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Liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) analysis of *Russelia equisetiformis* extract

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Plants produce phytochemicals to defend themselves from attacks of microorganisms. The presence of phytochemicals in *Russelia equisetiformis* was established by liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS). The plant material was extracted with methanol and analyzed by LC-ESI-MS/MS. This analysis revealed the presence of mostly antioxidant phenolic phytochemicals such as chlorogenic acid (1), methyl protocatechuate (2), *p*-coumaric acid (3), 4-hydroxybenzoic acid (4), gallic acid (5), caffeic acid (6), caftaric acid (7), syringic acid (8), and catechin (9). These results illustrate that the methanolic extract of *Russelia equisetiformis* can be used as a nutraceutical against oxidative stress inducing ailments.

Keywords: *Russelia equisetiformis*, ESI-MS/MS, methyl protocatechuate, caffeic acid, syringic acid, catechin

INTRODUCTION

Plants have been known to be the richest source of natural antioxidants and antimicrobial compounds [1,2]. In view of the importance of the plants as remedy for diseases, there is a need of proper evaluation of plants for their biological and phytochemical properties [3]. In medicinal plants, phenolic compounds are a large and diverse group of molecules, which include various families of phytochemicals. Phenolics are the most abundant secondary metabolites such as phenolic acids, condensed tannins, flavonoids, and lignins. All these phytoconstituents are involved in many processes in plants and animals. Among other phytoconstituents the phenolics and flavonoids are most interesting because of their various roles in plants protection, as well as in human health [4]. In plants, phenolics and flavonoids play several functions in flowers, seed pigmentation, fertility, reproduction, and in different reactions to defend against abiotic stresses like ultra violet light or biotic stresses such as pathogen attacks [5]. The phytochemicals found in plants are organic biomolecules known as naturally occurring antibiotics [6]. The synthetic antioxidants like butylated hydroxyanisol, butylated hydroxytoluene, and propyl gallate are used as food antioxidants but these antioxidants have adverse effects on health causing degenerative diseases and cancer [7].

Scrophulariaceae is a family of flowering plants and consists mostly of herbs or small shrubs, and rarely trees. The plants are annual herbs with flowers having bilateral, or rarely, radial symmetry [8]. This family also includes medicinal plants [9].

Russelia equisetiformis (Firecracker) is an evergreen, perennial shrub with an attractive look. Under optimum cultivation conditions, firecracker plants produce distinguished blossoms. *Russelia equisetiformis* is easily propagated from rooted cuttings. It grows up to four feet with red flowers, and much reduced leaves. *Russelia equisetiformis* is traditionally used as a medicinal plant and is considered to have analgesic, anti-inflammatory and membrane stabilizing ability [10]. We already analyzed plant extracts for antioxidant and antimicrobial properties [11]. In order to further extend the study, in this work the presence of secondary metabolites was evaluated by LC-ESI-MS/MS. The latter was carried out to analyze the extract of *Russelia equisetiformis* in order to ascertain the chemistry of secondary metabolites. This analysis may highlight the potential of *Russelia equisetiformis extracts*.

MATERIALS AND METHODS

The research work presented in this paper was conducted at the Department of Chemistry, Government College University, Faisalabad, Pakistan and the liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) assay was carried out at the

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General Procedure

Collection of Plant Material

The whole plants of *Russelia equisetiformis* belonging to the family *Scrophulariaceae* were collected by a team from the University of Agriculture, Faisalabad, Pakistan. The plant was identified by Dr. Mansoor Hameed (Taxonomist), Department of Botany, University of Agriculture, Faisalabad, Pakistan. A voucher specimen (No. 420) was deposited in the herbarium of the above department.

Extraction of phenolic compounds from Russelia equisetiformis for liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) analysis

Phenolic compounds in *Russelia equisetiformis* sample were extracted according to the already reported method [12] with minor modifications. 1.0 g of plant sample was mixed with 20 mL of absolute methanol for 10 min. After centrifugation at 2500 × *g* for 10 min, the supernatant was removed. The extraction was repeated thrice. Supernatants were combined, evaporated at 45°C to dryness using a rotary evaporator. The extract was stored at 4°C until use.

Analysis by liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS)

The phenolics in *Russelia equisetiformis* were analyzed by liquid chromatography combined with electrospray ionization mass spectrometry (LC-ESI-MS). The plant extract was filtered through a 0.45 µm membrane before analysis. Separation of phenolic compounds was performed on a Surveyor Plus HPLC system equipped with Surveyor Auto (Thermo Scientific, San Jose, CA, USA). The pump was equipped with a Luna RP C-18 analytical column (4.6×150 mm, 3.0 µm particle size) (Phenomenex, USA). Elution solvent consisted of LC-MS grade methanol and acidified water (0.5% formic acid v/v) as the mobile phases A and B, respectively. Solvent elution was performed in a gradient system running at a flow rate of 0.3 mL/min. The gradient elution was programmed as follows: from 10% to 30% A & 90 to 70% B from 0 to 10 min followed by 30 to 50% A & 70 to 50% B in the next 20 min. This flow was maintained till the end of analysis. 20 min re-equilibration time was used after each analysis. The column was maintained at 25°C and the injection volume was 5.0 µL. The effluent from the HPLC column was directed to the electron spray ionization mass spectrometer (LTQXL^{TM} linear ion trap Thermo Scientific, River Oaks Parkway, USA). The mass spectrometer was equipped with an ESI ionization source. Parameters for analysis were set using negative ion mode with spectra acquired over a mass range from *m/z* 260 to 800. The optimum values of the ESI-MS parameters were: spray voltage, +4.0 kV; sheath gas and auxiliary gas 45 and 5 units/min, respectively; capillary temperature 320°C; capillary voltage, -20.0 V, and tube lens voltage -66.51 V. The accurate mass spectral data of the molecular ions were processed through the Xcalibur Software (Thermo Fisher Scientific Inc, Waltham, MA, USA).

RESULTS AND DISCUSSION

Plants are being used throughout the history in conventional medicine. At present, about two-thirds to three-quarters of the world population is dependent on plant-based medicines for the treatment of many diseases. Therefore, there is an increasing interest to study the phytochemical and biological properties of plants. Phytochemical contituents found in plants have prominent pharmacological properties. In the present work, *Russelia equisetiformis* was analysed for phenolic compounds by LC-ESI-MS/MS.

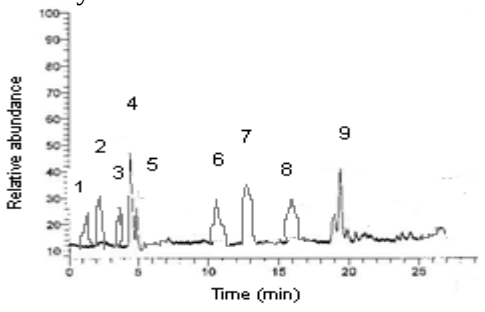

Fig. 1. The chromatogram of *Russelia equisetiformis* sample analyzed by liquid chromatography

The LC-ESI-MS/MS analysis (Figures 1-6) of *Russelia equisetiformis* sample showed the presence of phytochemicals such as chlorogenic acid (1), methyl protocatechuate (2), *p*-coumaric acid(3), 4-hydroxybenzoic acid (4), gallic acid (5), caffeic acid (6), caftaric acid (7), syringic acid (8), and catechin (9) (Table 1). The structures of the compounds identified are shown in Figure 2.

Table 1. Chemical compounds in *Russelia equisetiformis* extract determined by liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS)

Retention time (min)	Major MS/MS *m/z* (intensity)	Molar mass	Name of identified compound
2.14	353.1 (45), 191.1(42), 179(62), 173(100)	354.31	Chlorogenic acid (1)
3.10	167.1 (30), (152.1 (100), 108.0 (11)	168.12	Methyl protocatechuate (2)
3.66	163.05 (45), 119(100)	164.16	*p*-Coumaric acid (3)
4.60	137.25 (20), 121(60), 93(90)	138.12	4-Hydroxybenzoic acid (4)
4.70	169.15 (40), 125(100)	170.12	Gallic acid (5)
10.40	179.3 (100) 161.1 (10), 135.1 (27)	180.16	Caffeic acid (6)
11.80	311.15 (25), 178 (45), 148 (90)	312.22	Caftaric acid (7)
16.20	197.10 (35), 179.10 (60), 135.10 (100)	198.17	Syringic acid (8)
19.81	289.15 (35), 271.2 (15), 245.1 (45)	290.27	Catechin (9)

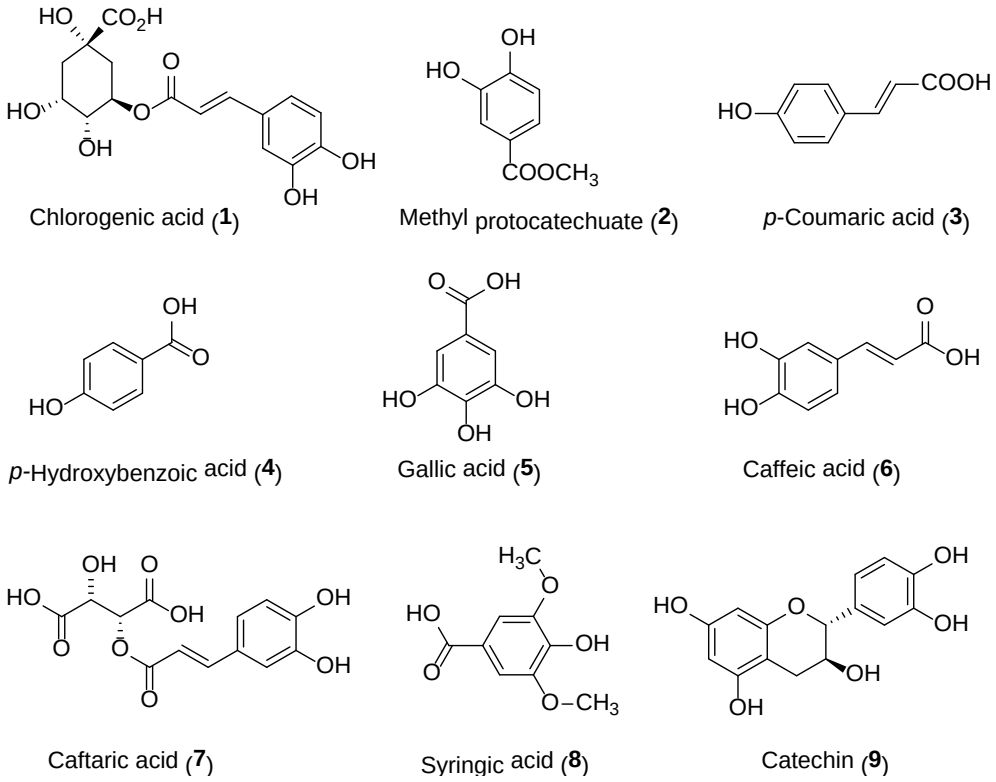

Fig. 2. Structures of the compounds in *Russelia equisetiformis* sample extract analysed by liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS)

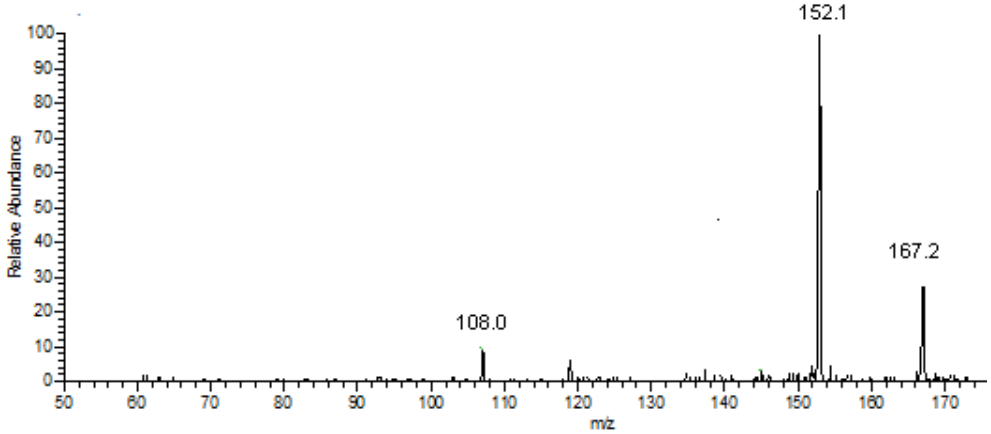

Fig. 3. A representative mass spectrum of methyl protocatechuate (2) in *Russelia equisetiformis* extract analysed by liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS).

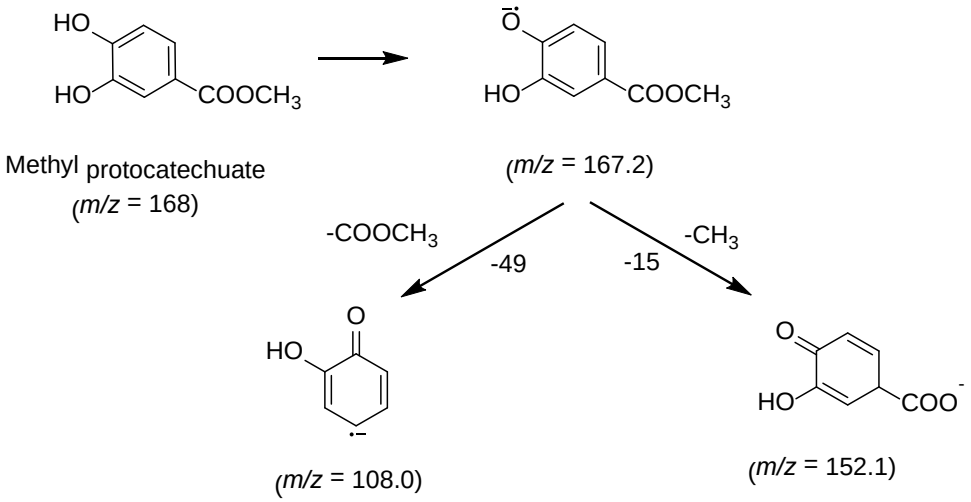

Fig. 4. Fragmentation pattern of the structure of methyl protocatechuate (2) in *Russelia equisetiformis* extract analysed by liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS)

The identification of compounds was made by comparison of retention times with the known standards analysed with the same solvent system and conditions. The ESI-MS/MS spectra of the analysed samples were also compared with the standards and data in the literature.

The presence of methyl protocatechuate (2) was confirmed by comparing the retention time with standards and ESI-MS/MS pattern in the chromatogram (Figure 3). The presence of a peak in the negative mode at *m/z* 167.2 corresponded to the molecular formula $C_8H_8O_4$.

The fragment ion peaks at *m/z* = 152.1 due to loss of CH_3 (–15) and at *m/z* = 108.0 due to loss of $COOCH_3$ (–49) confirmed the presence of methyl protocatechuate (2) (Figure 3).

Compound 6 has a molecular ion peak in negative ion mode at *m/z* = 179.3 which corresponds to the molecular formula of caffeic acid $C_9H_8O_4$, as evident from its mass spectrum (Figure 5). The fragment ion peak at *m/z* 135.1 was due to the loss of CO_2 (–44). The other fragment ion at *m/z* 161.1 was due to the loss of water molecule (–18). These fragments confirmed the presence of caffeic acid (6) (Figure 6). The use of mass spectrometry coupled to liquid chromatography is ideal for the assay of phenolics found in the plants. The advantage of LC-MS/MS is that separation and structural elucidation of compounds can be obtained in a continuous manner [13]. Ionization by electrospray is one of the extensively used methods for LC-ESI-MS/MS studies [14]. In the ESI negative ion mode, analysis of small molecules containing free carboxyl groups, yields mainly the ion $[M-H]^-$, relating to their carboxylate anion [15]. The identification of phenolic compounds in *Russelia equisetiformis* extract was based on chromatograms obtained by HPLC and ESI-MS/MS and comparison with literature data [16].

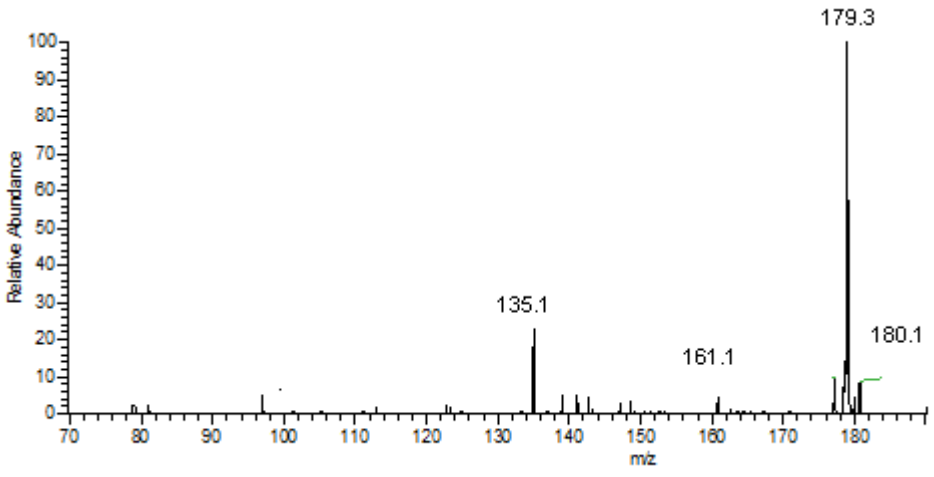

Fig. 5. Mass spectrum of caffeic acid (6) in *Russelia equisetiformis* extract analysed by liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS).

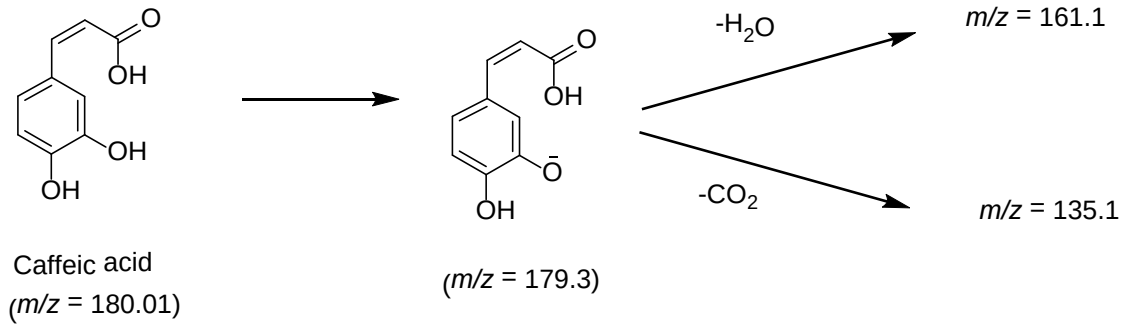

Fig. 6. Representative fragmentation pattern of caffeic acid (6) in *Russelia equisetiformis* extract analysed by liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS)

CONCLUSIONS

The liquid chromatography-mass spectrometry analysis of *Russelia equisetiformis* sample revealed the presence of phytochemicals such as chlorogenic acid (1), methyl protocatechuate (2), *p*-coumaric acid (3), 4-hydroxybenzoic acid (4), gallic acid (5), caffeic acid (6), caftaric acid (7), syringic acid (8), and catechin (9). This analysis may highlight the potential of *Russelia equisetiformis* extracts.

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АНАЛИЗ НА ЕКСТРАКТ ОТ *Russelia equisetiformis* ЧРЕЗ ТЕЧНА ХРОМАТОГРАФИЯ, СЪЧЕТАНА С ЕЛЕКТРОСПРЕЙ-ЙОНИЗАЦИЯ И МАС-СПЕКТРОМЕТРИЯ (LC-ESI-MS/MS)

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(Резюме)

Растенията произвеждат фито-химикализа собствена защите срещу микроорганизмите. Наличието на фито-химикали в *Russelia equisetiformis* е анализирано с помощта на течна хроматография, съчетана с електро-спрей йонизация и мас-спектрометрия. (LC-ESI-MS/MS). Растителната суровина се екстрахира с метанол и се анализира чрез LC-ESI-MS/MS. Анализът разкрива наличието главно на антиоксидантни фенолни фито-химикали, като хлорогенна киселина (1), метул-протокатехат (2), *p*-кумаринова киселина (3), 4-хидроксибензоена киселина (4), галова киселина (5), кафеена киселина (6), кафтарова киселина (7), сирингова киселина (8) и катехин (9). Тези резултати показват, че екстрактите от *Russelia equisetiformis* с метанол може да се използват за неутрализиране на окислителни стресове, причиняващи болести.