The separation and identification of phenolic acid and flavonoids from *Nerium indicum* flowers

A. Vinayagam* and P. N. Sudha

*Department of Chemistry, Sathyabama University, Chennai-600 119, India*  
E-mail: vinayaga_star@yahoo.co.in

*Department of Chemistry, DKM College for Women (Thiruvallur University, Chennai), Vellore-632 001, Tamilnadu, India*

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Abstract: Four major compounds were separated and identified from the methanol extracts of *Nerium indicum* flowers (Arali) using high performance liquid chromatography and mass spectral data. Through mass data, the chemical structures were elucidated as: *trans* 5-*O*-caffeoylquinic acid (1), quercetin-3-*O*-rutinoside (2), luteolin-5-*O*-rutinoside (3) and luteolin-7-*O*-rutinoside (4). In addition, the *cis* isomers of 5-*O*-caffeoylquinic acid in *Nerium indicum* flowers were confirmed by UV, HPLC and Mass. The structures of these compounds elucidated with the help of mass spectral data.

Keywords: *trans* 5-*O*-Caffeoylquinic acid, *cis* 5-*O*-caffeoylquinic acid, *Nerium indicum*, chromatography, mass data.

Introduction

Naturally occurring phenolic acids are phenylpropanoids with an aromatic ring and attached three carbon side chains. Caffeic, ferulic and *p*-coumaric acid, as hydroxyl cinnamic acids, are almost ubiquitous. Phenolic acids are distributed in nature in their free and bound forms, as esters and glycosides. Chlorogenic acids are a family of esters formed between *trans* cinnamic acids and (-)-quinic acid (1L-1(OH),3,4/5-tetrahydroxycyclohexanecarboxylic acid). A subgroup of chlorogenic acid is defined by the number and identity of the constituent cinnamic acids, and there are usually several isomers within each sub group. Many plants produce chlorogenic acids in which esterification occurs at positions 3, 4 and 5 of the quinic acid moiety. Esterification at position 1 is less frequent, but 1-acyl chlorogenic acids are found in some Asteraceae1–3.

Flavonoids and phenolic acids have protective role in carcinogenesis, inflammation, atherosclerosis, thrombosis and have high antioxidant capacity. Furthermore, flavonoids have been reported as aldose reductase inhibitors blocking the sorbitol pathway that is linked to many problems associated with diabetes4–8. Flavonoids interact with various enzymatic systems. Their inhibition of the enzymes cyclooxygenase and lipoxygenase results in a decrease of platelet activation and aggregation, protection against cardiovascular diseases, cancer chemoprevention and their anti-inflammatory activity9–13. Many other biological activities are attributed to flavonoids and phenolic acids: antiviral, antimicrobial, antihepatotoxic, antiosteoporotic, antiulcer, immunomodulatory, antiproliferative and apoptotic activity14–21.

The purpose of this research was to separate and identify phenolic compound and flavonoids from *Nerium indicum* flowers. A sensitive, accurate and specific method coupling high performance liquid chromatography (HPLC) with diode array detector (DAD) and electrospray ionization mass spectrometry (MS) was developed for the separation and identification of phenolic acid and flavonoids, in the methanolic extract of *Nerium indicum* flowers.

The molecular masses of phenolic acid and flavonoids were assigned by electrospray ionization using ion trap mass spectrometry in negative mode.

Experimental

General:

*trans* 5-*O*-Caffeoylquinic acid (1), quercetin-3-*O*-rutinoside (2), luteolin-5-*O*-rutinoside (3) and luteolin-7-
O-rutinoside (4) were separated from the plant material, its purity was checked by HPLC and structure elucidated by MS spectral data. Acetonitrile and methanol were HPLC grade from Merck (Darmstadt, Germany). Water was purified by a Milli-Q system from Millipore (Milford, USA).

Analyses were performed on Agilent 1200 chromatograph equipped with a diode array detector and mass detector in series (Agilent Technologies, Waldbronn, Germany). A PHENOMENEX LUNA-C18, 4.6 × 150 mm, particle size 5 μm with suitable guard column was employed for the separation. The binary mobile phase consisted of solvents A as 0.1% formic acid in water and solvents B as acetonitrile. The gradient elution started with 10% B and changed to 75% B in 20 min, then reached 95% B in 27 min. After each run the chromatographic system was set to 10% B in 4 min and equilibrated for 4 min. The flow rate was 1.0 mL/min and injection volume was 5 μL.

Separation of trans and cis isomer of 5-O-caffeoylquinic acid was attempted by using A ZORBAX SB PHENYL, 4.6 × 100 mm, particle size 1.8 μm with suitable guard column. The binary mobile phase consisted of solvents A as 0.1% formic acid in water and solvents B as acetonitrile. The isocratic elution started with 20% B and 80% A. The flow rate was 0.8 mL/min and injection volume was 5 μL.

Spectral data for all peaks were recorded in the range of 200-400 nm. The mass detector was an ion trap spectrometer (Agilent LC/MSD Trap XCT) equipped with an electrospray ionization interface and controlled by LC-MSD software. The ionization conditions were adjusted at 300 ºC and 3.5 kV for capillary temperature and voltage, respectively. The nebulizer pressure was 40 psi and the nitrogen flow rate was 8 L/min. Collision-induced fragmentation experiments were performed in the ion trap using helium as a collision gas, with voltage cycles from 0.3 up to 2 V. All mass spectrometry data were recorded in negative ion mode. The screening was performed in full scan covering the range from m/z 100 up to 1000; multiple reaction monitoring (MRM) mode.

UV spectra were recorded on Agilent UV-Vis 8453 diode array spectrophotometer.

Fig. 1. Structures of compounds from Nerium indicum flowers.

Plant material:
The flowers of Nerium indicum were collected from
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the surroundings of Vellore in March 2013 and identified by Dr. P. N. Sudha, Department of Chemistry, Thiruvallur University. The plant material was air dried, smashed into powder and stored in a desiccator.

**Extraction and isolation:**

The flowers of *Nerium indicum* (50 g) were extracted 3 times with MeOH under reflux for 3 h. Evaporation of the solvent under reduced pressure provided the MeOH

![Fig. 2. HPLC-DAD chromatogram of crude methanolic extract of *Nerium indicum*, λ = 325 nm.](image1)

![Fig. 2a. TIC/MS chromatogram of crude methanolic extract of *Nerium indicum* from HPLC-(−) ESI-MS.](image2)
extract (5.1 g). To separate the compound, liquid chromatography was used using acetonitrile-formic acid-water as mobile phase and the octadecylsilane as a stationary phase.

**Results and discussion**

*Optimization of chromatographic conditions:*

A method coupling high-performance liquid chroma-

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Fig. 3. HPLC-DAD chromatogram of trans and cis isomer of 5-O-caffeoyl quinic acid, $\lambda = 325$ nm.

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Fig. 3a. TIC/MS chromatogram of trans and cis isomer of chlorogenic acid from HPLC-$(-)$ ESI-MS.
tography (HPLC) with diode-array detector (DAD) and electrospray ionization mass spectrometry with an ion trap analyzer was optimized for the separation and identification of phenolic acid in the extract of *Nerium indicum* flowers. Different mobile phase compositions were screened to obtain chromatograms with good resolution within an acceptable time of analysis. Formic acid, as solvent A, and acetonitrile, as solvent B, were chosen for the gradient elution. Especially the formic acid and acetonitrile are volatile and thus compatible with LC/MS system. The formic acid (lower pH values) ensures better sample separation but shortens the HPLC column lifetime and affects ESI ionization. 325 nm were chosen as monitoring wavelengths according to absorption maxima of analytes.

Compound 1 was a 5-O-caffeoyl quinic acid, it gave an [M-H]⁻ ion peak at m/z 353 in the ESI mass spectrum as shown in Fig. 2a.

*Cis* isomer of 5-O-caffeoyl quinic acid, it gave an [M-H]⁻ ion peak at m/z 353 in the ESI mass spectrum as shown Fig. 3a. By using ZORBAX SB PHENYL column able to separate *cis* and *trans* isomer of 5-O-caffeoyl quinic acid. UV spectra also suggest that *trans* isomer is more absorption value compared to *cis* isomer as shown in Fig. 4.

ESI-MS of compound 2 showed a quasi-molecular ion [M-H]⁻ at m/z 609. Therefore compound 2 was determined as quercetin-3-O-rutinoside as shown in Fig. 2a. ESI-MS of compound 3, 4 showed a quasi-molecular ion [M-H]⁻ at m/z 593. So compounds 3, 4 were determined as luteolin-5-O-rutinoside and luteolin-7-O-rutinoside as shown in Fig. 2a.

**Conclusion**

Two phenolic acids (*trans* 5-O-caffeoylquinic acid and *cis* 5-O-caffeoylquinic acid) separated and identified. Three flavonoid glycosides (quercetin-3-O-rutinoside, luteolin-5-O-rutinoside and luteolin-7-O-rutinoside) were also identified from methanolic extract of *Nerium indicum* flowers using mass spectral data based on literature.

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