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Membrane stabilization and kinetics of carbohydrate metabolizing enzymes (α-amylase and α-glucosidase) inhibitory potentials of *Eucalyptus obliqua* L.Her. (Myrtaceae) Blakely ethanolic leaf extract: An *in vitro* assessment

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ABSTRACT

Eucalyptus spp. are important herbs finding therapeutic application in many parts of the world against a number of disorders including diabetes mellitus. Inhibitors of α-amylase and α-glucosidase offer an effective strategy to modulate levels of postprandial hyperglycemia via control of starch metabolism. The GC–MS analysis, membrane stabilization and mechanism(s) of inhibitory potential of *Eucalyptus obliqua* ethanolic extract on α-amylase and α-glucosidase were evaluated. The α-amylase inhibitory potential of the extract was investigated by reacting different concentrations of the extract with α-amylase and starch solution, while α-glucosidase inhibition was determined by pre-incubating α-glucosidase with different concentrations of the extracts followed by the addition of *p*-nitrophenylglucopyranoside. The mode(s) of inhibition of the enzymes were determined using the Lineweaver–Burke plot. The extract exhibited potent and moderate inhibitory potential against α-amylase and α-glucosidase, respectively. The inhibition was dose-dependent with respective half-maximal inhibitory concentration (IC_{50}) values of 2.19 and 0.45 mg mL^{-1}. The extract also had respective membrane stabilization potentials of 86.58% and 76% on bovine erythrocytes against hypotonic solution and heat-induced hemolysis. The effect elicited by the extract at the investigated doses could be ascribed to the presence of active metabolites as revealed by the analytical chromatogram. Overall, the non-competitive mechanism of action of *E. obliqua* extract is due to its inhibitory effects on both α-amylase and α-glucosidase. This concomitantly extenuates the rate of starch hydrolysis leading to ameliorated glucose levels, thus lending support to hypoglycemic candidature of *E. obliqua*.

1. Introduction

Diabetes mellitus (DM) is a complicated metabolic disorder that has gravely troubled human health and the overall quality of life. It is probably one of the oldest diseases known to man. It was first reported in Egyptian manuscript about 3000 years ago and expressively becoming the third greatest threat to human health after cancer, cerebrovascular disease, and cardiovascular disease (Vasim et al., 2012). Although DM is a combination of heterogeneous disorders commonly presenting with episodes of hyperglycemia, glucose intolerance, insulin resistance, and relative insulin deficiency (Mohan et al., 2007), its complications are usually attributable to either microvascular (retinopathy, neuropathy, and nephropathy) or/and macrovascular (heart attack, stroke, and peripheral vascular) ailments (Umar et al., 2010). In 2012, an estimated 1.5 million deaths were directly linked to DM and more than 80% of this occurred in developing countries (Vasim et al., 2012). Recent reports also estimated its global prevalence to be 9.0% among adults, and it is projected to be well above 15% before 2025 (Saravanan and Pari, 2015). The geometrical increases in the number of diabetics cannot be divorced from unhealthy life style, urbanization, aging, and deleterious impact of free radicals (Wild et al., 2004). Since free radicals have been implicated in the pathogenesis of DM, one of the logical approaches to manage its potential burden may be via antioxidant application. Antioxidants have been shown to prevent destruction of pancreatic β-cells by inhibiting auto-oxidation chain reaction, thereby halting progression to diabetes complications (Sabu and Kuttan, 2004; Liu et al., 2007). Due to their enormous antioxidative and pharmacological significance, medicinal plants are being extensively explored as therapeutic modality of choice against diabetes (Campos et al., 2003; Aslan et al., 2010). Besides ample advocation by the World Health Organization (WHO) on the relevance of botanicals to manage and treat DM (Wild et al., 2004), the overall increased admiration of phytotherapy for this disorder may be due to the limited efficacy and undesirable adverse effects associated with the orthodox antidiabetic drugs (Marles and Farnsworth, 1994). Interestingly, *Eucalyptus* spp. belong to the class of herbs with excellent antidiabetic and hypoglycemic potentials (Mahmoudzadeh-Sagheb et al., 2010; Baishakhi et al., 2014; Alis et al., 2015). *Eucalyptus obliqua* L.Her. (Myrtaceae) Blakely is an evergreen

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tall tree, native to Australia and with characteristic fast growing traits. It is a unique sp. of the eucalyptus family where other spp. derived their common feature of bearing oblique leaves. In addition to being ornamental, *E. obliqua* has been used to drain swamps and potent antimalarial bioactive principles have been isolated from its leaf extracts (Barry et al., 2015). Although, pharmacological significance of many eucalypts have been well documented (Lee and Shibamoto, 2001; Slee et al., 2006; Serafino et al., 2008; Sadlon and Lamson, 2010), *E. obliqua* is just receiving attention in this regard (Hideki et al., 2008).

Besides belonging to the family of botanicals with documented antidiabetic activity (Baishakhi et al., 2014), results of our systematic ethnobotanical survey of medicinal plants of the Local Government Areas of Kwara State, Nigeria, also revealed *E. obliqua* as one of the plants commonly and prominently used to manage and treat diabetes. In light of this, and coupled with no previous scientific reports on its mechanism of inhibitory potential on carbohydrate metabolizing enzymes, the presented study was designed to evaluate its α-amylase and α-glucosidase inhibitory potential via an *in vitro* model. In addition to its membrane stabilization effect, GC–MS analysis of the extract was also conducted with a view to providing detailed and comprehensive information on the phytoconstituents present therein.

2. Materials and methods

2.1. Chemicals and reagents

Acarbose and ibuprofen were procured from Bayer Medical Co., Germany and Ranbaxy Nigeria, Ltd., Nigeria, respectively. Porcine pancreatic α-amylase, rat intestinal α-glucosidase and *p*-nitrophenyl-α-D-glucopyranoside (pNPG) were products of Sigma-Adrich Co., St Louis, Missouri, USA, while soluble starch and dinitrosalicylic acid (DNS) were obtained from J. T. Baker Inc., Phillipsburg, USA. Water used was glass-distilled and other chemicals and reagents were of analytical grade.

2.2. Plant collection, authentication, and preparation of extract

Fresh leaves of *E. obliqua* were collected from the premises of Kwara State Ministry of Agriculture, Ilorin, Nigeria, and were authenticated at the Plant Sciences Department, University of Ilorin, Ilorin, Nigeria. A voucher specimen (UIH001/1178) was prepared and deposited at the University's Herbarium. The leaves were thoroughly rinsed, shade dried to constant weight, and thereafter pulverized (model MS-223; Blender/Miller III, Taiwan, China) to smooth powder. The powdered sample (300 g) was divided into three portions of 100 g each and extracted exhaustively with regular agitation in 1 L each of methanol, ethanol, and water, respectively. The resulting infusion in each case was filtered (Whatman no. 1 filter paper) and evaporated to dryness in a rotary evaporator (Cole Parmer SB 1100, Shangai, China) in respect of the organic solvent extracts, while the water extract was lyophilized using Virtis Bench Top lyophilizer (SP Scientific Series, USA). This yielded 10 g, 14 g, and 14.5 g of the methanol, ethanol, and water extracts, respectively.

About 15 μL of each extract was spotted on silica gel TLC plate, and the chromatograms obtained were thereafter developed in dichloromethane/methanol (8.5:1.5 v/v) system and sprayed with 0.2% 1,1-diphenyl-2-picrylhydrazyl (DPPH) in methanol for detection of antioxidant constituents. From the chromatograms (not shown), the ethanolic extract of *E. obliqua* revealed most prominent and highest number of antioxidant spots and was selected for the subsequent bioassays.

2.3. α-Glucosidase inhibition and kinetic assays

The α-glucosidase inhibitory activity was assayed following the method of Elsnoussi et al. (2012). Briefly, different concentrations (0.25–10.0 mg mL^{-1}) of sample were prepared in distilled water. Then, 50 μL from the stock solution was mixed with 100 μL of 0.1 M phosphate buffer (pH 6.9) containing 1.0 M of α-glucosidase solution. The mixtures were then incubated in 96-well plates at 25 °C for 10 min. Following this, 50 μL of 5 mM *p*-nitrophenyl-α-D-glucopyranoside solution in 0.1 M phosphate buffer (pH 6.9) was added to each well at timed intervals. The reaction mixtures were incubated at 25 °C for 5 min. The absorbance readings were thereafter taken at 405 nm using a microplate reader (Thermomax, USA) and the values compared with a blank which contained 50 μL of the buffer instead of the extract. Acarbose was prepared in distilled water at same concentrations as the extract and used as control. The experiments were conducted in triplicate and the α-glucosidase inhibitory activity was expressed as %inhibition using the expression:

$$\%\text{Inhibition} = [(\Delta A_{\text{control}} - \Delta A_{\text{extract}})/\Delta A_{\text{control}}] \times 100,$$

where $\Delta A_{\text{control}}$ and $\Delta A_{\text{extract}}$ are the changes in absorbances of the control and extract sample (relative to blank), respectively. Using standard calibration curve, the concentration of *E. obliqua* causing 50% inhibition (IC_{50}) of α-glucosidase activity was estimated.

For the enzyme kinetics on inhibition of α-glucosidase activity by *E. obliqua* ethanolic extract, the method of Sabiu et al. (2016) was adopted. Briefly, 50 μL of 5 mg mL^{-1} extract was pre-incubated with 100 μL of α-glucosidase solution for 10 min at 25 °C in one set of tubes. In another set of tubes, α-glucosidase was pre-incubated with 50 μL of phosphate buffer (pH 6.9). Fifty microliters of pNPG at concentrations (0.63–2.0 mg mL^{-1}) was added to both sets of reaction mixtures to start the reaction. The mixture was then incubated for 10 min at 25 °C, and 500 μL of Na_2CO_3 was added to stop the reaction. The amount of reducing sugars released was determined colorimetrically using a *p*-nitrophenol standard curve. Reaction rates (v) were thereafter calculated and double reciprocal plots of enzyme kinetics were constructed according to Lineweaver and Burk method to study the nature of inhibition. K_m and V_{max} values were also calculated from the Lineweaver–Burk plot (1/v versus 1/[S]) (Lineweaver and Burk, 1934).

2.4. α-Amylase inhibition and kinetic studies

Following the method of Sabiu et al. (2016), the α-amylase inhibitory activity and the mode of inhibition were evaluated. Briefly, varying concentrations (0.25–10.0 mg mL^{-1}) of the extract were prepared, and 500 μL of each was mixed with 500 μL of 0.02 M sodium phosphate buffer (pH 6.9) containing 0.5 mg mL^{-1} of α-amylase solution and incubated in test tubes at 25 °C for 10 min. After pre-incubation, 500 μL of 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9) was added to each tube at timed intervals. The reaction mixtures were incubated at 25 °C for 10 min and stopped with 1.0 mL of dinitrosalicylic acid color reagent. The tubes were then incubated in a boiling water bath for 5 min and subsequently cooled to room temperature. The reaction mixtures were then diluted with distilled water (15 mL), and the absorbance readings were measured at 504 nm using a spectrophotometer (Biochrom WPA Biowave II, Cambridge, England) and the values compared with a blank which contained 500 μL of the buffer instead of the extracts. Acarbose was prepared in distilled water at same concentrations as extracts and used as control. The experiments were conducted in triplicate, and the α-amylase inhibitory activity was expressed as %inhibition. The concentration of the extract causing 50% inhibition (IC_{50}) of α-amylase activity was estimated from its standard calibration curve.

For the kinetic experiments, extract was taken at its IC_{50} value and incubated with α-amylase, while the concentration of starch (substrate) was varied from 0.3 to 5 mg mL^{-1} and reaction allowed to proceed as highlighted above. The amount of reducing sugars released was determined spectrophotometrically using maltose standard curve and converted to reaction velocities (v). The Lineweaver–Burk double reciprocal plot (1/v versus 1/[S]) was constructed and the kinetics of