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Recovery of phenolics from the ethanolic extract of sugarcane (*Saccharum officinarum* L.) baggase and evaluation of the antioxidant and antiproliferative activities

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ABSTRACT

In this work, recovery of phenolics from sugarcane bagasse was performed and total phenolic content, total flavonoid content and antioxidant activities were measured. Four fractions were yielded after extraction with various solvents and ethyl acetate fraction was performed into further purification, producing 30 sub-fractions (G1–G30). Six compounds were isolated and their structures were identified by high resolution mass spectrometry (HR-MS) and high definition nuclear magnetic resonance (HD-NMR) spectroscopy as *p*-coumaric acid, tricin, luteolin, tricin 7-O-β-glucopyranoside, protocatechuic acid and diosmetin 6-C-glucoside which was identified in this species for the first time. Tricin expressed highest antioxidant activity in ORAC assay (9.76 ± 1.01 μmol TE/μmol) but no antioxidant activity in PSC and CAA assays. The structure-activity relationship was discussed to elucidate the different activities of the isolated phenolics in ORAC and CAA assays. Luteolin, *p*-coumaric acid and protocatechuic acid showed antiproliferative effect against MCF-7 cells with the EC_{50} at 28.45, 1856.90 and 3670.56 μM, respectively. This work reported the recovery of individual phenolics from bagasse, which suggested bagasse could be an abundant source of bioactive phenolics.

1. Introduction

Phenolics are secondary metabolites of plants, a diversified group of phytochemicals with various functions. It is widely accepted that phenolics, including phenolic acids, flavonoids, tannins, coumarins and stilbenes have free radicals scavenging (Malta et al., 2013), photoprotection (Chaiprasongsuk et al., 2016), antidiabetes (Shen et al., 2012; Xi and Liu, 2016) and anticancer (LS and NJA, 2016) properties, which may provide significant benefits for human wellbeing. Bioactive phenolic compounds have been found richly in a number of agricultural by-products, such as pine sawdust and almond hulls (Pinelo et al., 2004), apple peel (Wolfe et al., 2003; Wolfe and Liu, 2003) and citrus seed (Bocco et al., 1998).

Sugarcane (*Saccharum officinarum* L.) is one of the world's most important economic crops as the main feedstock for the production of sugar as well as ethanol (Del Río et al., 2015a). Previous studies have demonstrated that phenolic compounds are abundant in sugarcane and cane sugar manufacturing products (Zheng et al., 2017; Duarte-Almeida et al., 2011; Zhao et al., 2009). Bagasse, with a production of more than 2.8×10^8 t per year throughout the world (Pinheiro et al., 2017), is one of the most abundant by-products in sugarcane processing. In most cases, bagasse is used for electricity generation to power the sugar mills and the researches of bagasse are currently focused on biomass (such as lignins and celluloses) and bioenergy (bioethanol) production (Camargo et al., 2016; Del Río et al., 2015b; Ramadoss and Muthukumar, 2016). This would be of great help to solve problems of substances & energy shortage globally. But few of them paid attention to the bioactive compounds such as phenolics from bagasse. As reported previously, we found that sugarcane bagasse contained various phenolics such as quercetin, gallic, coumaric and ferulic acids and had the antioxidant, antihyperglycemic and antibiotic activities (Zheng et al., 2017; Zhao et al., 2015). Currently, no work attempted to recover

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individual phenolics yet from this common but valuable substance. Thus in this study, individual phenolic compounds were isolated by column chromatography and identified by high resolution mass spectrometry (HR-MS) and high definition nuclear magnetic resonance (HD-NMR) spectroscopy. Additionally, antioxidant and antiproliferative activities of the isolated phenolics were evaluated. Hopefully, our efforts could improve the further understanding and comprehensive utilization of sugarcane bagasse to better serve food, cosmetic and pharmaceutical industries.

2. Materials and methods

2.1. Plant material

Sugarcane (Guitang-11) bagasse was kindly provided by a sugar mill in Donta Group (Dongguan, China). The sample was dried, milled and passed through a 0.5 mm sieve, and kept dry at − 20 °C until use.

2.2. Preparation of extracts and isolation of compounds

The bagasse powder (21.32 kg) was extracted with 95% ethanol (EtOH) for 7 days at room temperature (25 °C) for three times. The EtOH solution was combined and evaporated *in vacuo* to obtain crude ethanolic extract (EF, 600 mL). The EF of bagasse was suspended in 600 mL of distilled water, and then sequentially extracted three times with petroleum ether, ethyl acetate (EtOAc) and *n*-butanol (*n*-BuOH) to produce petroleum ether fraction (PEF), EtOAc fraction (EAF), *n*-BuOH fraction (BuF) and aqueous fraction (AqF), respectively. The extracts were maintained at − 20 °C for further analysis.

EtOAc-soluble fraction (113.75 g) was submitted to a silica gel column (SGC, 10 × 170 cm) eluted successively with 4 BV of $CHCl_3$: CH_3COCH_3 (1: 0, 1: 0.05, 1: 0.1, 1: 0.2, 1: 0.4, 1: 0.8), $CHCl_3$: MeOH (1: 0.1, 1: 0.2, 1: 0.35), MeOH and H_2O. The thin layer chromatography (TLC) was carried out on precoated silica gel GF254 plates (Qingdao Haiyang Chemical Co., Ltd., Qingdao, China). Fractions of 1L were collected and combined after TLC analysis to yield 30 sub-fractions (G1– G30) and the yield was shown in Fig. S1. G9 was subjected to SGC and yielded C2 (11 mg). G10 was subjected to SGC and yielded C2 (13.7 mg). The sub-fractions of G10 (G10-1 and G10-2) were separated by a Sephadex LH-20 column (SLC) eluted with methanol and/or methanol: $CHCl_3$ (1:1, v: v) and yielded C1 (33.4 mg) and C2 (20.7 mg), respectively. G11 and G12 was combined and then subjected to SLC and the sub-fraction was purified by SLC for two times in succession and yielded C3 (14.9 mg) and C5 (17.7 mg). Likewise, C4 (14.2 mg) was separated by combining G21 and G22 together and purified by subjecting the sub-fraction to SLC eluted with methanol and/or methanol: $CHCl_3$ (1:1, v: v). G24 was subjected to SLC to afford C6 (less than 5 mg). The contents of the six compounds (C1– C6) in dried sugarcane bagasse were shown in Table S1.

2.3. Phytochemical investigations

2.3.1. Determination of total phenolic content (TPC) and total flavonoid content (TFC)

The TPC and TFC were measured using the method reported previously by our lab (Wang et al., 2016a). TPC of each sample was calculated by the regression equation of gallic acid calibration curve. The results were expressed as milligrams gallic acid equivalents per 100 mg of dry weight (DW) of extracts (mg GAE/100 mg, DW). TFC of each sample was calculated by the regression equation of catechin calibration curve. The results were expressed as the milligrams of catechin equivalents/100 mg of DW of extracts (mg CE/100 mg, DW).

2.3.2. Identification of compounds

The isolated compounds from sugarcane bagasse were identified by HR-MS and HD-NMR. The HR-MS was performed on a quadrupole time-of-flight (qTOF) mass spectrometer (MaXis Impact, Bruker, Germany) fitted with an electrospray interface (ESI) source operated in positive ion mode (HR-ESI-MS). The experiment was carried out with ion scanning from 50 to 1000 *m/z*, nebulizer pressure at 0.6 bar, nitrogen as the dry gas, flow rate at 3.0 L/min and temperature at 200 °C. HD-NMR experiments were performed on an AVANCE III HD 600 spectrometer (Bruker, Germany) operating at 600 MHz (^{1}H) and 151 MHz (^{13}C). MeOH-d_4 (δ_H 3.31 and δ_C 49.15) and DMSO-d_6 (δ_H 2.50 and δ_C 39.51) were used as references.

2.4. The biological assays

2.4.1. Measurement of antioxidant activity by oxygen radical absorption capacity (ORAC) assay and peroxyl radical scavenging capacity (PSC) assay

The antioxidant activity of sugarcane bagasse extracts were determined by ORAC assay as described by Wen et al. (2016). The antioxidant activity of the isolated compounds was quantified by ORAC assay and PSC assay as described by Wen et al. (2016). In ORAC assay, all the regents used in this experiment were prepared freshly and dissolved in phosphate buffer (75 mM, pH 7.4). Trolox (used as the standard) or samples was diluted to series concentrations with phosphate buffer and was transferred 20 μL into a black walled 96-well plate (Corning Scientific, Corning, NY, USA), followed by the addition of 200 μL fluorescein sodium salt (0.96 μM). After the incubation for 20 min at 37 °C, 20 μL of 119 mM ABAP was added into each well. Fluorescence intensity was measured every 5 min for 35 cycles at excitation wavelength of 485 nm and emission wavelength of 535 nm using a multi-mode microplate reader (Filter Max F5, Molecular Devices, USA). Vitamin C (Vc, for crude extracts) or Quercetin (for isolated compounds) was taken as the positive reference and the ORAC values were calculated by extrapolation based on a calibration curve. The results were expressed as μmol Trolox equivalents per milligram of DW of extracts (μmol TE/mg, DW) or μmol Trolox equivalents per μmol of isolated compounds (μmol TE/μmol).

In PSC assay, 100 μL of isolated compounds or VC solutions were added in a black 96-well microplate. Then 100 μL of 33.15 μM DCFH and 50 μL of 200 mM ABAP were added. The microplate was placed in the microplate reader mentioned above at 37 °C for 40 min and the fluorescence was measured at the excitation wavelength of 485 nm and emission wavelength of 535 nm. Quercetin was taken as the positive reference and the half maximal effect concentration (EC_{50}) was measured. The PSC value was calculated by dividing the EC_{50} of VC by the EC_{50} of the sample and final PSC value of each compound was expressed as μmol VC equivalents per μmol of tested compounds (μmol VCE/μmol).

2.4.2. Measurement of antioxidant activity by cellular antioxidant activity (CAA) assay

Human liver cancer cell line HepG2 was purchased from ATCC company and was grown in Williams' Medium E (WME), supplemented with 5% fetal bovine serum (FBS), 2 mM L-glutamine, 10 mM Hepes, 5 μg/mL insulin, 0.05 μg/mL hydrocortisone, 50 units/mL penicillin, 100 μg/mL gentamycin and 50 μg/mL streptomycin. The cells were maintained at 37 °C in 5% CO_2 in an incubator.

Cells between 12 and 35 passage were used to conduct the CAA assay as reported by Zhu et al. (2015). HepG2 cells were seeded at a density of 6.0×10^4 cells/well on a black walled 96-well microplate with 100 μL complete medium in each well and maintained at 37 °C for 24 h. After that, the medium was removed and each well was washed by 100 μL of phosphate buffered saline (PBS). Then, the cells were treated for 1 h with different (non-cytotoxic) concentrations of tested compounds in treatment medium (100 μL) containing with 50 μM DCFH-DA. After complete removal of the medium, certain cells were not washed by 100 μL of PBS (no PBS wash protocol) and certain cells were washed by PBS (PBS wash protocol), followed by the addition of