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The influence of procyanidins isolated from small-leaved lime flowers (*Tilia cordata* Mill.) on human neutrophils

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ABSTRACT

Linden flower is a wildly used plant material among patients in the treatment of common cold symptoms and mucosa inflammations. However, the structure and bioactivity of flavan-3-ol derivatives present in infusions from flowers of *Tilia cordata* have not been studied so far. The aim of current study was to isolate and identify main procyanidins present in the flowers of small-leaved lime and to evaluate their influence on the inflammatory response of human neutrophils ex vivo. The chemical structure of isolated compounds was established by 1D and 2D NMR experiments. The bioactivity of obtained compounds was tested in human neutrophils model. Cytotoxicity and influence of compounds on apoptosis was established by flow cytometry. The levels of produced cytokines were established by ELISA after stimulation with lipopolysaccharide (LPS). The inhibition of the production of reactive oxygen species was checked by luminol-dependent chemiluminescence method after N-formylmethionyl-leucyl-phenylalanine (f-MLP) induction. The phytochemical work resulted in the isolation of 10 compounds. Compounds were identified as oligomeric procyanidins and their precursor epicatechin. The potential anti-inflammatory activity of compounds was evaluated in the concentration range 5–20 μM. All compounds were able to decrease the production of ROS from f-MLP-stimulated neutrophils. Most of compounds were able to inhibit the LPS-induced release of IL-8. Some trimeric and tetrameric derivatives were also able to decrease the production of MIP-1β. Obtained results partially support the traditional usage of infusion from lime flowers in the treatment of symptoms of inflammation and irritation of mucosa in common cold, pharyngitis and tonsillitis.

1. Introduction

Small-leaved lime (*Tilia cordata* Mill., Malvaceae) is a deciduous tree growing to 20–40 m tall with simple alternate mostly hairless leaves native to Europe and Western Asia. It is widely cultivated as an ornamental tree planted in parks and gardens to form avenues. It has creamy yellow flowers in clusters of five to eleven that appear in June [1]. According to the European Pharmacopoeia 9.0 whole, dried inflorescence of *T. cordata* is one of the source of drug remedy called lime flower (*Tiliae flos*) [2]. Lime flower also has its monography in ESCOPE [3,4]. In the literature there are many studies focused on phytochemistry of lime flower obtained from wide variety of *Tilia* species. However, there are no reports on chemical composition of small-leaved lime flowers. Several groups of compounds were identified as constituents of lime flower including flavonoids (quercetin and kaempferol glycosides), mucilage (mainly galactomannans), volatile oil, phenolic acids and procyanidins [5]. For a long time, lime flower was considered as a typical flavonoid and mucilage containing plant material. Recent HPLC-DAD-MS study by Karioti et al. revealed that lime flower (*T. platyphyllos* Scop.) extracts contain significant amounts of condensed tannins (procyanidins) including dimeric, trimeric, tetrameric and pentameric compounds [6]. The knowledge on accurate chemical structure of those compounds is limited. Infusions of lime flower are used worldwide as a medicinal plant material to treat throat irritations, common cold and other upper respiratory tracts disorders. Tinctures from lime flower are used as spasmolytic and mild sedative remedy [5,7]. Lime flower is also a diaphoretic agent and it also has a topical application as an itch-relieving drug in the treatment of different dermatological conditions [8]. Beneficial effects in the sore throat (associated with i.e. tonsillitis and/or pharyngitis) and upper respiratory disorders of *Tiliae flos* is often

Abbreviations: DEX, dexamethasone; PMN, polymorphonuclear cells; Q, quercetin; ROS, reactive oxygen species

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linked to the high content of mucilage, however the potential positive effect of other compounds occurring in high quantities in this plant material such as flavonoids and procyanidins should not be neglected.

Neutrophils, also known as polymorphonuclear cells (PMN), constitute the first line of defence of the innate immune system. After infiltration to the inflamed site they generate reactive oxygen species (ROS) and release proteases (elastase, metalloproteinases MMPs), chemokines and cytokines (interleukins IL-8, IL-1β). Neutrophils phagocyte infectious agents such as bacteria and eliminate them by intense oxidative burst. During chronic inflammatory processes neutrophils activated by a great variety of stimuli increase their pro-inflammatory attributes. It has also been shown that activated neutrophils may migrate from the vessels to the surface of pharynx and tonsils where they induce the host dependent inflammatory response against pathogens [9]. It is claimed that overstimulation of PMNs initiates further non-infectious inflammatory response. Thus, the reduction of leukocytes functions seems to be necessary. In the literature there are some data indicating that lime flower tea can be used to relieve the throat irritation and reduce the inflammation in the course of tonsillitis and pharyngitis.

The aim of current study was to isolate and identify main procyanidins present in the flowers of small-leaved lime and to evaluate their influence on the viability and inflammatory response of human neutrophils ex vivo.

2. Materials and methods

2.1. Chemicals and general procedures

Roscovitine (98% purity), luminol, f-MLP (N-formylmethionyl-leucyl-phenylalanine), Hanks' balanced salt solution (HBSS), L-glutamine, fetal bovine serum (FBS), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), propidium iodide (PI) and RPMI 1640 medium were purchased from Sigma-Aldrich GmbH (Steinheim, Germany). LPS (lipopolysaccharide) was purchased from Merck Millipore (Billerica, MA, USA). Annexin V and ELISA sets were purchased from BD (Franklin Lakes, USA). Dexamethasone (**DEX**) and quercetin (**Q**) (> 95% HPLC purity) were purchased from Sigma-Aldrich GmbH. Phosphate-buffered saline (PBS) was purchased from ThermoFisher Scientific (Waltham, MA, USA). Methanol, acetone and acetonitrile for isolation and HPLC was purchased from Avantor (Gliwice, Poland). Ultra-pure water was produced with Merck Millipore Simplicity UV system.

Preparative HPLC was performed using Shimadzu LC10vp chromatographic system (Kyoto, Japan) equipped UV–Vis detector, fraction collector and a Zorbax SB-C_{18} column (150 mm × 21.2 mm × 5 μm, Agilent, CA, USA; mobile phase: 0.1% HCOOH in water (A) and 0.1% HCOOH in acetonitrile (B), flow rate: 7 ml/min; gradient elution). Used linear gradients are reported in the subsection below. After preparative HPLC fractions containing purified compounds were evaporated to get rid of organic solvent and water residue was freeze-dried.

Mass spectra of isolated compounds were measured using LR-MS Amazon SL ion trap instrument (Bruker, Bremen, Germany). UV–Vis spectra of compounds were measured with Shimadzu UV-160A.

1D and 2D NMR spectra were recorded on Bruker Avance III 600 spectrometer (Bruker BioSpin, Germany), operating at 600.14 and 150.92 MHz for ^{1}H and ^{13}C, respectively, and equipped with a 5 mm BBFO probe head. Chemical shifts were referenced to the residual solvent signals: 3.31 and 49.50 ppm for ^{1}H and ^{13}C in methanol-d_4, respectively.

2.2. Plant material

The flowers of *Tilia cordata* Mill. were collected in Warsaw, Banacha Str. (N52°12′37.1″; E20°59′05.4″). Collected material was identified based on morphological characters by Sebastian Granica Ph. D. according to Rutkowski [10]. Voucher specimen is available at the Department of Pharmacognosy and Molecular Basis of Phytotherapy, Medical University of Warsaw (No. 20150624_B) Plant material was dried in shade and finely grounded before extraction.

2.3. Isolation of procyanidins from small-leaved lime flowers

The plant material (1320 g) was extracted with acetone:methanol:-water (3:1:1, *v/v/v*) using ultrasonic bath. The extraction was carried out exhaustively – 5 times; 2 l of the mixture each time, sonication for 30 min each time. Combined extracts were evaporated at 45 °C in order to get rid of organic solvents. The water residue was shaken with solvents of rising polarity (6 × 600 ml for each solvent) in the following order: chloroform ($CHCl_3$), diethyl ether (Et_2O), ethyl acetate (EtOAc) and *n*-butanol saturated with water (*n*-BuOH). After evaporation raw $CHCl_3$, Et_2O and EtOAc fractions were obtained – 27.3, 2.9 and 22.7 g respectively. The *n*-BuOH fraction was dissolved in water and freeze-dried to give 45.8 g of dried weight.

The diethyl ether (Et_2O) was adsorbed at silica gel (ca. 3 g) and subjected to silica gel column (27 cm × 5 cm) eluted with mixtures of chloroform-methanol in 12 steps from 0:100 to 100:0 to obtain 175 fractions 21 ml each. Fractions were pooled into 7 main fractions (E_1–E_7) based on TLC profile (silica gel plate developed with ethyl acetate:water:formic acid, 18:1:1, derivatized with 1% Naturstoffreagenz A). Fraction E_5 (1.3 g) was subjected to preparative HPLC on Zorbax SB-C_{18} (0–60 min, 0–60% B) to obtain compounds **A** (23 mg, 22.8–24.5 min).

The ethyl acetate fraction (AcOEt) was adsorbed on silica gel (ca. 25 g) and subjected to silica gel column (25 cm × 5 cm) eluted with mixtures of chloroform-methanol in 11 steps (300 ml per step) from 0:100 to 100:0 to obtain 180 fractions 20 ml each. Fractions were pooled into 7 main fractions (Z_1–Z_7) based on TLC profile (silica gel plate developed with ethyl acetate:water:formic acid, 18:1:1, derivatized with 1% Naturstoffreagenz A). Fraction Z_4 (5.2 g) was separated on Sephadex LH-20 column (60 cm × 3.5 cm) eluted with pure methanol to obtain 140 fractions 6 ml each. Based on TLC profile (conditions as above) fractions were pooled into 7 main fractions (ZS_1–ZS_7). Fraction ZS_7 (207 mg) were subjected to preparative HPLC on Zorbax SB-C_{18} (0–65 min, 3–26% B) to obtain compound **E** (12 mg, 47.5–48.5 min). Fraction Z_5 (7.9 g) was separated on Toyopearl HW-40F column (50 cm × 3.5 cm) eluted with 70% methanol to obtain 235 fractions 5 ml each. Based on TLC profile (silica gel plate developed with ethyl acetate:water:formic acid, 18:1:1 (*v/v/v*), derivatized with 1% vanillin in concentrated sulphuric acid) fractions were pooled into 22 main fractions (ZSS_1–ZSS_{22}). Fraction ZSS_{13} and ZSS_{14} (together 505 mg) were subjected to preparative HPLC on Zorbax SB-C_{18} (0–65 min, 3–26% B) to obtain compounds **C** (11 mg, 19.5–20.0 min) and **B** (6.0 mg, 32.8–33.8 min).

The *n*-BuOH fraction was suspended in water and subjected to Diaion HP-20 column chromatography (column 25 cm × 5 cm) eluted with water-methanol mixtures from 0% of MeOH to 100% of MeOH (11 steps – 0%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% and 100% of MeOH; 1 l per step, flow rate around 50 ml/min) to give 220 fractions. Based on TLC profile (silica gel plate developed with ethyl acetate:water:formic acid, 18:1:1 (*v/v/v*), derivatized with 1% vanillin in concentrated sulphuric acid) fractions were pooled into four main fractions (D_1–D_4). Fraction D_1 (5.6 g) was dissolved in 70% MeOH and subjected to Toyopearl HW-40F column (55 cm × 2.2 cm) eluted with 70% MeOH to give 588 fractions 10 ml each. Fractions were pooled into 22 main fractions (DT_1–DT_{22}) based on TLC profile (conditions as above). Fractions DT_{10} and DT_{11} (together 157 mg) were subjected to preparative HPLC on Zorbax SB-C_{18} (0–60 min, 0–60% B) to obtain compound **F** (38 mg, 38.0–39.5 min). Fractions DT_{14} and DT_{15} (together 262 mg) were subjected to preparative HPLC on Zorbax SB-C_{18} (0–65 min, 3–26% B) to obtain compounds **H** (15 mg, 35.0–36.0 min) and **G** (30 mg, 40.5–42.5 min). Fractions DT_{18} and DT_{19} (together 217 mg) were subjected to preparative HPLC on Zorbax SB-C_{18}