Research Article

α-Amylase and α-Glucosidase Inhibitory Activities of Phenolic Extracts from *Eucalyptus grandis* × *E. urophylla* Bark

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This study evaluated the inhibitory effects of different extracts and fractions from *Eucalyptus grandis* × *E. urophylla* bark (EB) against α-glucosidase and α-amylase enzyme activities. The ethyl acetate extract (EB-E) showed the highest activity among others. Seven fractions were derived from EB-E; among them EB-E-7 showed the highest significant inhibition of both enzymes, with IC 

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 of 1.40±0.18 and 1.72±0.12 μg/mL, respectively. EB-E and its active fraction EB-E-7 showed highest contents of total phenolics: 178.79±4.68 and 920.4±5.46 mg GAE g

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, respectively. HPLC-MS analysis of EB-E-7 revealed the presence of ellagic acid, quercetin-glucuronide, quercetin-3-α-rhamnopyranoside, and ellagic acid rhamnoside as major compounds, together with smaller concentrations of myricetin-rhamnose,isorhamnetin-hexoside, myricetin-3-α-arabinofuranoside, and isorhamnetin. Therefore, the phenolic compounds from *Eucalyptus grandis* × *E. urophylla* bark potently inhibited α-amylase and α-glucosidase activity, having potential in prevention of hyperglycemia.

1. Introduction

Nowadays, 114 million people in China are diagnosed with type 2 diabetes [1]. Defects in insulin secretion and deteriorating glucose regulation are the main contributing factors to postprandial hyperglycemia [2, 3]. Serum glucose is obtained from hydrolysis of dietary starch which is regulated by the activity of α-amylase and α-glucosidase. The enzyme activities were inhibited by natural inhibitors that decrease glucose absorption and postprandial hyperglycemia [4]. One tactic of managing diabetes is the control of serum glucose by inhibiting the activities of α-amylase and α-glucosidase [5, 6]. Thus, there is a sustained requirement for natural α-amylase and α-glucosidase inhibitors that have better specificity and fewer side effects. Natural resources containing α-glucosidase and α-amylase inhibitors have been a research target. Inhibitors from *Eisenia bicyclis*, Irish seaweed, and Alaskan seaweed have all been recently reported as one of the biochemical mechanisms accountable for the reduction of serum glucose levels. Polyphenolic constituents from seaweeds have shown strong inhibitory activity against both α-glucosidase and α-amylase [4, 7, 8].

*Eucalyptus* species, some of which have been utilized in traditional medicine, are mainly used in industry for timber and paper pulp production [9, 10]. *Eucalyptus grandis* × *E. urophylla* is a preferred genotype in South America (3.75 million ha of plantations) [11], due to their fast growing and short rotation periods and favorable pulping and bleaching ability [12]. *Eucalyptus* bark is a waste material from the pulp industry. It is either thrown away or simply burned for energy production. The exploitation of valuable chemical constituents from the industrial waste material is a strategy that has been already implemented in some pulp industries. Previous studies have reported that the bark of *Eucalyptus* species is an abundant source of valuable bioactive compositions such as phenolic compounds [13], tannins [14], flavonoids, and monoterpenes [15], as well as high value triterpenic acids [11, 16, 17]. However, to the
best of our knowledge, information concerning the phenolic composition of *E. grandis* × *E. urophylla* is scarce [12]. Most published studies have just reported the presence of these compounds in leaves [18, 19], yet little research has been undertaken to evaluate effect of phenolic compounds of *E. grandis* × *E. urophylla* bark extracts (EB) on carboxylic enzymatic activity of α-amylase and α-glucosidase.

The aim of this study is to evaluate the potential of EB as a source of valuable phenolic compounds with biological activity. In this study, EB was extracted and bioassayed in order to identify the chemical composition of extracts that hold potential for diabetic care through their inhibition of carboxylic enzyme activity of α-glucosidase and α-amylase.

### 2. Experimental

#### 2.1. Materials

*Eucalyptus grandis* × *E. urophylla* bark samples (EB) were selected from 4-year-old trees in Guangxi state-owned seven-slope Forest Farm. The barks were cut into pieces, air-dried, and ground into less than 1 mm granulometry.

n-Hexane, n-butanol, ethyl acetate, methanol (HPLC grade), formic acid, Tris-HCl buffer pH 7.5 (PB), and LC-MS grade solvents were purchased from Fisher Scientific (USA). p-Nitrophenyl-α-D-glucopyranoside (PNPG), α-glucosidase (75 U/mg), α-amylase, carobose, starch substrate, quercetin, gallic acid, and ellagic acid were obtained from Sigma-Aldrich (USA).

#### 2.2. Extraction and Isolation

The powdered bark of *E. grandis* × *E. urophylla* (1.0 kg) was extracted with 80% methanol in water (1:12 w/v, 3x), three times at 50°C in an ultrasonic bath, and then filtered with Büchner funnel. The combined aqueous methanol extracts were concentrated under reduced pressure to obtain an aqueous fraction (crude extract). This aqueous fraction was diluted with ddH2O and liquid-liquid extracted successively with hexane, ethyl acetate, and n-butanol (1:3 v/v, 5x) to afford 4 different solvent partitions (H, E, B, and W, resp.). Solutions were concentrated with rotary evaporator to give residues and then freeze-dried to afford H extract (11.5 g), E extract (38.6 g), B extract (56.2 g), and W extract (74.7 g), respectively. Dry extracts were stored in the refrigerator until bioactive experiments and separation were conducted.

The ethyl acetate extract (EB-E) (0.750 g) was separated with a Grace Reveleris® PREP LC (Columbia, MD, USA) with an Alltech Econosil C18 column (250 mm × 10 mm × 10 μm) and eluted using a solvent system made of water with 0.1% TFA and acetonitrile. Based on the UV and ELSD profiles, eluates were collected and combined into 7 fractions: EB-E-1 (0.337 g), EB-E-2 (0.123 g), EB-E-3 (0.024 g), EB-E-4 (0.016 g), EB-E-5 (0.025 g), EB-E-6 (0.045 g), and EB-E-7 (0.052 g).

#### 2.3. Total Phenolic Content

The total phenolic content (TPC) of bark extracts and fractions was determined by the Folin-Ciocalteu method which was described in a previous study [20]. Briefly, samples solutions in methanol (0.5 mL) were mixed with 10-fold-diluted Folin-Ciocalteu reagent (2.5 mL) and 2 mL of 7.5% sodium carbonate. The mixture was incubated for 30 min at room temperature before the absorbance was measured at a wavelength of 760 nm, using a UV-Vis 2450 spectrophotometer (Shimadzu, Japan). A mixture of water and reagents was used as a blank. Gallic acid was used for the calibration curve and results were expressed as gallic acid equivalent (mg GAE/g extract or fraction). The analyses were carried out in triplicate and the average value was calculated in each case.

#### 2.4. Characterization of Phenolic Compounds

LC-MS analysis was performed using a Shimadzu LC-MS-IT-TOF instrument (Shimadzu, Tokyo, Japan) equipped with a Prominence HPLC system (SIL-20A HT autosampler, LC-20AD pump system, and SDP-M20A photo diode array detector). The LC separation was performed using C18 reverse-phase column (Shim-pack XR-ODS column, 50 mm × 3.0 mm id × 2.2 μm; Shimadzu Scientific Instruments Inc., Columbia, MD, USA) and a binary solvent system comprised of water with 0.1% formic acid (A) and 100% methanol (B). Compounds were separated into the ion source at a flow rate of 0.35 mL/min with the following gradient: 5–10% B (0–5 min), 10–15% B (5–15 min), 15–30% B (15–25 min), 30–60% B (25–35 min), hold 60% B (35–40 min), and 60–5% B (40–43 min). Prior to the next injection, the column was reequilibrated for 5 min at initial conditions [4]. The heat block and curved desolvation line (CDL) were maintained at 200°C. Nitrogen was used as nebulizer and drying gas with the flow rate was set at 1.5 L/min. The ESI source voltage was set at 4.5 kV and the detector was set at 1.5 V. The instrument was calibrated to <5 ppm error in mass accuracy with an external standard of sodium TFA solution. Ionization was performed using a conventional ESI source in positive and negative ionization modes. Data was acquired from m/z 150–2500. Shimadzu's LC-MS Solution software was used for system control and data analysis. Compound identification was based on retention times, UV spectra, and molecular formulae generated based on m/z in the negative ion mode. Identity of isolated compounds was confirmed by the aid of NMR spectra, literature, and available authentic samples.

#### 2.5. α-Amylase Inhibitory Activity

The α-amylase inhibitory bioactive assay was carried out as described by Kellogg et al. [4]. 35 μL of inhibitor, different extracts, and fractions of EB or solvent (control) were added to different well of the 96-well microplate with 5 μL 1% (w/v) starch solution. The mixture was reacted at 37°C for 5 min, 10 μL enzyme solution was added to each well and incubated at 37°C for 10 min, and then 150 μL Lugol's solution was added to each well. The absorbance value at 595 nm was measured using the microplate reader against a blank (without enzyme). All samples were prepared in triplicate. EB samples were replaced by the acarbose as the positive control. In (1), A<sub>c</sub> is an absorbance value of the uninhibited enzyme (solvent replacing EB extract and inhibitor of the mixture), A<sub>e</sub> is an absorbance value of the enzyme with the EB samples, and A<sub>0</sub> is an absorbance value of solvent (no enzyme present). IC<sub>50</sub> values were obtained from different concentration sample solutions.
The inhibitory activity of the different sample from EB was calculated by the following equation:

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inhibitory\ \text{activity}\% = \frac{(A_c - (A_1 - A_0))}{A_c} \times 100\%. \quad (1)
\]

### 2.6. α-Glucosidase Inhibitory Activity

The bioassay method of multowell plate system was applied for α-glucosidase inhibitory activity assay as described by Kellogg et al. [4]. 20 μL of inhibitor, different extracts, and fractions of EB or solvent (control) were added to each well of the 96-well microplate with 100 μL PNPG solution, 1 mmol/L in PB. The mixture was incubated for 5 min at 30 °C. 100 μL enzyme solution was added to each well; then the absorbance value at 405 nm was measured for 30 min in the microplate reader against a blank (without enzyme). All samples were prepared in triplicate. EB samples were replaced by the acarbose as the positive control. In (1), \(A_c\) is an absorbance value of the uninhibited enzyme (solvent replacing EB extract and inhibitor of the mixture), \(A_1\) is an absorbance value of the enzyme with the EB samples, and \(A_0\) is an absorbance value of solvent (no enzyme present).

The inhibitory activity of the different sample from EB was calculated by the following equation:

\[
inhibitory\ \text{activity}\% = \frac{(A_c - (A_1 - A_0))}{A_c} \times 100\%. \quad (2)
\]

### 2.7. Statistics

All assays were performed in triplicate. Statistical analyses were suggested as means ± SD by Origin 8 software. The IC\(_{50}\) value was calculated by logarithmic transformation. Significant differences p value was p < 0.05. All data were presented as means ± SD (n = 3).

### 3. Results and Discussion

#### 3.1. Extraction Yield and Total Phenolic Content

The extraction yields of 80% aqueous methanol \(E.\) grandis \(× E.\) urophylla bark extract analyzed, recovery of different solvent fractions (hexane, ethyl acetate, n-butanol, and water, resp.), recovery of fractionation by PREP LC, and the respective total phenolic content (TPC) determined by Folin-Ciocalteu method are listed in Table 1. The crude extract constituted 19.39% of the extraction reached 93.35%.

#### 3.2. Fractionation of the Active Extract EB-E

The EB-E extract suggested the highest inhibition to α-amylase and α-glucosidase activities. EB-E was further separated on PREP LC to generate 7 fractions based on the UV and ELSD profiles (Figure 1). Collected fractions were assayed for their TPC. The TPC in EB-E-7 fraction (920.4 ± 5.46 mg GAE g\(^{-1}\)) was the highest among the 7 collected fractions. Total recovery of EB-E fractionations by PREP LC was 82.80% (Table 1).

| Fraction | TPC (mg GAE g\(^{-1}\)) | Recovery |
|----------|------------------------|----------|
| EB-E     | 920.4 ± 5.46           | 82.80%   |
| EB-E-1   |                        |          |
| EB-E-2   |                        |          |
| EB-E-3   |                        |          |
| EB-E-4   |                        |          |
| EB-E-5   |                        |          |
| EB-E-6   |                        |          |
| EB-E-7   | 920.4 ± 5.46           | 82.80%   |

#### 3.3. Phenolic Compounds Identification

The effective phenolic fraction EB-E-7 suggested the highest inhibiting α-amylase and α-glucosidase activity among the assayed fractions. Phenolic compounds in fraction EB-E-7 were identified according to their UV and MS spectra and compared with literature reports by HPLC-UV, HPLC-ESI-MS/MS, and available authentic references (Figure 2 and Table 2). LC-MS analysis in the negative ion mode was applied to identify the molecular weights and corresponding formulae of compounds in EB-E-7 generated by PREP LC. The results of identified compounds of EB-E-7 and their responding retention time, λ\text{max} of UV spectrum, mass spectra, and structures are listed in Table 2. LC-MS of phenolic compounds has strong deprotonated molecular ions [M-H]\(^−\) in the negative mode.

Peak 1 showed [M-H]\(^−\) ion peak at m/z 449 and a fragment ion at m/z 317 [M-C\(_6\)H\(_7\)O\(_4\)]\(^−\) was consistent with myricetin-3-α-arabinoside (structure identification based on LC-MS and NMR data) [21]. The TIC spectra acquired from Peak 2 indicated intense [M-H]\(^−\) ion peak at m/z 477 and a fragment ion at m/z 315 [M-C\(_6\)H\(_7\)O\(_4\)]\(^−\) consistent with isorhamnetin-hexoside [13]. Peak 3 was assigned to quercetin-glucuronide with [M-H]\(^−\) at m/z 477 yielding a product ion at m/z 301, which corresponds to quercetin aglycone after the loss of a glucuronide moiety (−176 Da) [20]. Peak 4 gave [M-H]\(^−\) ion peak at m/z 447 and had...
Table 1: Quantitative analysis of extracts and fractions from \textit{E. grandis} × \textit{E. urophylla} bark.

| Sample                      | Preparation yields (%) | Total phenolic content (mg GAE g\(^{-1}\) extract/fraction) |
|-----------------------------|------------------------|-------------------------------------------------------------|
| Bark crude extract          | 19.39                  | 401.24 ± 4.32                                               |
| EB-hexane extract (EB-H)    | 1.15                   | 2.45 ± 0.12                                                 |
| EB-ethyl acetate extract (EB-E) | 3.86              | 178.79 ± 4.68                                               |
| EB-butanol extract (EB-B)   | 5.62                   | 137.31 ± 3.84                                               |
| EB-water extract (EB-W)     | 7.47                   | 76.23 ± 2.34                                                |
| Total recovery              |                        | 93.35                                                       |

Fractions from EB-E

| Fraction   | Preparation yields (%) | Total phenolic content (mg GAE g\(^{-1}\) extract/fraction) |
|------------|------------------------|-------------------------------------------------------------|
| EB-E-1     | 44.91                  | 47.52 ± 1.18                                                |
| EB-E-2     | 16.34                  | 45.86 ± 1.16                                                |
| EB-E-3     | 3.14                   | 178.08 ± 5.98                                               |
| EB-E-4     | 2.11                   | 456.25 ± 8.62                                               |
| EB-E-5     | 3.32                   | 470.65 ± 7.78                                               |
| EB-E-6     | 6.05                   | 186.18 ± 5.42                                               |
| EB-E-7     | 6.93                   | 920.40 ± 5.46                                               |

Total recovery 82.80

\(^a\) Gallic acid was used for the calibration curve and results were expressed as gallic acid equivalent (mg GAE g\(^{-1}\) of extract).

All values are expressed as mean ± standard deviation (\(n=3\)) of three independent samples. RSD < 5%.

Calibration formula used: \(y = 1.355x - 0.0419\), \(r^2 = 0.9994\); \(y\) is absorbance value; \(x\) is concentration in mg mL\(^{-1}\).

Table 2: Chemical profile of the identified phenolic compounds in EB-E-7 fraction from \textit{Eucalyptus grandis} × \textit{E. urograndis} bark by HPLC-ESI-IT-TOF-MS.

| Comp. number | \(t_R\) (min) | \(\lambda_{max}\) (nm) | MS (m/z) | MS\(^2\) ions (m/z) | Chemical name                      |
|--------------|---------------|------------------------|----------|---------------------|-----------------------------------|
| 1            | 28.4          | 261, 356               | 449      | 317                 | Myricetin-3-\(\alpha\)-arabinoside |
| 2            | 29.1          | 256, 366               | 477      | 315                 | Isorhamnetin-hexoside             |
| 3            | 29.4          | 253, 357               | 477      | 301                 | Quercetin-glucuronide             |
| 4            | 29.7          | 256, 352               | 447      | 315                 | Quercetin-3-\(\alpha\)-rhamnoside |
| 5            | 31.2          | 254, 374               | 447      | 301                 | Ellagic acid rhamnoside           |
| 6            | 32.0          | 254, 369               | 301      | 301, 257            | Ellagic acid                     |
| 7            | 33.5          | 256, 356               | 463      | 317                 | Myricetin-rhamnoside             |
| 8            | 34.6          | 249, 365               | 315      | 300, 271            | Isorhamnetin                     |

3.4. Enzyme Inhibition of Different Solvent Extracts of EB. The inhibitory effects of different solvent extracts of EB against \(\alpha\)-amylase and \(\alpha\)-glucosidase were indicated using PNPG and Lugol’s solution as colorimetric indicators, respectively. At 4 mg/mL, different solvent partition extracts from crude methanol extract of EB significantly decreased both \(\alpha\)-amylase and \(\alpha\)-glucosidase activity (Figures 3(a) and 3(b)). The ethyl acetate extract depressed enzyme activity to less than 20%; therefore, this extract was used for further separation.

Analysis of the different solvent partitions of EB illustrated that the medium-polarity ethyl acetate fraction (EB-E) played an important role in the \(\alpha\)-glucosidase and \(\alpha\)-amylase inhibitory activity (Figures 3(a) and 3(b)). EB-E was further fractionated by Revealeris\textsuperscript{®} PREP LC to afford 7 fractions (Figure 1). Each fraction was screened for inhibiting enzyme activity at 2 mg/mL. Among the 7 EB-E fractions, EB-E-7 exhibited the highest inhibitory effects of \(\alpha\)-glucosidase, decreasing activity to 2.25% ± 0.07% of the control (Figure 3(d)), yet this fraction moderately inhibited \(\alpha\)-amylase activity. In contrast with the uninhibited control, a residual activity was 11.78% ± 0.73% (Figure 3(c)). Table 1 showed...
that the TPC of EB-E was the highest among different solvent extracts, and EB-E-7 fraction had the highest total phenolic content (920.4 ± 5.46 mg GAE g⁻¹) between 7 fractions. The phenolic content of the 4 extracts and 7 fractions showed positive correlation with their enzyme inhibitory activities.

3.5. Comparison with Inhibitory Activity of Acarbose. The active fractions of EB-E demonstrated dose-dependent inhibitory effect of α-amylase or α-glucosidase (Figures 4(a) and 4(b)). Inhibition of EB-E-4, EB-E-5, and EB-E-7 was compared to that of acarbose which was an effective inhibitor against α-amylase and α-glucosidase. Figure 4 displayed the IC₅₀ value of three EB-E fractions for inhibiting α-amylase and α-glucosidase activity. The IC₅₀ values of EB-E-7, EB-E-5, and EB-E-4 inhibiting α-glucosidase were 1.40 ± 0.04, 2.51 ± 0.06, and 4.01 ± 0.07 µg/mL, respectively, obviously less than that for acarbose (Figure 4(b)). In this research, the IC₅₀ value of acarbose was 109.65 ± 1.75 µg/mL, similar to other researches [8]. The dramatically reduced IC₅₀ value of the EB-E fractions showed that EB-E fractions are effective inhibitors of α-glucosidase.

In the same way, EB-E-7 fraction was the most active against α-glucosidase, also highly active at inhibiting α-amylase. Compared with acarbose, EB-E-7 fraction indicated obviously lower IC₅₀ values. The IC₅₀ value of EB-E-7 against α-amylase was 1.72 ± 0.08 µg/mL, while EB-E-5 and EB-E-4 had IC₅₀ values of 3.91 ± 0.16 and 6.98 ± 0.32 µg/mL, in comparison with 117.84 ± 0.78 µg/mL for acarbose (Figure 4(a)). EB-E-7 (TPC 920.4 ± 5.46 mg GAE g⁻¹) showed the highest efficiency for inhibition of α-amylase and α-glucosidase enzyme activities. The study suggested that the active phenolic fractions of Eucalyptus bark have analogous inhibition of α-amylase and α-glucosidase. Therefore, the phenolic compounds from Eucalyptus grandis × E. urograndis bark potently inhibited α-amylase and α-glucosidase activity associated with hyperglycemia.
Figure 4: IC_{50} values (µg/mL) for Eucalyptus bark inhibition of α-amylase (a) and α-glucosidase (b). The active phenolic fractions of Eucalyptus bark (EB-E-4, EB-E-5, and EB-E-7) were assayed at a range of concentrations (1.00–150.00 µg/mL). Acarbose was utilized as a positive control. RSD < 5%.

4. Conclusions

This report, for the first time, evaluated the inhibitory activities of different solvent extracts and polyphenol-rich fractions of Eucalyptus grandis × urophylla bark (EB) in vitro against α-glucosidase and α-amylase enzymes. Phytochemical and bioactivities analyses of Eucalyptus grandis × E. urophylla bark (EB) revealed the presence of 8 phenolic compounds in EB-E-7 fraction isolated from the ethyl acetate extract (EB-E) by semipreparative HPLC. The total phenolic content (TPC) of EB-E-7 fraction accounted for 920.4 ± 5.46 mg GAE g⁻¹ of fraction. EB-E extract and EB-E-7 phenolic fraction significantly inhibited α-amylase and α-glucosidase activities. Therefore, the phenolic compounds from Eucalyptus grandis × E. urophylla bark potently inhibited α-amylase and α-glucosidase activity associated with hyperglycemia. The results of this research showed that Eucalyptus grandis × E. urophylla bark represents a rich source for value-added products with potential for drug industries that may contribute to diabetic care.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

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