Chemical Constituents of *Lavatera trimestris* L. – Antioxidant and Antimicrobial Activities

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Nine phenolic compounds, such as *cis-*/*trans-p*-coumaric acid, *cis-*/*trans-p*-coumaric acid methyl ester, glucose ester of *cis-*/*trans-p*-coumaric acid, caffeic acid methyl ester, kaempferol 7-*O*-β-D-glucoside and kaempferol 3-*O*-β-D-glucoside, were isolated from *Lavatera trimestris* flowers by chromatographic techniques and their structures were elucidated by spectral means (NMR). All compounds were tested for their antioxidant activity, while the methanolic extract was tested also for its antimicrobial activity. Also several non-polar constituents have been identified using GC and GC/MS methods. This is the first time that phenolic esters and non-polar constituents were identified in the flowers of *L. trimestris* L.

Key words: Lavatera trimestris L., Flavonoids, Phenolic Acids

Introduction

Lavatera L. (Malvaceae) is a genus of 21–23 mostly well marked species of herbs, shrubs and tree-like shrubs, occurring in both the Old and New Worlds (Ray, 1995). Several species of the *Lavatera* genus have been used in traditional medicine. The leaves of *L. arborea* have been used in Peru for the treatment of vaginitis and in wound healing (Rojas *et al.*, 2003), also therapeutic properties of *L. olbia*, like laxative, hepatic and anti-inflammatory, are well known in Sardinia (Ballero *et al.*, 2001). Leaves of *L. cretica* show a great anti-insect activity (Pascual-Villalobos and Robledo, 1999) and both *L. arborea* and *L. cretica* have been used in folk veterinary phytotherapy (Viegi *et al.*, 2003). Compounds responsible for these activities are mainly flavonoids and phenolic acids occurring in high amount in the Malvaceae family.

Flavonoids have been shown to act as scavengers of various oxidizing species, might reduce the risk of cardiovascular diseases and stroke while they also have antibacterial, antiviral, diuretic, spasmolytic and oestrogenic activities (Harborne and Williams, 2000; Robak and Gryglewski, 1996; Peterson and Dwyer, 1998). Phenolic acids also show a broad spectrum of pharmacological activities such as anti-inflammatory, antioxidant, bacteriostatic and immuno-stimulating ones (Borkowski, 1993; Fernandes *et al.*, 1996).

Lavatera trimestris L. is a species that occurs in Poland, especially on lowlands. Some phenolic acids and flavonoids have been identified previously (Głowniak *et al.,* 2005) using TLC and HPLC methods. The aim of this study was the isolation and structure elucidation of its main compounds, to assay their biological activity as well as to analyse non-polar compounds by GC and GC/MS.

Experimental

General

NMR spectra were recorded on Bruker DRX 400 and Bruker AC 200 spectrometers [^{1}H (400 MHz) and ^{13}C (50 MHz)]; chemical shifts are expressed in ppm downfield to TMS. The 2D NMR experiments were performed using standard Bruker microprograms. TLC analyses were carried out using glass pre-coated silica gel 60 F_{254} sheets (E. Merck, Germany).

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Plant material

Flowers of *L. trimestris* L. were collected in 2003 in Medicinal Plant Garden, Deptartment of Pharmacognosy, Medical University of Lublin, Poland. Plant material was collected during the flowering period. Flowers were air-dried at room temperature and powdered to a homogeneous size in a mill according to the standard accepted for flowers (Plata, 2002). A voucher specimen was deposited in the herbarium of Department of Pharmacognosy.

Extraction

Dried flowers (100 g) were first extracted exhaustively with chloroform in a Soxhlet apparatus to remove chlorophyll and other ballast compounds. The purified material was dried and then extracted with methanol (78 °C). The methanol extract was concentrated.

Chromatographic separation

Column chromatography with silica gel 60 (230–400 mesh; E. Merck) and polyamide (MN SC6; Macherey Nagel, Düren, Germany) were used. The concentrated methanolic extract was applied to a silica gel column and eluted with a gradient of CH_2Cl_2 and MeOH in the order of increasing polarity to give 37 fractions. Some of the richest fractions (22–29) containing mostly flavonoids and phenolic acids with similar chemical compositions were collected and further separated on a second polyamide column with mixtures of MeOH and H_2O. 17 fractions were obtained.

The most interesting fractions were separated by preparative TLC on silica gel ($200 \times 200 \times 0.5$ mm, Kieselgel 60, Merck).

The structures of nine, pure isolated compounds were established by spectroscopic methods, including 1D-NMR (^1H, ^{13}C and DEPT) and 2D-NMR experiments (HMQC, HMBC, COSY, NOESY, COSY-LR) and mass spectroscopy. NMR data were recorded on a Bruker AC 200 at 50 MHz and on a Bruker Avance 400 instrument at 400 MHz.

Gas chromatography

GC analyses were carried out on a Perkin-Elmer Clarus 500 gas chromatograph, fitted with a HP 5MS (30 m × 0.25 mm, 0.25 μm film thickness) capillary column. The column temperature was programmed from 60–280 °C at a rate of 3 °C/min. The injector and detector temperatures were 230 °C and 300 °C, respectively. Helium was used as the carrier gas at a flow rate of 1 mL/min.

GC/MS analyses

The GC/MS analyses were carried out using a Hewlett Packard 6890–5973 GC-MS system operating on EI mode [equipped with a HP 5MS (30 m × 0.25 mm, 0.25 μm film thickness) capillary column]. He (1 mL/min) was used as carrier gas. The initial temperature of the column was 60 °C and then it was heated to 280 °C at a rate of of 3 °C/min. The identification of the components was based on comparison of their mass spectra with those of Wiley and NBS libraries (Massada, 1976) and those described by Adams (2001), as well as on comparison of their retention indices (Van den Dool and Kratz, 1963) obtained using *n*-alkanes (C_9–C_{25}) with literature values (Adams, 2001). The identity of all compounds was performed by comparison of the expected molecular mass with the results obtained from the CI spectra.

Tested material

The isolated constituents, *trans*-*p*-coumaric acid (**1**), *cis*-*p*-coumaric acid (**2**), *trans*-*p*-coumaric acid methyl ester (**3**), *cis*-*p*-coumaric acid methyl ester (**4**), *trans*-1-*p*-coumaroyl-β-D-glucose ester (**5**), *cis*-1-*p*-coumaroyl-β-D-glucose ester (**6**), caffeic acid methyl ester (**7**), kaempferol 7-*O*-β-D-glucoside (**8**), kaempferol 3-*O*-β-D-glucoside (**9**), were tested for their antioxidant activity using two methods (Rancimat and DPPH$^\bullet$). The methanolic extract of the plant was tested for its antimicrobial activity.

Hydrogen donation ability

The hydrogen donation ability of the pure compounds was determined according to the DPPH$^\bullet$ method (Yen and Hsieh, 1995).

Rancimat test

The antioxidant effect was studied according to the Rancimat method (Lalas and Tsaknis, 2002) using a Rancimat 679 apparatus (Metrohm LTD, Herisau, Switzerland). The conditions were 90 °C and 15 L/h.

Antimicrobial activity

The antibacterial activities of the methanolic extract were determined using the dilution technique

by measuring the minimal inhibitory concentration (MIC) (expressed in mg/mL) against two Gram-positive: *Staphylococcus aureus* (ATCC 25923) and *Staphylococcus epidermidis* (ATCC 12228), and four Gram-negative bacteria: *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 25922), *Enterobacter cloacae* (ATCC 13047) and *Klebsiella pneumoniae* (ATCC 13883), as well as against three human pathogenic fungi: *Candida albicans* (ATCC 10231), *C. tropicalis* (ATCC 13801) and *C. glabrata* (ATCC 28838), as it has been described previously (Melliou and Chinou, 2005).

Results and Discussion

The chemical analysis led to the isolation and identification of nine phenolic compounds: *cis*-/*trans*-*p*-coumaric acid, *cis*-/*trans*-*p*-coumaric acid methyl ester, glucose ester of *cis*-/*trans*-*p*-coumaric acid, caffeic acid methyl ester, kaempferol 7-*O*-*β*-D-glucoside and kaempferol 3-*O*-*β*-D-glucoside.

As far as we know the genus *Lavatera* wasn't examined broadly. There is only one short report about the presence of *p*-coumaric, ferulic, caffeic and resorcilic acids and some flavonoids which weren't identified (Matławska *et al.*, 1997). Głowniak *et al*. (2005) confirmed the presence of *p*-coumaric, ferulic and caffeic acids using TLC, 2D-TLC and RP-HPLC methods. Also *p*-hydroxybenzoic, protocatechuic, gallic, vanillic, isovanillic, syringic, ellagic, and chlorogenic acids were identified. Seven glycosides were identified on the basis of chromatographic tools as hiperoside, isoquercetine, quercitrine, rutoside, kaempferol 3-rhamnoglucoside, luteoline 7-glucoside, isorhamnetine 3-glucoside. Our investigation led to the isolation and structure elucidation of esters of phenolic acids. TLC identification of these compounds wasn't possible because of the lack of standards. Also kaempferol glucosides were isolated and examinated for the first time in *L. trimestris*. Isolation of the pure compounds led to the determination of biological activity like antibacterial and antioxidant activity.

In an earlier survey on the *Lavatera* genus Matławska *et al.* (1999) identified in flowers of *Lavatera thuringiaca* kaempferol 3-*O*-*β*-D-glucoside, kaempferol and quercetin 3-*O*-rutinoside, *cis*-/*trans*-tiliroside and *p*-coumaric acid. This result fits well with the view that *p*-coumaric acid and kaempferol glucoside could occur regularly in the flowers of *Lavatera.* Thus further experiments are required.

Also several non-polar constituents have been identified using GC/MS methods such as: hydrocarbons (C_{15}–C_{29}), sitostenone, eicosyl stearate, stearyl arachidate, isopropyl myristate, hexahydrofarnesyl acetone and methyl esters of some non-polar acids such as: tetra-, heptadecanoic, palmitic, linoleic, stearic, lignoceric, eico-, doco-, and tricosanoic acids.

L. trimestris L. is a plant rich in phenolic acid derivatives and flavonoids with notable antioxidant activity (see Table I). In contrast to the observed activity in the DPPH$^{\bullet}$ test all tested compounds were inactive in the Rancimat test. Besides, the methanolic extract of the plant exhibited an interesting antimicrobial spectrum of activities against all tested microbials with MIC values ranging from 2.75–4.20 mg/mL (Table II). To our knowledge it is the first time that an extract of *Lavatera* has been tested for its antimicrobial activities and exhibited also an interesting antimicrobial profile, similar to Polish Malva's flowers used

Table I. Antiradical activity (% disappearance of DPPH$^{\bullet}$) of various compounds.

Tested compound	Disappearance level (%)	mg/mL
trans-*p*-Coumaric acid (**1**)	63.58 (0.40)	138
cis-*p*-Coumaric acid (**2**)	63.48 (0.37)	135
trans-*p*-Coumaric acid methyl ester (**3**)	63.59 (0.61)	128
cis-*p*-Coumaric acid methyl ester (**4**)	63.56 (0.65)	126
trans-1-*p*-Coumaroyl-*β*-D-glucose ester (**5**)	60.32 (0.60)	127
cis-1-*p*-Coumaroyl-*β*-D-glucose ester (**6**)	60.30 (0.62)	125
Caffeic acid methyl ester (**7**)	50.0 (0.31)	10
Kaempferol 7-*O*-*β*-D-glucoside (**8**)	60.88 (0.50)	176
BHT	86.11 (0.38)	164
BHA	89.60 (0.34)	164

Values are means of triplicate determinations and standard deviation is given in parenthesis.

Table II. Antimicrobial activities of the methanolic extract of *Lavatera trimestris* (MIC values in mg/mL).

	L. trimestris	Intraconazole	5-Flucytocine	Netilmicin
S. aureus	2.75	–	–	4×10^{-3}
S. epidermidis	3.15	–	–	4×10^{-3}
P. aeruginosa	3.88	–	–	8.8×10^{-3}
E. cloacae	3.75	–	–	8×10^{-3}
K. pneumoniae	4.20	–	–	8×10^{-3}
E. coli	2.95	–	–	10×10^{-3}
C. albicans	3.84	1×10^{-3}	0.1×10^{-3}	–
C. tropicalis	3.27	0.1×10^{-3}	1×10^{-3}	–
C. glabrata	3.00	1×10^{-3}	10×10^{-3}	–

Values are means of triplicate determinations.

in traditional medicine. In an earlier study an ethanolic extract from leaves of *Lavatera arborea* L. showed neither antibacterial nor antifungal activity against four bacteria (*Bacillus subtilis, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa*) and four fungi (*Candida albicans, Trichophyton mantagrophytes, Microsporum gypseum, Sporothix schenckii*) (Rojas *et al.*, 2003).

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