

UHPLC-ESI-MS/MS analyses for quantification of phenolic compounds of *Nepeta nuda* subsp. *Lydiae*

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ARTICLE INFO

Article history:

Received on: 07/09/2016

Revised on: 08/10/2016

Accepted on: 02/11/2016

Available online: 29/11/2016

Key words:

Nepeta nuda, phenolic acid, chlorogenic acid, rosmarinic acid, quinic acid.

ABSTRACT

Phenolic compounds of *Nepeta nuda* subsp. *Lydiae* leaves were identified by UHPLC-ESI-MS/MS. To the best of our knowledge, phenolic compounds in *N. nuda* subsp. *Lydiae* are reported for the first time. Chlorogenic acid, rosmarinic acid and quinic acid, were found to be the most abundant compound in the methanol extract of *N. nuda* subsp. *Lydiae*, among the twenty-seven compounds studied by LC-MS/MS. Also, smaller amounts of kaempferol, p-coumaric acid, tr-caffeic acid, apigenin, luteolin and rhamnetin were identified, quantitatively. In conclusion, leaves of *N. nuda* subsp. *Lydiae* have high potential of phenolic contents that mainly attributed with biological activities.

INTRODUCTION

The genus *Nepeta* L. (Lamiaceae), herbaceous perennials, rarely annual, and often pleasantly aromatic herbs; stems erect or procumbent, eglandular or glandular, found in temperate Asia, Europe, North Africa, in mountains of tropical Africa and include about 250 species in the world (Davis, 1982; Ozhatay *et al.*, 2009). Several *Nepeta* species are used in folk medicine as diuretic, diaphoretic, antitussive, antispasmodic, antiasthmatic, febrifuge, emmenagogue, and sedative agents; furthermore, many of these species are often pleasantly aromatic, rich in essential oils, and of potential economic interest (Tzakou *et al.*, 2000; Rapisarda *et al.*, 2001). *N. nuda* subsp. *Lydiae* is varies from other subspecies in terms of some characters; flower colour, stem indumentum, inflorescence and calyx characters (Davis, 1982). Phenolic compounds are secondary metabolites that widely existed throughout the plants, are known to have antioxidant properties. Phenolic compounds play important roles in neutralizing free radicals as well as to prevent lipid

peroxidation chain reactions (Pacífico *et al.*, 2015). Liquid chromatography with various detection techniques has been widely used to determine phenolic acids. Because of the high sensitivity and selectivity, HPLC coupled to ESI-MS has received considerable attention for the determination of phenolic acids (Yang *et al.*, 2016). Thus, we aimed to determine the phenolic contents of *N. nuda* subsp. *Lydiae* by using UHPLC-ESI-MS/MS technique. According to the results, chlorogenic acid (1325 ± 65 ppb), rosmarinic acid (238 ± 12 ppb) and quinic acid (224 ± 11 ppb) were identified as the major phenolic compounds.

MATERIALS AND METHODS

Plant material

The plant material was collected by Dr. Omer Kilic from a quercus forest (1600-1700 m) in Bingol, a southeast city of Turkey. The voucher specimens are deposited at the Department of Biology, Hacettepe University and at the Department of Park and Garden Plants of Bingol University, Turkey. The identification of plant sample was made by Dr. Kilic, according to volume 7 of Flora of Turkey (Davis, 1982). The leaves of plant were dried in air and collected until used.

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Chemicals

Standard phenolic compounds of LC-MS/MS (quinic acid, malic acid, tr-aconitic acid, gallic acid, chlorogenic acid, protocatechuic acid, tannic acid, tr-caffeic acid, vanillin, p-coumaric acid, rosmarinic acid, rutin, hesperidin, hyperoside, 4-OH benzoic acid, salicylic acid, myricetin, fisetin, coumarin, quercetin, naringenin, hesperetin, luteolin, kaempferol, apigenin, rhamnetin, chrysin) were purchased from Sigma-Aldrich (Germany) and Fluka (Germany). The other chemicals were purchased from Sigma-Aldrich (Germany).

Preparation of plant extract for LC-MS/MS

The air-dried and powdered plant materials (100 g) were extracted three times with 300 mL of methanol for 24 h at room temperature. The solvent was removed under vacuum at 30°C in a rotary evaporator until dry methanol extracts were obtained (Yield:15.6%). Dry filtrates were diluted to 1000 mg extract/L and filtrated with 0.2 µm microfiber filter prior to LC-MS/MS analysis.

LC-MS/MS instrumentation and chromatographic conditions

LC-MS/MS analyzes of the phenolic compounds were performed by using a Nexera model Shimadzu UHPLC coupled to a tandem MS instrument. The liquid chromatography was equipped with LC-30AD binary pumps, DGU-20A3R degasser, CTO-10ASvp column oven and SIL-30AC auto sampler. The chromatographic separation was performed on a C18 reversed-phase Inertsil ODS-4 (150 mm × 4.6 mm, 3 µm) analytical column.

The column temperature was fixed at 40°C. The elution gradient consisted of mobile phase A (water, 5 mM ammonium formate and 0.1% formic acid) and mobile phase B (methanol, 5 mM ammonium formate and 0.1% formic acid). The gradient program with the following proportions of solvent B was applied t (min), B%: (0, 40), (20, 90), (23.99, 90), (24, 40), (29, 40). The solvent flow rate was maintained at 0.5 mL/min and injection volume was settled as 4 µL.

MS instrumentation

MS detection was performed using Shimadzu LCMS 8040 model triple quadrupole mass spectrometer equipped with an ESI source operating in both positive and negative ionization modes. LC-MS/MS data were collected and processed by Lab Solutions software (Shimadzu, Kyoto, Japan).

The multiple reaction monitoring (MRM) mode was used to quantify the analytes: the assay of investigated compounds was performed following two or three transitions per compound, the first one for quantitative purposes and the second and/or the third one for confirmation. The optimum ESI conditions were determined as DL temperature; 250°C, heat block temperature; 400°C, nebulizing gas flow (nitrogen); 3 L/min and drying gas flow (nitrogen); 15 L/min (Ertas *et al.*, 2015).

RESULTS AND DISCUSSION

Phenolic compounds contain one or more hydroxyl group with an aromatic structure and they exist in different species of plants. The phenolic compounds have antioxidant activities because of their redox properties, which allow them to act as reducing agents, hydrogen donors and quenchers of singlet oxygen (Turan *et al.*, 2016; Isik *et al.*, 2015). Also, the structure of aromatic ring and the number of hydroxyl groups of phenolic compounds are important factors on antioxidant abilities. Phenolic compounds can interrupt chain oxidation reactions by donation of a hydrogen atom or chelating metals. Therefore identification and quantification of phenols from different source is increasingly becoming important due to their antioxidant potential. It was reported that compounds with structures containing functional hydroxyl groups can show metal chelation activity (Fiorucci *et al.*, 2007). It was indicated that many phenolic compounds such as; curcumin, L-adrenaline, L-carnitine and quercetin bounded ferrous ions (Fe²⁺) through groups and chelated metal ions (Gulcin 2012). Many methods such as; spectrophotometric, chromatographic and enzymatic methods have been used for determination of the phenolic acids of plants or foods. Liquid chromatography with various detection techniques has been widely used to determine phenolic acids. Because of the high sensitivity and selectivity, liquid chromatography coupled to ESI-MS has received considerable attention for the determination of phenolic acids (Sandin-Espana *et al.*, 2016). In this study, we identified the phenolic acids of *N. nuda* subsp. *Lydiae* by UHPLC-ESI-MS/MS. We preferred to use methanol extract of *N. nuda* subsp. *Lydiae* for this purpose because it was previously stated that methanol has a protective role and prevents phenols from artificial oxidation by enzymes such as phenoloxidase.

The datas of phenolic compounds were shown on Table 1 as well as the UHPLC-ESI-MS/MS chromatograms of standards and plant extract were shown in Fig. 1A and Fig. 1B, respectively. According to the analysis of UHPLC-MS/MS, some chromatograms of plant extract were detected; peak 1 exhibited a negative molecular ion at [MS-H]⁺ at m/z of 190.95 corresponding to quinic acid, the peak 5 has shown an m/z of 353 which is the indication of chlorogenic acid, the peak 8 has shown an m/z of 178.95 corresponding to tr-caffeic acid, the peak 10 has shown an m/z of 136.95 corresponding to p-coumaric acid, the peak 11 has shown an m/z of 151.05 corresponding to rosmarinic acid, the peaks 23, 24 have shown m/z of 284.95 corresponding to luteolin and kaempferol, the peak 25 has shown an m/z of 268.95 corresponding to apigenin, the peak 26 has shown an m/z of 314.95 corresponding to rhamnetin.

In order to establish the relationship between peak area and concentration, linear regression analysis was made for investigated standards. The ranges of calibration curves were 250-10000 µg/mL for quinic acid, malic acid, tr-aconitic acid, chlorogenic acid, vanillin, rosmarinic acid, rutin; 25-1000 µg/mL for gallic acid, tr-caffeic acid, 4-OH benzoic acid, salicylic acid, quercetin, naringenin, hesperetin, luteolin, kaempferol, apigenin,

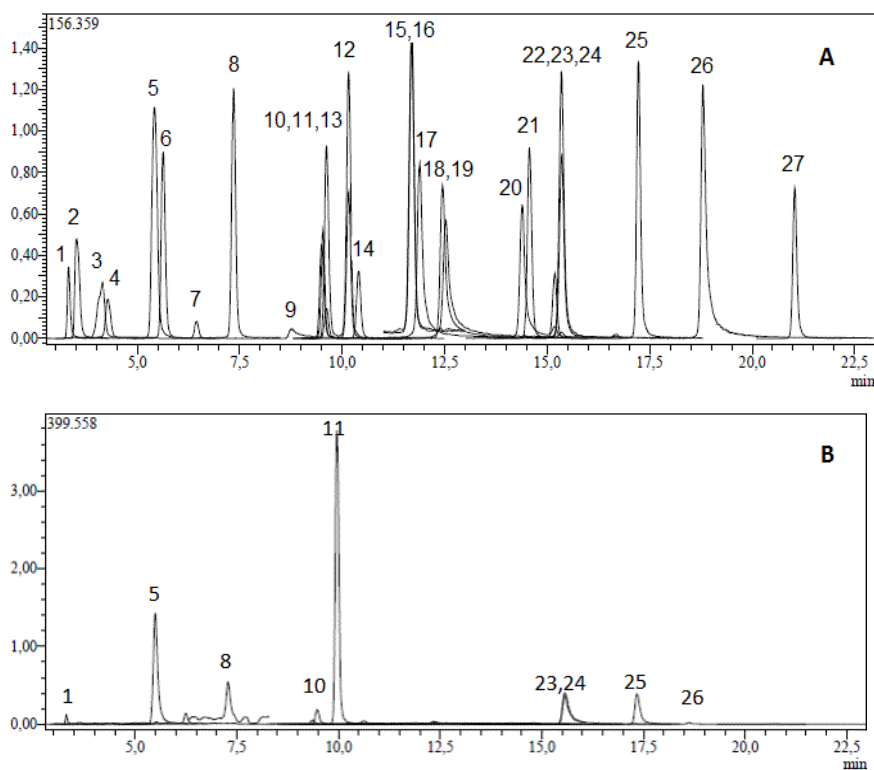


Fig. 1: UHPLC-ESI-MS/MS chromatograms of standard phenolic compounds (A) and methanol extract of *N. nuda* subsp. Lydiae (B): (1:quinic acid, 2:malic acid, 3:tr-aconitic acid, 4:gallic acid, 5:chlorogenic acid, 6:protocatechuic acid, 7:tannic acid, 8:tr-caffeic acid, 9:vanillin, 10:p-coumaric acid, 11:rosmarinic acid, 12:rutin, 13:hesperidin, 14:hyperoside, 15:4-OH benzoic acid, 16:salicylic acid, 17:myricetin, 18:fisetin, 19:coumarin, 20:quercetin, 21:naringenin, 22:hesperetin, 23:luteolin, 24:kaempferol, 25:apigenin, 26:rhamnetin, 27:chrysin).

rhamnetin, chrysin and 100-4000 $\mu\text{g/mL}$ for protocatechuic acid, tannic acid, p-coumaric acid, hyperoside, fisetin, coumarin.

The linearity was estimated via linear regression analysis and expressed as correlation coefficient (R). The result showed that the peak area is strongly related to concentration (Table 1). Correlation coefficient (R) was higher than 0.99 for all standard except for hyperoside (R=0.95). We also calculated LOD and LOQs which are ranged 0.05-25.8 and 0.17-74.5, respectively. To quantify each phenolic compound in the sample, the individual peak areas of each component from UHPLC-MS/MS chromatogram profile were compared with the areas of standards at known concentrations. The amount of each compound was expressed as μg analyte/kg (ppb) dried plant material.

According to the results; chlorogenic acid (1325 ± 65 ppb), rosmarinic acid (238 ± 12 ppb) and quinic acid (224 ± 11 ppb) were detected as major phenolic compounds as well as smaller amounts of kaempferol (89 ± 5 ppb), p-coumaric acid (86 ± 4 ppb), tr-caffeic acid (73 ± 4 ppb), apigenin (56 ± 3 ppb), luteolin (19 ± 1 ppb), and rhamnetin (9 ± 1 ppb) identified, quantitatively (Table 1). Chlorogenic acid, rosmarinic acid and quinic acid were detected as main phenolics from *N. nuda* subsp. Lydiae by UHPLC-ESI-MS/MS method. These phenolic compounds contain hydroxyl groups which have significant roles on biological properties. Biological activities of phenolic compounds usually depend on not only the numbers of hydroxyl

groups but also the positions of the hydroxyl groups and presence of other functional groups (Bursal, 2013). Chlorogenic acid is a natural dietary flavanone which has been identified in different species of plants. Chlorogenic acid has been recognized as a potent antioxidant, anti-inflammatory and anticarcinogenic agent. In a previous report, high amounts of chlorogenic acids were estimated by the chromatography analysis from green coffee beans. Also, high antioxidant capacities of different coffee brews determined by Folin-Ciocalteu, ABTS and DPPH in vitro assays (Jeszka-Skowron *et al.*, 2016). So, it could be a relation between concentration of chlorogenic acid and antioxidant capacity. Rosmarinic acid is a natural polyphenolic acid, an ester of caffeic and 3,4-dihydroxyphenyllactic acids. Rosmarinic acid was isolated and identified as one of the main component of several medicinal plants. It was reported that rosmarinic acid had several biological activities, namely antioxidant, anti-inflammatory, antibacterial, antiviral, antitumor, hepatoprotective and cardioprotective properties (Petersena and Simmonds, 2003). In a previous study, it was reported that the a methanol extract of *Thymus nummularius* contained high amounts of rosmarinic acid and it had a very rich phenolic content (Ertas *et al.*, 2015). Quinic acid is obtained from cinchona bark, coffee beans, and other plant products. It is a constituent of tannins. It was reported that quinic acid had been found on the extract of *Caesalpinia spinosa* (Garro Galvez *et al.*, 1997).

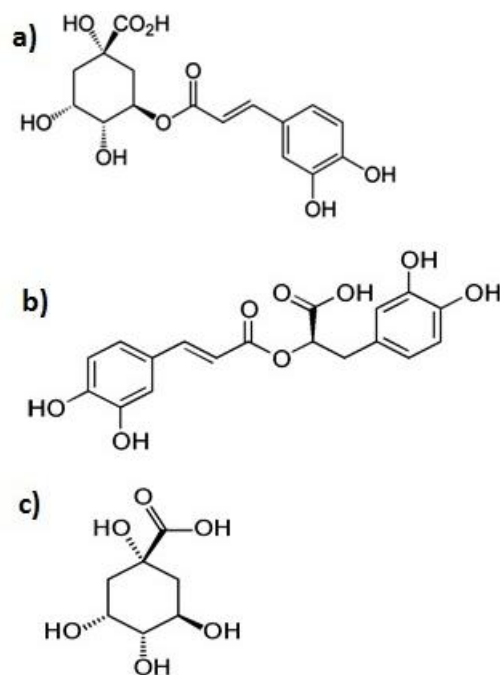


Fig. 2: Chemical structure of main phenolics from *N. nuda* subsp. *Lydiae*; **a)** chlorogenic acid, **b)** rosmarinic acid, **c)** quinic acid

Table 1: LC-MS/MS parameters of selected compounds and amount of *N. nuda* subsp. *Lydiae*.

No	Analytes	^a RT	^b Parent ion (m/z)	Ionization Mode	^c R ²	^d RSD%	Linearity Range (µg/L)	^e LOD/LOQ (µg/L)	Recovery (%)	^f U	^g Amount
1	Quinic acid	3.32	190.95	Neg	0.9927	0.0388	250-10000	22.3 / 74.5	103.3	4.8	224±11
2	Malic acid	3.54	133.05	Neg	0.9975	0.1214	250-10000	19.2 / 64.1	101.4	5.3	ND
3	tr-Aconitic acid	4.13	172.85	Neg	0.9933	0.3908	250-10000	15.6 / 51.9	102.8	4.9	ND
4	Gallic acid	4.29	169.05	Neg	0.9901	0.4734	25-1000	4.8 / 15.9	102.3	5.1	ND
5	Chlorogenic acid	5.43	353	Neg	0.9932	0.1882	250-10000	7.3 / 24.3	99.7	4.9	1325±65
6	Protocatechuic acid	5.63	152.95	Neg	0.9991	0.5958	100-4000	25.8 / 85.9	100.2	5.1	ND
7	Tannic acid	6.46	182.95	Neg	0.9955	0.9075	100-4000	10.2 / 34.2	97.8	5.1	ND
8	tr- Caffeic acid	7.37	178.95	Neg	0.9942	1.0080	25-1000	4.4 / 14.7	98.6	5.2	73±4
9	Vanillin	8.77	151.05	Neg	0.9995	0.4094	250-10000	10.1 / 33.7	99.2	4.9	ND
10	p-Coumaric acid	9.53	162.95	Neg	0.9909	1.1358	100-4000	15.2 / 50.8	98.4	5.1	86±4
11	Rosmarinic acid	9.57	358.9	Neg	0.9992	0.5220	250-10000	10.4 / 34.8	101.7	4.9	238±12
12	Rutin	10.18	609.1	Neg	0.9971	0.8146	250-10000	17.0 / 56.6	102.2	5.0	ND
13	Hesperidin	9.69	611.1	Poz	0.9973	0.1363	250-10000	21.6 / 71.9	100.2	4.9	ND
14	Hyperoside	10.43	463.1	Neg	0.9549	0.2135	100-4000	12.4 / 41.4	98.5	4.9	ND
15	4-OH Benzoic acid	11.72	136.95	Neg	0.9925	1.4013	25-1000	3.0 / 10.0	106.2	5.2	ND
16	Salicylic acid	11.72	136.95	Neg	0.9904	0.6619	25-1000	4 / 13.3	106.2	5.0	ND
17	Myricetin	11.94	317	Neg	0.9991	2.8247	100-4000	9.9 / 32.9	106.0	5.9	ND
18	Fisetin	12.61	284.95	Neg	0.9988	2.4262	100-4000	10.7 / 35.6	96.9	5.5	ND
19	Coumarin	12.52	146.95	Poz	0.9924	0.4203	100-4000	9.1 / 30.4	104.4	4.9	ND
20	Quercetin	14.48	300.9	Neg	0.9995	4.3149	25-1000	2.0 / 6.8	98.9	7.1	ND
21	Naringenin	14.66	270.95	Neg	0.9956	2.0200	25-1000	2.6 / 8.8	97.0	5.5	ND
22	Hesperetin	15.29	300.95	Neg	0.9961	1.0164	25-1000	3.3 / 11.0	102.4	5.3	ND
23	Luteolin	15.43	284.95	Neg	0.9992	3.9487	25-1000	5.8 / 19.4	105.4	6.9	19±1
24	Kaempferol	15.43	284.95	Neg	0.9917	0.5885	25-1000	2.0 / 6.6	99.1	5.2	89±5
25	Apigenin	17.31	268.95	Neg	0.9954	0.6782	25-1000	0.1 / 0.3	98.9	5.3	56±3
26	Rhamnetin	18.94	314.95	Neg	0.9994	2.5678	25-1000	0.2 / 0.7	100.8	6.1	9±1
27	Chrysin	21.18	253	Neg	0.9965	1.5530	25-1000	0.05 / 0.17	102.2	5.3	ND

^a: Retention time, ^b: Molecular ions of the standard compounds (mass to charge ratio), ^c: Coefficient of determination, ^d: Relative standard deviation, ^e: Limit of detection/Limit of quantification, ^f: Percent relative uncertainty at 95% confidence level (k=2), ^g: Quantitative results of real samples in µg analyte/kg (ppb).

CONCLUSION

The phenolic composition of *N. nuda* subsp. *Lydiae* was investigated by UHPLC-ESI-MS/MS analyses. Chlorogenic acid, rosmarinic acid and quinic acid are found to be the highest amounts of phenolic acids. The effective biological activities of *N. nuda* subsp. *Lydiae* might be due to its rich amounts of phenolic compounds. As a result, leaves of *N. nuda* subsp. *Lydiae* have high potential to be used in food industry and pharmaceutical industry.

ACKNOWLEDGMENT

We would like thank to Dr. Omer Kilic for supplying and identification of plant sample.

Financial support and sponsorship: We gratefully acknowledge financial support from the Dicle University (ZGEF.15.002).

Conflict of Interests: There are no conflicts of interest.

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How to cite this article:

Aras A, Bursal E, Dogru M. UHPLC-ESI-MS/MS analyses for quantification of phenolic compounds of *Nepeta nuda* subsp. *Lydiae*. *J App Pharm Sci*, 2016; 6 (11): 009-013.