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ScienceDirect
Food Science and Human Wellness 5 (2016) 85–94
Food Science and Human Wellness
www.elsevier.com/locate/fshw

Bioactive constituents from the leaves of *Quercus phillyraeoides* A. Gray for α-glucosidase inhibitor activity with concurrent antioxidant activity

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Received 6 October 2015; received in revised form 18 February 2016; accepted 28 February 2016
Available online 5 March 2016

Abstract

Several α-glucosidase inhibitory constituents were isolated from the methanolic extract of the leaves of *Quercus phillyraeoides* A. Gray (*Q. phillyraeoides*) using a bioassay-guided fractionation technique. Further separation and purification of the methanol-soluble fraction led to the isolation of constituents with moderate and strong inhibitory activities against α-glucosidase: β-sitosterol-D-glucoside (**1**) and condensed tannin fractions (**2**, **3**, **4**, **5**, and **6**). Compound **1** and fractions **2**–**6** had inhibitory concentration (IC_{50}) values against α-glucosidase from *Saccharomyces cerevisiae* of 118.8, 2.79, 2.78, 3.10, 2.60, and 3.14 μg/mL, respectively, while quercetin as the standard had an IC_{50} value of 4.80 μg/mL. Furthermore, the significant antioxidant activities were evaluated using several assays, such as the DPPH radical scavenging, hydrogen peroxide radical scavenging, reducing power, and β-carotene-linoleate bleaching assays, and the results suggested that the isolated constituents showed their possible application for treating the hyperglycemia-induced oxidative stress. The results of the present study showed the potential of *Q. phillyraeoides* as a rich source of natural antidiabetic medicine.

Keywords: *Quercus phillyraeoides* A. Gray; α-Glucosidase inhibitor; Antioxidative activity; Lineweaver–Burk plot

1. Introduction

Diabetes mellitus (DM) is a common chronic disease that has become a serious health issue due to its associated complications. There are two types of DM: type 1 DM, caused by the destruction of pancreatic beta cells, resulting in an insulin deficiency, and type 2 DM results from defects in insulin secretion or insulin resistance [1,2]. DM is characterized by high blood glucose levels, which lead to complications such as hypertension, neuropathy, nephropathy, retinopathy, and diabetic foot ulcers [3]. Type 2 DM is the most frequently encountered form of DM, accounting for more than 80% of all cases [4]. The number of DM patients has markedly increased in the past few years and is estimated to rise to 366 million by 2030 [5].

Current DM treatments are based on the use of synthetic drugs, which are often associated with a number of serious adverse effects [6]. Therefore, the development of better pharmaceuticals as alternatives for the treatment of DM without any side effects is urgently needed. Natural compounds from plants have attracted much attention because they become alternatives to the currently used synthetic DM drugs. Ethnobotanical studies have identified approximately 1200 plants in the world with antidiabetic potential [7]. Several studies also suggested the application of medicinal plant extracts to antidiabetic treatments due to fewer side effects than those of synthetic medicines [8,9]. Another advantage is that natural compounds may be safely consumed in the daily diet, thereby reducing the risk of DM [10].

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Peer review under responsibility of Beijing Academy of Food Sciences.

http://dx.doi.org/10.1016/j.fshw.2016.02.004

However, free radicals have also been suggested to cause DM [11]. Although free radicals typically originate from the surrounding environment, several physiological and biochemical processes in the human body also produce reactive oxygen species, such as the superoxide radical, hydroxyl radicals, and peroxyl radicals, as by-products [12]. Therefore, the search for antidiabetic and antioxidant drug constituents from plants has been attracting increasing attention in recent years.

Quercus phillyraeoides A. Gray (*Q. phillyraeoides*) of the family *Fagaceae* is an evergreen tree that is distributed in the limestone mountains and acid bed rocks of East Asia (Korea, China and Japan). The leaves of *Quercus* species have been used in Korean folk medicine for dysentery, diarrhea, hemorrhage, dermatitis, and the exclusion of extravasated blood [13]. Previous phytochemical studies on *Q. phillyraeoides* led to the identification of several tannins from the leaves of *Q. phillyraeoides* [14]. However, the bioactivities of *Q. phillyraeoides* have not yet been examined.

The objective of the present study was to identify the active constituents from the leaves of *Q. phillyraeoides* that are responsible for inhibitory activity against α-glucosidase and subsequently evaluate the kinetics responsible for this enzyme inhibitory activity. An *in vitro* assay of α-glucosidase inhibitory activity was conducted using an α-glucosidase enzyme obtained from *Saccharomyces cerevisiae* (*S. cerevisiae*) yeast. The antioxidant properties of isolated constituents were also evaluated using several assays as an assessment to alleviate oxidative stress related to DM. These assays may be used for preliminary observations in the evaluation of pharmacological activities and also to verify the medicinal effects of these active constituents isolated from plants.

2. Materials and methods

2.1. General instrumentation and reagents

An analysis using gas chromatography (GC) was conducted on a GC-FID 2014 model (Shimadzu, Japan). The electron ionization mass spectra (EI-MS) of isolated constituents were recorded on a GC Mass Spectrometer (GC-MS QP 2010 Plus, Shimadzu, Japan) and Fast Atomic Bombardment Mass Spectrometer (FAB-MS, Shimadzu, Japan). TLC was run on silica gel 60 F_{254} pre-coated plates (Merck 5554) and spots were detected using UV light.

All chemicals used were purchased from commercial available sources and were used without further purification. α-Glucosidase [(EC 3.2.1.20)] type I from *S. cerevisiae*, *p*-nitrophenyl α-D-glucopyranoside (*p*-NPG), 1,1-diphenyl-2-picrylhydrazyl (DPPH), β-carotene, potassium ferricyanide [$K_3Fe(CN)_6$], trichloroacetic acid, ferric chloride ($FeCl_3$), hydrogen peroxide, and bis(trimethylsilyl) acetamide (BSA) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Quercetin, Tween 40, and gallic acid were purchased from Sigma–Aldrich Co., Ltd. (Tokyo, Japan). All solvents used in this study (methanol, ethanol, toluene, pyridine, ethyl acetate, chloroform, *n*-hexane, and acetone) were purchased from Wako Pure Chemical Industries, Ltd.

2.2. Plant material

The leaves of *Q. phillyraeoides* were collected from a site in Ehime University, Matsuyama, Japan, in September 2014. Voucher specimens have been deposited in the Department of Plant Chemistry, Faculty of Agriculture, Ehime University, Japan. The leaves were dried naturally.

2.3. Extraction and isolation procedures

The dried leaves of *Q. phillyraeoides* were powdered and extracted twice with methanol (1:8, w/w) at room temperature for 3 days. The methanol filtrate was concentrated using rotary evaporator under reduced pressure. The methanolic extract was partitioned successively using solvents with increasing polarity from *n*-hexane, chloroform, ethyl acetate (EtOAc), and methanol (MeOH) to obtain *n*-hexane-, chloroform-, ethyl acetate-, and methanol-soluble fractions. All extracts were screened for α-glucosidase inhibitory activity, with the methanol-soluble fraction exhibiting the highest activity. The active methanol soluble fraction (90.3 g) was separated by column chromatography over a silica gel (100 mesh). The column was eluted with solvents of increasing polarities and a stepwise elution from *n*-hexane (100%), ethyl acetate (EtOAc, 50%) in *n*-hexane, EtOAc (100%), and EtOAc with variable concentrations in methanol (MeOH) to MeOH 100% to obtain six fractions (F1–F6). Fraction F4, which exhibited the highest α-glucosidase inhibitory activity among the fractions examined, was further separated by silica gel column chromatography using solvents with increasing polarities and a stepwise elution from *n*-hexane (100%), ethyl acetate (EtOAc, 50%) in *n*-hexane, EtOAc (100%), and EtOAc with variable concentrations in methanol (MeOH) to MeOH 100% to obtain five fractions (F41-F45). Compound **1** (15.2 mg) was isolated as a white buff powder from fraction F42 by silica column chromatography followed by the recrystallization of compound **1** from methanol. Fractions **2** to **6** (81.7 mg, 15.1 mg, 54.9 mg, 553 mg, and 313 mg, respectively) were isolated from F44 using Sephadex LH-20 column chromatography by eluting with methanol and water in gradients.

Acid hydrolysis of **1**: Compound **1** (1.5 mg) was refluxed with 2 N HCl in aq. MeOH for 4 h to give β-sitosterol and D-glucose, which were confirmed with an available standard using thin layer chromatography. Furthermore, the β-sitosterol was further confirmed using GC analysis.

Thiolysis of fractions **2**–**6**: A solution of the fraction in methanol (4000 ppm, 50 μL) was reacted with 2 N HCl in methanol (50 μL), followed by the addition of benzyl mercaptan (BM) in methanol (100 μL; 5:95, v/v). The solution was heated at 90 °C for 5 min [15]. The reaction was stopped by placing the solution in an ice bath. Prior to the GC–MS analysis, the trimethylsilane (TMS) derivation of the result of the thiolysis product was conducted by reacting the sample with pyridine (10 μL) and bis(trimethylsilyl) acetamide (BSA, 20 μL).