{Percentage of inhibition} \% = \left(1 - \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{extract}}}{\text{Abs}_{\text{control}}} \right) \times 100
\]

where Abscontrol represents the absorbance value of control and Absextract denotes the absorbance value of *E. kingiana* extracts. The concentration of *E. kingiana* extracts required to inhibit 50% of α-glucosidase inhibition (IC50) was further calculated from the plot of percentage inhibition versus extract concentrations.

2.7. Mode of α-amylase inhibition

The kinetic inhibition of *E. kingiana* extract with the lowest IC50 value on α-amylase activity was performed accordingly (Kazeem et al., 2013) with slight modifications. In brief, two sets of tubes (A and B) were prepared. For set A, 100 μL of α-amylase solution was added to 250 μL of the extract. For set B, same volume of α-amylase was added to the tubes containing 250 μL of phosphate buffer. Both tubes were subjected to incubation for 10 min at 25 °C. Concentration of starch solution was ranging from 0.30 mg/mL to 5.00 mg/mL. To start the reaction, 250 μL of starch solution was added to both tubes. The tubes were incubated for 10 min at 25 °C prior to the addition of 500 μL DNS reagent. Concentration of reducing sugars formed was calculated from the maltose standard curve and converted to reaction rates. Subsequently, the Lineweaver-Burk plot was constructed from Michaelis-Menten kinetics graph to determine inhibition type of extract on α-amylase activity.

2.8. Mode of α-glucosidase inhibition

The kinetic inhibition of *E. kingiana* extract with the lowest IC50 on α-glucosidase activity was determined with slight modifications (Kazeem et al., 2013). Firstly, two sets of tubes (A and B) were prepared. For set A, 100 μL of α-glucosidase solution was added to 50 μL of the extract. For set B, same volume of α-glucosidase solution was added to the tubes containing 50 μL of phosphate buffer. Both tubes were incubated for 10 min at 37 °C. Concentrations of pNPG solution were in a range of 0.63 mg/mL to 2.00 mg/mL. To start the reaction, 50 μL of pNPG solution was added to every tube. Then, the tubes were incubated for 10 min at 37 °C prior to the addition of 500 μL Na2CO3. The concentration of reducing sugars formed was calculated from the p-nitrophenol standard curve and further converted to reaction rates. Lineweaver-Burk plot was plotted based on the Michaelis-Menten kinetics graph to determine the inhibition type of extract on α-glucosidase activity.

2.9. Total phenolic content

The total phenolic content of all extracts was performed according to the Folin-Ciocalteu spectrophotometric method as previously described (Velioglu et al., 1998) with some modifications. In brief, Folin-Ciocalteu’s reagent was prepared in ten-fold dilutions. A total volume of 2.5 mL of Folin-Ciocalteu’s reagent was added to the tubes containing 0.5 mL of extract (0.2 mg/mL). The mixture was maintained at room temperature for 5 min prior to the addition of 2 mL of 7.5% Na2CO3 solution. Then, distilled water was added to the mixture. The tubes were maintained at room temperature for 90 min. The absorbance at 765 nm was measured using microplate reader. A gallic acid calibration curve was used to determine the total phenolic content which expressed as mg of gallic acid equivalents per gram of extracts (mg GAE / g extract). The following Eq. (3) was applied to calculate total phenolic content:

\[
\text{Total phenolic content} (\text{mg GAE} / \text{g extract}) = c \times \frac{V}{M}
\]

where c represents the concentration determined from calibration curve (mg/mL), V represents the volume of *E. kingiana* extract used in the assay (mL) and M represents the mass of extracts used during the assay (g).
2.10. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity assay

The free radical scavenging activity of *E. kingiana* extracts were carried out using DPPH free radical assay with minor modifications (Sahoo et al., 2013). DPPH solution was freshly prepared in methanol. A volume of 1.5 mL of extracts at different concentrations was prepared and 1.5 mL of DPPH solution (0.1 mM/L) was added to it. The tubes were shaken vigorously for 2 min by using vortex mixer. The mixture was maintained at room temperature for 30 min in the dark environment. The absorbance reading was recorded at 517 nm using microplate reader. Control was prepared by replacing the extract with methanol. Ascorbic acid was included as positive control. The following Eq. (4) was used to determine the percentage of DPPH free radical inhibition:

\[
\text{Scavenging activity} \% = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{extract}}}{\text{Abs}_{\text{control}}} \times 100
\]  

where \(\text{Abs}_{\text{control}}\) represents the absorbance of the control and \(\text{Abs}_{\text{extract}}\) represents the absorbance of *E. kingiana* extracts. The IC\(_{50}\) value was determined from GraphPad Prism analysis to identify the concentration of *E. kingiana* extracts required to cause a 50% scavenging activity.

2.11. Ferric reducing antioxidant power (FRAP) assay

The total antioxidant power of extracts was measured using FRAP assay with some modifications (Benzie and Strain, 1996). The presence of antioxidants in the extract contributes to the reduction of ferric form, resulting in an intense blue color complex (Wojdylo et al., 2007). The FRAP reagent consists of acetate buffer, 2,4,6-tris (2-pyridyl)-s-triazine (TPTZ), and ferric chloride (FeCl\(_3\),6H\(_2\)O) in ratio 10:1:1 respectively. The reagent was placed in the water bath at 37 °C until the experiment was started. To prepare 1 L of 300 mM of acetate buffer, 3.10 g of sodium acetate was dissolved in 500 mL of distilled water then followed by the addition of 16 mL of glacial acetic acid. The mixture was added with the corresponding amount of distilled water and the pH of the mixture was adjusted to 3.6. The 10 mM TPTZ solution was prepared in 40 mM hydrochloric acid (HCl) and 20 mM FeCl\(_3\),6H\(_2\)O was prepared in distilled water. Then, 3 mL of FRAP reagent was added to the tubes containing 100 μL of *E. kingiana* extracts (0.03125 mg/mL) and vortexed. The mixture was placed in a water bath at 37 °C for 30 min in dark condition prior to measure the absorbance reading at 593 nm. Blank solution was prepared by replaced the FRAP reagent with distilled water. Ascorbic acid was included as a positive control in this assay. The concentration of FRAP content in the *E. kingiana* extract was expressed as mM of ferrous equivalent Fe (II) per gram of extracts. Ferrous sulphate standard curve was plotted in the concentration range of 0–1.0 mM.

2.12. Cell culture and cultivation

The Caco-2 (ATCC® HTB-37™) human intestinal cell monolayers were purchased from the American Type Culture Collection (ATCC) and maintained at 37 °C in humidified incubator with 5% CO\(_2\). The cells were cultivated and grown in complete growth medium consisting of Dulbecco’s Modified Eagle’s Medium (DMEM) containing 15 mM glucose supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 0.1 mM non-essential amino acids, and 0.1 mM glutamine. The model of Caco-2 cells monolayer were sub-cultured when reaching more than 90% confluency and media were refreshed regularly for every 48 h.

2.13. Cell viability assay

The 3-(4,5-dimethylthiazol-3-yl)-2,5-diphenyl-2H-tetrazolum bromide (MTT) assay was employed to determine the percentage viability of cells upon treatment with extracts (Abu Bakar and Tan, 2017). In this assay, the *E. kingiana* extracts that exhibit significant inhibition of carbohydrate-hydrolyzing enzyme and antioxidant capabilities were selected for cytotoxic evaluation. Briefly, the stock solution was prepared by mixing all extracts with distilled DMSO and sterilized by filtration. In brief, Caco-2 cells were plated into each well of 96-well plates at a density of 1 × 10\(^4\) cells/mL using complete growth medium. A volume of 200 μL of the diluted cell suspension was added to each well and incubated for 24 h. Then, the complete growth media in each well was replaced with 100 μL of serum-free media and incubated for 3 h. Different concentrations (0.025–0.1 mg/mL) of selected *E. kingiana* extracts were added to each well prior to another 45 h incubation. Then, the cells were treated with 20 μL of MTT reagent (5 mg/mL in phosphate-buffered saline) and re-incubated for another 4 h. The supernatant was removed from the wells and 200 μL of DMSO was added to dissolve the purple precipitate. The plate was incubated for 30 min after gently shaken for 15 min. The absorbance reading at 540 nm was recorded using a microplate reader (Halo MPR-96, Dynamica Australia). The following Eq. (5) was used to calculate the percentage of cell viability:

\[
\text{Cell viability} \% = \frac{\text{Abs}_{\text{extract}}}{\text{Abs}_{\text{control}}} \times 100
\]  

where \(\text{Abs}_{\text{extract}}\) denotes the absorbance of the extract and \(\text{Abs}_{\text{control}}\) denotes the absorbance of the control.

2.14. Glucose uptake inhibitory assay in Caco-2 human intestinal cell monolayers

The assessment of glucose uptake inhibition in Caco-2 human intestinal cell monolayers was determined as previously described with several modifications (Malunga and Eck, 2016). In brief, cells were cultivated and subcultured on 24-well plates at the density of 2.5 × 10\(^5\) cells/well for 14 days with medium renewal every 2 days at 37 °C in 5% CO\(_2\) atmosphere. After 2 weeks, the cells were placed in serum-free for 24 h prior to glucose uptake studies. Further, the media was removed, and monolayer cells were rinsed 3 times with phosphate-buffered saline (PBS). Following this, the cells were incubated with preincubation buffer consisting of HEPES buffer with 5 mM glucose for 40 min at 37 °C in 5% CO\(_2\) atmosphere. Subsequently, to initiate the glucose uptake experiment, the preincubation buffer was replaced with 50 μL HEPES buffer (pH 7.4 and glucose free) containing \(^{3}H\) 2-deoxyglucose (5 mM in glucose-free HEPES buffer) in the presence of *E. kingiana* extracts. The cells were then incubated at room temperature in the dark environment for 15 min. The glucose uptake activity was stopped by adding 100 μL of ice-cold preincubation buffer immediately after transport buffer was removed. To observe such dependent effect of *E. kingiana* extracts on the glucose transporter in the presence of sodium, the cells were lysed with 60 μL lysis buffer (20 mg SDS in 1 mL 0.2 M NaOH) and further incubated at room temperature for 2 h. The cells were vortexed, sonicated, collected, and aliquot of cell lysates (45 μL) was added to a scintillating vial for scintillation spectrometry analysis. Protein content of the cell lysates was quantified using BCA Protein Assay Kit (Bio-Rad, USA). The quantification of \(^{3}H\) 2-deoxyglucose concentration was normalized to protein content. The glucose uptake was expressed as counts per minute (cpm) /mg protein relative to untreated control. Cells incubated with DMSO were considered as control. Meanwhile, the inhibitory effect of *E. kingiana* extracts on glucose uptake activity under sodium independent condition was similarly performed except for HEPES buffer without sodium was used. Accordingly, the validity of this assay was cross-checked by observing the linearity of the uptake rates of glucose without *E. kingiana* extracts.

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2.15. Statistical analysis

All the experiments were analyzed in triplicates and the results are presented as mean ± standard error mean (SEM). Statistical analyses were performed by t-test. Differences were considered significant at p value less than 0.05. The IC\textsubscript{50} value was calculated by using GraphPad Prism Version 7.0.

3. Results

3.1. Percentage yield of \textit{E. kingiana} bark and leaf extracts

The secondary metabolites present in the plant can be extracted using appropriate solvents at different polarities. The efficiency of solvent to extract secondary metabolites from plant can be measured by using percentage yield (Murugan and Parimalazhagan, 2014). Fig. 1 showed the percentage yield of \textit{E. kingiana} crude extracts (bark and leaf) after extraction with ethyl acetate and methanol. The result showed that LM extract obtained the highest yield at 12.75%, followed by BM and LEA extracts at 6.17% and 3.86%, respectively. The lowest yield was obtained by BEA extract, with 1.61%.

3.2. Preliminary phytochemical analysis of \textit{E. kingiana} bark and leaf extracts

The phytochemical analysis of \textit{E. kingiana} extracts by using different solvents was shown in Table 1. It can be observed that BEA and BM extracts showed the presence of rich variety of phytochemicals such as tannins, saponins, flavonoids, and terpenoids compared to all other extracts. However, LEA extract had minimum presence of these phytochemicals. In general, flavonoids, saponins and tannins were detected in all extracts except LEA extract, whereas terpenoids were only present in both bark extracts.

3.3. Inhibitory effect of \textit{E. kingiana} bark and leaf extracts against \(\alpha\)-amylase and \(\alpha\)-glucosidase activities

The bark and leaf extracts of \textit{E. kingiana} extracted from two different solvents (methanol and ethyl acetate) were evaluated for their \(\alpha\)-amylase and \(\alpha\)-glucosidase inhibition activities along with acarbose as a positive control. As presented in Table 2, the inhibitory activity of the extracts and acarbose were depicted based on the calculated IC\textsubscript{50} values.

Fig. 2 A and B showed the percentage of inhibition for \(\alpha\)-amylase and \(\alpha\)-glucosidase inhibitory activity of \textit{E. kingiana} bark extracts, respectively in comparison to a positive control, acarbose. The tested concentration had pronounced effect on the percentage of enzyme inhibition, which demonstrated a dose-dependent curve. The highest \(\alpha\)-amylase inhibitory activity was exhibited by BEA extract with the lowest IC\textsubscript{50} value at 2.32 \(\mu\)g/mL and followed by BM extract at IC\textsubscript{50} = 8.30 \(\mu\)g/mL. Both bark extracts were significantly potent than acarbose (IC\textsubscript{50} value at 36.19 \(\mu\)g/mL). A significant difference was observed for both bark extracts of \textit{E. kingiana} compared to acarbose. Likewise, BEA extract also displayed the most potent \(\alpha\)-glucosidase inhibition activity as evidenced by its lowest IC\textsubscript{50} value at 1.83 \(\mu\)g/mL, whereas the less effective activity was obtained from BM extract with IC\textsubscript{50} value at 47.20 \(\mu\)g/mL. These IC\textsubscript{50} values were then compared with acarbose and significant differences were observed in both bark extracts. This suggested that both bark extracts possessed stronger \(\alpha\)-amylase and \(\alpha\)-glucosidase inhibitory activities than acarbose.

The inhibition activity of \textit{E. kingiana} leaf extracts against \(\alpha\)-amylase and \(\alpha\)-glucosidase were shown in Fig. 2 C and D, respectively. A concentration-dependent curve was observed. The inhibitory activity of LM extract based on IC\textsubscript{50} against \(\alpha\)-amylase (IC\textsubscript{50} = 49.4 \(\mu\)g/mL) was more effective than LEA extract (IC\textsubscript{50} = 58.93 \(\mu\)g/mL). However, the inhibition potency of these extracts was considered to be less effective than acarbose, with IC\textsubscript{50} value at 36.19 \(\mu\)g/mL. For \(\alpha\)-glucosidase inhibition activity, highest inhibitory activity with IC\textsubscript{50} value at 32.06 \(\mu\)g/mL was observed in LM extract. By contrast, LEA extract exhibited weaker inhibitory activity with IC\textsubscript{50} value at 716 \(\mu\)g/mL. In this regard, LM extract can be regarded as a mild \(\alpha\)-amylase and \(\alpha\)-glucosidase inhibitor.

3.4. Effect of BEA extract of \textit{E. kingiana} on the inhibition mode of \(\alpha\)-amylase and \(\alpha\)-glucosidase

The BEA extracts with lowest IC\textsubscript{50} value among all the extracts was chosen accordingly to be evaluated for the mode of enzyme inhibition. As shown in Fig. 3 A and B, the inhibition mode of \(\alpha\)-amylase of BEA extract was determined using Michaelis-Menten and Lineweaver-Burk plots, respectively. The graph plot showed a near competitive inhibition of the \(\alpha\)-amylase activity. The Vmax and Km values of such extract were found to be increased from 0.241 mg/mL.min to 0.525 mg/mL.min and 1.405 mg/mL to 9.676 mg/mL, respectively. Such increase in Km and Vmax values indicated that the extract exhibited competitive mode of inhibition against \(\alpha\)-amylase activity.

As depicted in Fig. 3 C and D, the mode of \(\alpha\)-glucosidase inhibition by BEA extract was determined using the Michaelis-Menten and Lineweaver-Burk plots. It was observed that the Vmax value of BEA extract was found to be decreased from 0.019 mg/mL.min to 0.017 mg/mL.min whereas the Km value was remained unaffected. The decreased in Vmax values with unaffected Km value proved that the BEA extract showed a non-competitive mode of inhibition against \(\alpha\)-glucosidase.
3.5. Total phenolic content of E. kingiana extracts

The total phenolic content of the E. kingiana bark and leaf extracts was determined from gallic acid standard curve. As presented in Table 3, the total phenolic content among the E. kingiana extracts was in the following order: BM extract (10.16 mg GAE/g) > LM extract (7.59 mg GAE/g) > BEA extract (4.32 mg GAE/g) > LEA extract (0.13 mg GAE/g). These results revealed that the levels of phenolic compounds were considerably higher in methanol extracts than in ethyl acetate extracts of E. kingiana bark and leaf.
3.6. Effect of E. kingiana extracts on free radical scavenging activity

Next, the DPPH free radical scavenging analysis of bark and leaf parts of E. kingiana extracts were evaluated. As depicted in Fig. 4, all extracts scavenged the DPPH radicals in a dose-dependent manner. The results showed an increase in DPPH radicals scavenging ability with an increase in extract concentrations, suggesting concentration-dependent effect. All extracts showed no significant differences compared to the control, ascorbic acid (AA). However, BM extract showed a significant effect on DPPH radical scavenging activity among other E. kingiana extracts (see Fig. 4A).

Table 4 showed the IC<sub>50</sub> values of DPPH assay of E. kingiana extracts. It was found that the radical scavenging activity of BM extract was higher compared to other extracts as evidenced by lowest IC<sub>50</sub> values (1.18 µg/mL). In following, LM and BEA extracts showed significant scavenging activity with IC<sub>50</sub> value of 3.18 µg/mL whereas LEA extract had the highest IC<sub>50</sub> (119.40 µg/mL) which indicated its poor scavenging activity. The IC<sub>50</sub> value of BM extract was close to ascorbic acid (0.90 µg/mL), thus suggesting a potential source of good antioxidant compounds.

3.7. Effect of E. kingiana extracts on reducing antioxidant power

The reducing power of all E. kingiana extracts was shown in Table 5. FRAP values of all extracts were calculated from ferrous sulphate calibration graph. In general, the result indicated that all E. kingiana extracts have lower antioxidant capacity than ascorbic acid. The highest reducing antioxidant power was exhibited by BM extract which exhibited by high level of phenolic content. The weakest reducing power was demonstrated by LEA extract.

3.8. Effect of E. kingiana extracts on cell viability of Caco-2 cell lines

The cell viability analysis using MTT assay was employed to evaluate the cytotoxic effects of E. kingiana extracts at different concentrations (0.025–0.1 mg/mL) in Caco-2 cell monolayers. Untreated cells were considered as control. Interestingly, it is worth noting that both BEA and BM extracts displayed no cytotoxicity properties relative to the untreated control cells even at higher concentration (0.1 mg/mL) following 48 h exposure (see Fig. 5). This is a good indication that the optimal concentrations used in the E. kingiana extracts in other biological assays in the present study are considerably safe and did not cause significant cytotoxicity on human intestinal cell. In this regard, we therefore selected the lowest concentrations (0.025 and 0.05 mg/mL) of both BEA and BM extracts of E. kingiana to be further evaluated for glucose uptake inhibition in Caco-2 cells monolayer.

Table 5 FRAP reducing ability of E. kingiana extracts. Data are means ± standard error means (N = 3).

<table>
<thead>
<tr>
<th>Sample</th>
<th>FRAP (mM Fe&lt;sup&gt;2+&lt;/sup&gt; equivalent/g extract)</th>
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<tr>
<td>BM</td>
<td>118.92 ± 9.31*</td>
</tr>
<tr>
<td>BEA</td>
<td>95.14 ± 7.74*</td>
</tr>
<tr>
<td>LM</td>
<td>102.38 ± 0.52*</td>
</tr>
<tr>
<td>LEA</td>
<td>52.22 ± 1.56*</td>
</tr>
<tr>
<td>AA</td>
<td>174.59 ± 5.82*</td>
</tr>
</tbody>
</table>

* significantly different (p < 0.05) compared to ascorbic acid.

3.9. Effect of BEA and BM extracts on glucose uptake inhibition in Caco-2 cell lines

Caco-2 human intestinal cell monolayers are widely utilized as a cellular model for intestinal absorption studies (Pico and Martínez, 2019). To further elucidate the specific mechanistic evidence of BEA and BM extracts on intestinal cellular glucose uptake inhibition, we investigated the effect of these two extracts on glucose uptake inhibitory potential in an intact human intestinal epithelial cell lines under both sodium-dependent and sodium dependent conditions. As illustrated in Fig. 6, BEA and BM extracts of E. kingiana at concentrations of 0.025 mg/mL and 0.05 mg/mL exhibited significant inhibition on the glucose uptake activity under sodium-dependent condition compared to untreated control. Nevertheless, both BEA and BM extracts at tested con-

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Table 3 Total phenolic content from leaf and bark extracts of E. kingiana. Data are means ± standard error means (N = 3).

<table>
<thead>
<tr>
<th>Extract</th>
<th>Total phenolics content (mg GAE /g extract)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BM</td>
<td>10.16 ± 0.06</td>
</tr>
<tr>
<td>BEA</td>
<td>4.32 ± 0.05</td>
</tr>
<tr>
<td>LM</td>
<td>7.59 ± 0.13</td>
</tr>
<tr>
<td>LEA</td>
<td>0.13 ± 0.01</td>
</tr>
</tbody>
</table>

GAE = Gallic acid equivalent.

Fig. 4. The DPPH free radical scavenging activity of bark (A) and leaf (B) extracts of E. kingiana. AA: ascorbic acid. Error bars represent standard error of means of N = 6.
centrations had no significant inhibitory effect on glucose uptake activity relative to untreated control under sodium-independent condition (sodium-free media).

4. Discussion

One of the common strategies for controlling DM is to reduce the increased blood glucose levels by slowing down glucose absorption. This can be achieved by inhibition of the carbohydrate-hydrolyzing enzymes such as α-amylase and α-glucosidase. This in turns can prolong the carbohydrate digestion time and thereby reducing the rate of glucose absorption. Consequent upon this, elevated postprandial hyperglycemia can be diminished (Safamansouri et al., 2014). Accordingly, an attempt to find other alternative drugs derived from natural sources with increased potency and lesser adverse effects is of great interest. In this research work, we investigated for the first time the attributive effect of E. kingiana extracts on carbohydrate-hydrolyzing enzymes, antioxidant activities and intestinal glucose uptake inhibition in human intestinal Caco-2 cell monolayers.

Most of the phytochemicals are health-benefiting compounds and known to contribute to multiple biological activities. For instance, terpenoids and flavonoids had been reported as powerful antioxidants which possess antibacterial, anti-inflammatory and anticancer activities (Iqbal et al., 2015). Saponins which can function as surface-active compounds were used in the treatment of hyperglycemia, anti-inflammatory and act as antioxidants. Tannins are known to have anti-mutagenic, antibacterial, antitumor activity and can act as antioxidants to inhibit the generation of hydroxyl radical (Iloki-Assanga et al., 2015). In the present study, the results indicated that both bark extracts of E. kingiana (BM and BEA) contain most of the phytochemical compounds tested and may contribute synergistically in treatment of hyperglycemia-associated oxidative stress complications.

The current study showed that bark extracts of E. kingiana had potential α-amylase and α-glucosidase inhibition activity. Specifically, BEA extract was more potent than other E. kingiana extracts in inhibiting these enzymes activity. This may be due to the solubility of bioactive compounds extracted was higher in BEA extract than other E. kingiana extracts which responsible for the enzyme inhibition activity (Kazeem et al., 2013). As shown by the IC50 values, BEA extract was a mild inhibitor of α-amylase and a strong inhibitor of α-glucosidase. This is in accordance with a previous report where a strong α-amylase inhibition can lead to the build-up of indigestible starch in the colon which induce abnormal colonic fermentation (Etxeberria et al., 2012). Thus, the mild inhibitor of α-amylase coupled with stronger α-glucosidase inhibition can efficiently control the postprandial spike in blood glucose concentrations with fewer side effects (Kazeem et al., 2013).

Several phytochemical compounds such as tannins and flavonoids can act as antioxidants to protect the function of pancreatic beta cell and thus reducing the progression of diabetes (Kazeem et al., 2013). Phytochemical variation of E. kingiana extracts in contributing...
inhibitory effect might be due to difference in chemical compounds extracted with different polarities of solvents (Qayyum et al., 2016). Phytochemicals such as polyphenolics and flavonoids have been reported to have features of reducing blood glucose level (Ponnumary et al., 2011). This indicated that the presence of phytochemicals in BEA extract such as flavonoids, terpenoids, saponins and phenols could be one of the contributing factors that lead to the enzyme inhibition activity. The synergistic interaction of all compounds present in the extract could give rise to the maximum therapeutic efficiency in lowering the blood glucose level. In line with this, it was proved that the biological properties of plant extracts are closely associated with the number of phytochemicals present (Ullrich-Merzenich et al., 2010).

In the kinetic study to evaluate the enzymatic model of inhibition in the steady state, BEA extract exhibited a near competitive inhibition of the α-amylase activity. It was believed that such components in the extract might have same functional group or structure to the substrate which caused substrate to move from the active site of the enzyme (Khacheba et al., 2014). This indicates that the active components in the BEA extract competes with the substrate for binding site of the enzyme. The increase in Vmax value, possibly due to the presence of another activator in BEA extract which distorted the conformation binding of active site, resulting in weaker enzyme-substrate binding (increased in Km). On the other hand, the activator does not distort the binding during transition state, leading to increase in Vmax. Consequently, this can delay the breakdown of complex carbohydrates to simple monosaccharides (Kazeem et al., 2013). Meanwhile, BEA extract displayed a non-competitive inhibition on α-glucosidase. The active components in the BEA extract (inhibitor) presumably did not compete with substrate for the active site, thus Km value was unaffected. At the same time, such inhibitors may have some effects on the functional enzyme, thus leading to decreased Vmax (Pelley, 2011). The substrate can bind normally to α-glucosidase since the active components of the extract binds to a site other than the active site of enzyme. However, this caused a conformational change to the enzyme. Hence, the enzyme was non-functional if the inhibitor was bound with it (Zhang et al., 2015).

Phenolic compounds are among the secondary metabolites present in plants which possess various biological activities such as anti-inflammation, antiaging or anticancer activities (Yadav and Agarwala, 2011). They contain the conjugated benzene ring structure and hydroxyl groups which are responsible for antioxidant activity. Furthermore, it has been reported that these compounds contribute to radicals scavenging capacity (Bouazzzi et al., 2015). The effectiveness of the hydrogen atom to be donated from the hydroxyl group in the aromatic ring to free radicals mainly depend on the arrangement and number of hydroxyl group present in phenolic compounds. Besides, phenolic compounds can act as reducing agents by donating their electrons or hydrogen atoms, quenchers of singlet oxygen to those compounds with possessing metal chelating potential (Iqbal et al., 2015). As shown in Table 3, the current study revealed that BM extract contained higher value of phenolic compounds in comparison to other E. kingiana extracts. This is due to the high polarity of methanol that can extract more phenolic compounds than ethyl acetate did (Hyun et al., 2016; Senguttuvan et al., 2014). It can be verified that the strong scavenging capacity of methanol extracts was closely associated with higher phenolic content present in these solvents. Therefore, the strong scavenging capacity of methanol extracts could be related to the hydrogen donating capability from the phenolic compounds. Several studies reported that the antioxidant activity of plant extracts are contributed by the richness of phenolic content (Iloki-Assanga et al., 2015). On the other hand, the presence of flavonoids in E. kingiana methanolic extracts may also contribute to a high antioxidant activity. Flavonoids are the most important and widely distributed phenolic compounds. They contain hydroxyl group which possess scavenging ability by neutralizing the free radicals in the body which could lead to the formation of reactive oxygen species (Pietta, 2000). Flavonoids can also act as antioxidants by reducing the molecular damage caused by oxidative stress, that might be beneficial in the treatment of type 2 DM (Sarian et al., 2017).

In the current study, methanol extracts of E. kingiana exhibited a higher reducing power than ethyl acetate extracts. We deduced that variation in reducing power of E. kingiana extracts could be attributed to polarities in different solvent systems which may influence the amount and type of antioxidant compounds in the extracts that can dissolved by the solvent. Overall these findings are in accordance with a previous report in which methanol extracts of Cinnamomum verum exhibited high antioxidant activity (Maximba and Wale, 2015). The present findings revealed that the antioxidant capacity of E. kingiana extracts from family Lauraceae could possibly correlated with the presence of phenolic compounds, which contribute their antioxidant activities through hydrogen atom donation to interrupt the formation of free radical chain. Furthermore, the result of FRAP found clear support for the above-mentioned findings of free radical scavenging activity, further suggesting the good antioxidant potential of the E. kingiana bark methanolic extracts. Therefore, the findings presented in the current study indicate that the high antioxidant potential of BM extract most likely is due to presence of phenolic compounds.

Accordingly, specific evaluation on the cytotoxicity properties of plant extracts is required to provide safety and toxicological information on the dose-dependent effect on such cellular model. In the present study, Caco-2 cell monolayers were utilized as a cellular model. It was found that both BEA and BM extracts at different concentrations tested (0.025–0.1 mg/mL) did not exhibit significant decrease in cell viability compared to untreated cells, thus suggesting non-toxic conditions of E. kingiana extracts at these concentrations on Caco-2 cell monolayers. Compelling evidence suggests that intestinal glucose absorption across the apical membrane was mainly mediated by a concerted action of SGLT1 and GLUT2 (Kellnet et al., 2008; Pico and Martinez, 2019). To better elucidate the mechanistic effect of such extract on intestinal glucose absorption, we further demonstrated two distinct glucose uptake pathways; sodium-dependent (SGLT1 and GLUT-mediated uptake) and sodium-independent (GLUT-mediated component) in Caco-2 cell monolayers. Several reports showed that several dietary polyphenols and phytochemicals that are frequently found in plants, fruits and vegetables such as polyphenol-glucosides, (−)-epigallocatechingallate, (−)-epicatechingallate and (−)-epigallocatechin possessed inhibitory activities against glucose transporters in human intestinal cells by decreasing glucose uptake activity (Johnston et al., 2005; Pico and Martinez, 2019). In the present study, the bark extracts of E. kingiana were significantly decreased the glucose uptake of Caco-2 cells compared to untreated control under sodium-dependent condition (total glucose uptake). However, no differential effect was observed on glucose uptake inhibition by BEA and BM extracts under sodium-dependent condition (sodium mediated glucose uptake). It is possible, therefore that this cellular glucose uptake inhibition by E. kingiana extracts is likely to be mediated by SGLT1 in correlation to no inhibitory effect on glucose uptake under sodium-free condition which indicate no interaction with GLUT2. Further studies with more defined analyses on relative gene and protein expression of these glucose transporters in Caco-2 cell monolayers are imperatively required to better explicate the mechanism of action of these crude extracts.

With the increasing prevalence of metabolic diseases such as DM and
other associated comorbidities, such efforts are being actively engaged towards identifying the better alternative natural therapies for preventing and treating hyperglycemia-associated oxidative stress conditions. To the best of our knowledge, we are the first to discover the attributive properties of *E. kingiana* bark extracts against carbohydrate-hydrolyzing enzymes, antioxidant capabilities and glucose absorption in Caco-2 human intestinal cells monolayer. Nevertheless, our study has several limitations. As bark extracts of *E. kingiana* revealed the promising antidiabetic and antioxidant potential, further investigations on the isolation and identification of bioactive components which responsible for such attributive effects are imperatively needed. Besides, further mechanistic studies in elucidating the biological properties of *E. kingiana* extracts and their mechanism of actions in various cellular models and animal studies are desirable to provide concrete evidence on its attributive effect in attenuating hyperglycemia-induced oxidative stress complications.

5. Conclusion

In summary, the present study systematically evaluated the *in vitro* efficacy of *E. kingiana* extracts on carbohydrate-hydrolyzing enzymes, antidiabetic activities with inhibitory potential on glucose uptake in human intestinal Caco-2 cells monolayer. Importantly, the key finding from the present study is the inhibitory potential of these bark extracts of *E. kingiana* to affect intestinal glucose absorption, ostensibly via an interaction with SGLT1, thus potentially reducing the risk of postprandial hyperglycemia. Taken together, it can be concluded that bark extracts (ethyl acetate and methanol) of *E. kingiana* exert promising in vitro anti-hyperglycemic and antioxidant properties, which can be further utilized as a potential candidate for treatment of hyperglycemia–associated oxidative stress complications.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcb.2020.101594.

References


