Research Paper

Separation and Identification of Phenolic Acid and Flavonoids from *Nerium indicum* Flowers

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Four major compounds were separated and identified from the methanol extracts of *Nerium indicum* flowers (Arali) using HPLC and mass spectral data. Through mass data, the chemical structures were elucidated as: trans5-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 Mass, HPLC and UV. The structures of these compounds confirmed with the help of liquid chromatography mass spectrometry.

Key words: *trans*-5-O-caffeoylquinic acid, *cis*-5-O-caffeoylquinic acid, *Nerium indicum*, chromatography, mass data

Naturally occurring phenolic acids are phenylpropanoids with an aromatic ring and attached three carbon side chains. Caffeic, ferulic and p-coumaric acid, as hydroxycinnamic 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-tetrahydroxycyclohexanecarboxyl ic 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 subgroup. 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 Asteraceae[1-3].

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 diabetes[4-8]. Flavonoids interact with various enzymatic systems. Their inhibition of the enzymes cyclooxygenase and lipooxygenase results in a decrease of platelet activation and aggregation, protection against cardiovascular diseases, cancer chemoprevention and their antiinflammatory activity[9-13]. Many other biological activities are attributed to flavonoids and phenolic acids: antiviral, antimicrobial, antihepatotoxic, antiosteoporotic, antiulcer, immunomodulatory, antiproliferative and apoptotic activity[14-22].

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.

MATERIALS AND METHODS

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).

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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.

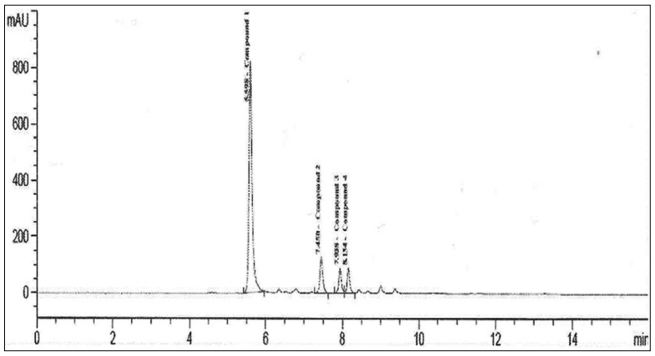

Fig. 1: HPLC-DAD chromatogram of crude methanolic extract of *Nerium indicum* flowers.
Compound 1 (trans 5-O-caffeoylquinic acid) with t_R of 5.5 min, compound 2 (quercetin-3-O- rutinoside) with t_R of 7.4 min, compound 3 (luteolin-5-O-rutinoside) with t_R of 7.9 min and compound 4 (luteolin-7-O-rutinoside) with tR of 8.1 min.
***DAD1, Sig=325.00, 4.00 Ref=off, EXT of 2210NIF.D**

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 LCMSD software. The ionization conditions were adjusted at 300° 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.

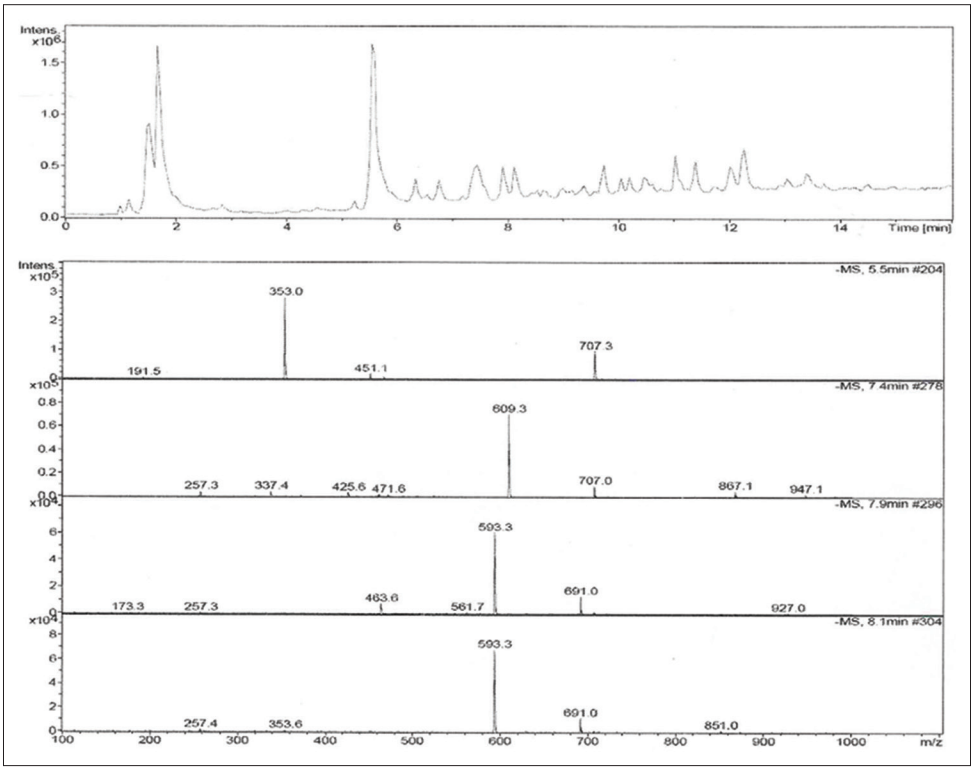

Fig. 2: TIC/MS chromatogram of crude methanolic extract of *Nerium indicum* flowers from HPLC-(-) ESI-MS.
m/z value of 353.0 with tR of 5.5 min, m/z value of 609.3 with t_R of 7.4 min, m/z value of 593.3 with t_R of 7.9 min and m/z value of 593.3 with t_R of 8.1 min.

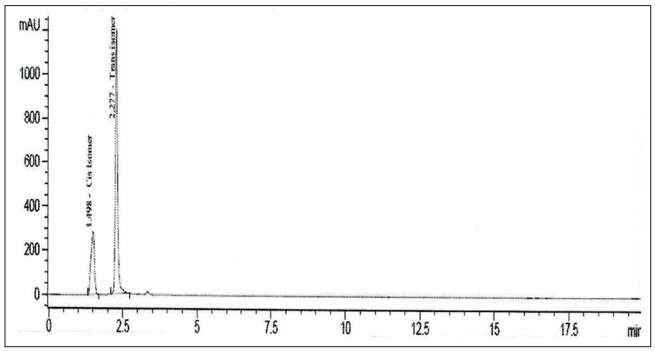

Fig. 3: HPLC-DAD chromatogram of cis and trans isomer of 5-O-caffeoyl quinic acid.
Cis isomer with t_R of 1.4 min and trans isomer with t_R of 2.2 min.

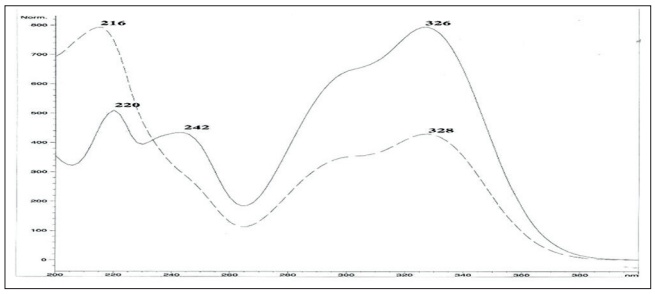

Fig. 5: Overlay UV spectrum of cis and trans isomer of 5-O-caffeoyl quinic acid.
Cis isomer with absorption maximum at 216 and 328 nm similarly trans isomer with absorption maximum at 220, 242 and 326 nm.

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.

Plant material:
The flowers of *Nerium indicum* were collected from the surroundings of Vellore, in November 2013, and identified at the 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 extract (5.1 g). To separate the compound, preparative liquid chromatography was done using acetonitrile-formic acid-water as mobile phase and the octadecylsilane as a stationary phase.

RESULTS AND DISCUSSION

A method coupling HPLC with diode-array detector (DAD) and electrospray ionization mass spectrometry

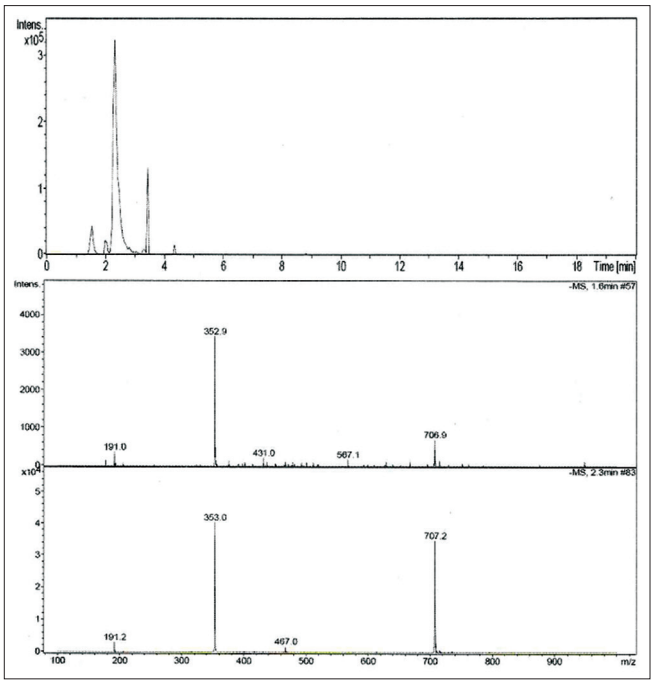

Fig. 4: TIC/MS chromatogram of cis and trans isomer of 5-O-caffeoyl quinic acid from HPLC-(-) ESI-MS.
Cis isomer with m/z value of 362.9 and trans isomer with m/z value of 363.0.

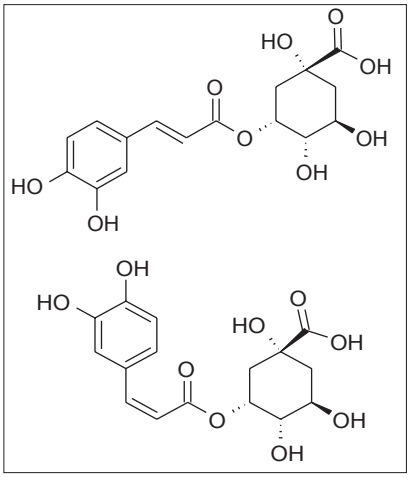

Fig. 6: Structures of trans 5-O-caffeoylquinic acid and cis 5-O-caffeoylquinic acid.

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. Formic acid (lower pH values) ensures better sample separation but shortens the HPLC column

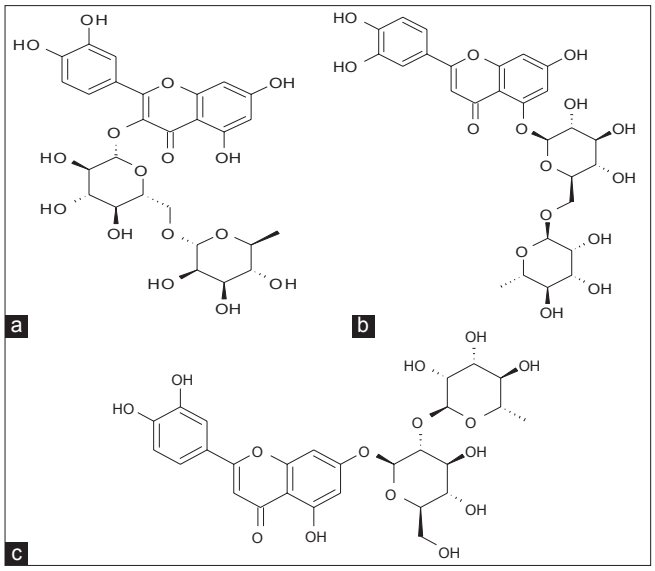

Fig. 7: Structure of flavonoids.
a. quercetin-3-O-rutinoside, b. luteolin-5-O-rutinoside, c. luteolin-7-O-rutinoside.

lifetime and affects ESI ionization. 325 nm were chosen as monitoring wavelengths according to absorption maxima of analytes. A typical chromatogram showing the separation of 4 components is presented in fig. 1.

Compound 1 observed as a [M-H] - ion peak at m/z 353 in the ESI mass spectrum (fig. 2) and based on literature data[23] compound 1 was confirmed as a 5-O-caffeoyl quinic acid. By using Zorbax SB Phenyl column able to separate *cis* and *trans* isomer of 5-O-caffeoyl quinic acid (fig. 3) gave an [M-H]-ion peak at m/z 353 in the ESI mass spectrum (fig. 4). UV spectra also suggest that trans isomer is more absorption value compared to cis isomer (fig. 5). Mass spectral value of compound 2 showed a quasi-molecular ion [M-H] - at m/z 609, compared with literature data[24] compound 2 was identified as a quercetin-3-O-rutinoside (fig. 2).

ESI-MS of compound 3, 4 showed a quasi-molecular ion [M-H] - at m/z 593, both are same mass value only position is different and based on literature data[25] compounds 3 and 4 identified as luteolin-5-O-rutinoside and luteolin-7-O-rutinoside (fig. 2).

Based on the above data two phenolic acids, trans 5-O-caffeoylquinic acid and cis 5-O-caffeoylquinic acid (fig. 6). Three flavonoid glycosides, quercetin-3-O-rutinoside, luteolin-5-O-rutinoside and luteolin-7-O-rutinoside (fig. 7) were identified from crude methanolic extract of *Nerium indicum* flowers.

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