Journal of Ethnopharmacology 191 (2016) 21–28

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jep

In vitro and *in vivo* anti-diabetic and hepatoprotective effects of edible pods of *Parkia roxburghii* and quantification of the active constituent by HPLC-PDA

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ARTICLE INFO

Article history:
Received 31 December 2015
Received in revised form
25 April 2016
Accepted 4 June 2016
Available online 6 June 2016

Keywords:
Diabetes
α-Glucosidase
α-Amylase
Anti-hyperglycemic
Parkia roxburghii

ABSTRACT

Ethnopharmacological relevance: *Parkia roxburghii* G. Don. is a traditional medicinal plant and its pods are extensively used as food and medicine. It is believed by the traditional healers to have medicinal properties to treat diabetes, hypertension and urinary tract infections (Jamaluddin et al., 1994).
Materials and methods: The methanolic extract of pods of *P roxburghii* and fractions were screened for their α-glucosidase and α-amylase inhibitory activity. Anti-hyperglycemic effects were studied on streptozotocin (45 mg/kg b.w.) induced diabetes in albino rats (seven groups, n=7 n=6), using different doses for 14 days. Plasma glucose concentration (HbA_{1c}) was analysed using whole blood, while SGOT, SGPT, TG, TC and uric acid were analysed using serum, employing commercial kits. Quantitative analysis of the major active constituent was carried out by HPLC-PDA.
Results: Bioactivity guided chemical investigation of the edible pods of *P roxburghii* identified sub-fraction EA-Fr 5 which significantly inhibited α-glucosidase (IC_{50} 0.39 ± 0.06 μgmL^{-1}), reduced the blood glucose level to normal, and lowered the elevated levels of liver function enzymes SGOT and SGPT in STZ-induced diabetic rats. EA-Fr 5 was found to contain epigallocatechin gallate (**1**) and hyperin (**2**) which exhibited significantly higher α-glucosidase inhibitory potency with IC_{50} 0.51 ± 0.09 and 0.71 ± 0.03 μM respectively. EA-Fr 5 contained 379.82 ± 2.90 mg/g of EGCG, the major active constituent which manifests a broad spectrum of biological activities.
Conclusion: The present investigation for the first time reports the occurrence of EGCG and hyperin in *P roxburghii* and substantiates the traditional use of pods of *P roxburghii* as dietary supplement for management of diabetes with significantly promising α-glucosidase inhibitory potency and anti-hyperglycemic as well as hepatoprotective effects.

1. Introduction

Diabetes mellitus is now considered as epidemic and Asia has been considered as the global epicentre (Chen, 2012). Around 382 million people worldwide and 63 million in India have been suffering from diabetes (Zimmet et al., 2014; Kaveeshwar and Cornwall, 2014). Food habit plays a significant role in the metabolic syndrome and disease processes, particularly in diabetes mellitus type 2 (DM type 2).

DM is a lifestyle dependent metabolic disorder. In dieting, controlling blood glucose levels in everyday life is especially important. Blood glucose levels are greatly affected by the saccharides contained in food. These are decomposed by the actions of α-glucosidase and α-amylase, which are digestive enzymes (Loo and Huang, 2007). Therefore, to prevent or treat obesity and diabetes, it is very important to develop inhibitors to control the activity of these enzymes (Israili, 2011).

Acarbose, isolated from *Actinoplanes* strain, has been the best known α-glucosidase and α-amylase inhibitor (Kaissi and

Abbreviations: DM, diabetes mellitus; HPLC, high performance liquid chromatography; PDA, photodiode array detector; TLC, thin layer chromatography; NMR, nuclear magnetic resonance; LCMS, liquid chromatography mass spectrometry; STZ, streptozotocin; DMSO, dimethylsulfoxide; PNPG, *p*-nitrophenyl-α-D-glucopyranoside; DNS, 3,5-di-nitro salicylic acid; OCED, Organisation for Economic Co-operation and Development; CPCSEA, Committee for the Purpose of Control and Supervision of Experiments on Animals; SGOT, serum glutamic oxaloacetic transaminase; SGPT, serum glutamic pyruvic transaminase; TG, triglyceride; TC, total cholesterol; MSL, mean sea level

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http://dx.doi.org/10.1016/j.jep.2016.06.015
0378-8741/

Sherbeeni, 2011). But it has limitations with several side effects due to strong inhibition of pancreatic α-amylase (Bischoff, 1994). There is a need to search for safe, more affordable and widely available or easy to produce medication for treatment of DM type 2. Therefore investigating the efficacy of indigenous plants could be a better alternative. Tremendous efforts are now being made towards the development of effective α-glucosidase and α-amylase inhibitors from natural sources (Matsui et al., 2006; Mata et al., 2013).

Fruits (pods) of *Parkia roxburghii* (Mimosaceae), popularly known as *Yongchak* in the North East (NE) India, are traditionally used as a food and medicine, and are known to be effective in the management and treatment of diabetes. A favourite nutritious, leguminous vegetable food item, it is a tree that grows luxuriantly in NE India and South East Asia (Firake et al., 2013). Pods of *P roxburghii* have been used to treat various diseases like kidney disorder, urinary tract infection, diabetes, hypertension and headache (Buring and Berg, 2013; Ong et al., 2011; Samuel et al., 2010). It has also been reported for antioxidant (Ali et al., 2011), anticancer (Aisha et al., 2009), antimutagenic (Tangkanakul et al., 2011) and antimicrobial (Sakunpak and Panichayupakaranant, 2012) potential. The therapeutic potential of *P roxburghii* has mainly been evaluated with its crude extract only rather than pure compounds. The isolation of stigmast-4-en-3-one, β-sitosterol and stigmasterol from the related species *P speciosa* has been reported so far with their hypoglycemic effect (Jamaluddin et al., 1994, 1995). The anti-diabetic effects of *P roxburghii* and its active constituent(s) have been sporadically reported. So far most of the therapeutic evaluation has been carried out with extracts only. The current study was designed to investigate the anti-diabetic effects of pods of *P roxburghii*, and to isolate and quantify the active constituent(s). The limited information on anti-diabetic effects of *P roxburghii* and its active constituent(s) encouraged the present study.

2. Materials and methods

2.1. Materials, reagents and instruments

Pods of *Parkia roxburghii* G. Don. were collected from Bishnupur, Manipur, India (N24°34'12.2″, E93°38'22.8″ and Elevation 857 MSL) and authenticated by Dr. B. Thongam, Scientist, IBSD, Imphal. A voucher specimen (No. IBSD/M-216) was deposited in the IBSD herbarium.

Organic solvents, AR or HPLC grade as required for experiments, were purchased from MerckMillipore India. Merck silica gel (100–200 mesh size) was used for column chromatography. Thin layer chromatography (TLC) plates pre-coated with silica gel 60 F254 (0.25 mm, normal phase, Merck) were used for TLC. α-Glucosidase (Maltase, EC 3.2.1.20), *p*-nitrophenyl-α-D-glucopyranoside and streptozotocin (STZ) were purchased from Sisco Research Laboratory (SRL). α-Amylase (porcine pancreas, EC 3.2.1.1), starch, and 3,5-dinitrosalicylic acid (DNS) were purchased from Sigma. Kits for biochemical analysis (SGOT, SGPT and HbA_{1c}) were purchased from ERBA diagnostics, Mannheim GmbH, Germany. NMR spectra were recorded on a Bruker Avance 500 MHz instrument with TMS as an internal standard and chemical shifts are expressed in δ values. Agilent 6520 Accurate mass Q-TOF/LC-MS was used to determine molecular weight. Absorbance was measured by Thermo Scientific Multiskan spectrometer.

2.2. Extraction and purification of active constituents

Fruits of *P roxburghii* (3.10 kg) were properly washed with tap water, chopped into small pieces, air dried in shade and ground to yield 1.2 kg of powder which was extracted at room temperature with 5 L of methanol three times, 24 h each time. The extracts were combined and the solvent was evaporated under reduced pressure at 46 °C by using rotary evaporator to get 335 g of crude methanol extract. About 115 g of methanolic extract was suspended in water (300 mL) and fractionated successively with ethyl acetate (500 mL × 2) and *n*-butanol (500 mL × 2). All the fractions were evaporated to yield 26.0 g of ethyl acetate fraction (EA), 42.5 g of *n*-butanol fraction (Bu) and 20 g of water fraction (W). The fractions were tested for their α-glucosidase inhibition potential where EA proved significantly more active. It was sub-fractionated on silica gel column with increasing polarity of solvents in the order Petroleum ether (PE): CHLoroform (CHL) 4:1 (400 mL, Fr-1), PE: CHL 1:1 (400 mL, Fr-2), CHL (800 mL, Fr-3), CHL: Methanol (MeOH) 9:1 (400 mL, Fr-4), CHL: MeOH 3:1 (1000 mL, Fr-5), and CHL: MeOH 7:3 (800 mL, Fr-6). The ethyl acetate sub-fraction 5 (EA-Fr 5) was found to be the most active fraction against α-glucosidase and was subjected to re-chromatography on silica gel column followed by semi-preparative HPLC to yield the compounds **1** and **2** (Supplementary A1). The compounds were characterized as epigallocatechin gallate (EGCG) and hyperin respectively by spectroscopic analysis.

2.3. Preparation of enzyme and sample solutions

Stock solution of α-glucosidase enzyme (0.5 U/mL) was prepared in 0.1 M phosphate buffer of pH 6.8 and diluted for assay to the required concentration with the same buffer. A stock solution (4 U/mL) of α-amylase enzyme was prepared by dissolving the enzyme in ice cold water. Plant extract, fractions and compounds were dissolved in dimethyl sulfoxide (DMSO) to obtain stock solutions and diluted with water to desired test concentration for assay, maintaining DMSO concentration below 1% v/v (Supplementary A2).

2.4. α-Glucosidase inhibitory assay

α-Glucosidase inhibitory activities were determined spectrophotometrically in a 96-well microplate reader according to a reported pre-incubation based method by using *p*-nitrophenyl-α-D-glucopyranoside (PNPG) as substrate (Kumar et al., 2013). Briefly, 25 μL of enzyme solution (0.5 U/mL of α-glucosidase in 0.1 M phosphate buffer, pH 6.8) was mixed with 25 μL of the test sample and incubated for 10 min at 37 °C. Then 25 μL of PNPG solution (0.5 mM PNPG in 0.1 M phosphate buffer, pH 6.8) was added to the mixture and further incubated at 37 °C for 30 min. The reaction was terminated by the addition of 100 μL of 0.2 M Na_2CO_3 solution and the absorbance was measured using a multi-well plate reader at 405 nm. Acarbose was used as positive control and the uninhibited enzyme was taken as negative control (DMSO control). The assay was performed in three independent experiments.

2.5. α-Amylase inhibitory assay

α-Amylase inhibitory activities were determined following the reported method (Kumar et al., 2013) with some modifications. Briefly, 50 μL of test solution in 20 mM phosphate buffer of pH 6.9 containing 6.7 mM NaCl and 50 μL of α-amylase solution (containing 4 μmL^{-1} of the enzyme in ice cold water) were mixed together and allowed to incubate for 10 min at 25 °C to inhibit the enzyme. Then 50 μL of starch solution (0.5% w/v) as substrate prepared in the same buffer was added to the mixture and allowed to react for 10 min at 25 °C. The reaction was stopped using 100 μL DNS color reagent (48 mM 3,5-di-nitro salicylic acid and 1.063 M sodium-potassium tartrate in 2 M NaOH), and the mixture was further incubated at 85 °C in water bath. After 15 min, the