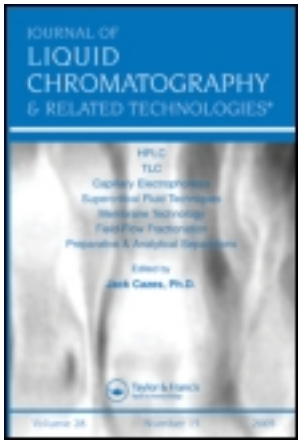

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SIMULTANEOUS DETERMINATION OF ROSMARINIC ACID, SALVIANOLIC ACID B AND CAFFEIC ACID IN SALVIA LERIIFOLIA BENTH. ROOT, LEAF AND CALLUS EXTRACTS USING A HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH DIODE-ARRAY DETECTION TECHNIQUE

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SIMULTANEOUS DETERMINATION OF ROSMARINIC ACID, SALVIANOLIC ACID B AND CAFFEIC ACID IN *SALVIA LERIIFOLIA* BENTH. ROOT, LEAF AND CALLUS EXTRACTS USING A HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH DIODE-ARRAY DETECTION TECHNIQUE

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□ Salvia leriifolia *Benth. (Lamiaceae) is a medicinal plant with analgesic, hypoglycemic and anti-inflammatory properties. Many of the beneficial effects of* Salvia *spp. are attributed to the phenolic compounds. The present study aimed to determine the amounts of rosmarinic acid, salvianolic acid B and caffeic acid in the roots, leaves and calli of* S. leriifolia *using a simple, rapid and efficient HPLC method. In addition, the effect of different combinations of 2,4-dichlorophenoxyacetic acid and kinetin–as growth regulators–on phenolic composition was evaluated. The results indicated that the 3 phenolic acids were well separated using the applied gradient HPLC system [C18 (5 μm) column; phosphoric acid 1% and methanol as mobile phase]. Limit of detection and quantification values for rosmarinic acid, caffeic acid and salvianolic acid B were 0.2 and 0.5, 1.0 and 2.0, and 0.5 and 1.0 μg/mL, respectively. Inter- and intra-day variations for rosmarinic acid, caffeic acid and salvianolic acid B were 2.8% and 3.4%, 2.4% and 3.5%, and 4.8% and 4.7%, respectively. Recovery values for the 3 phenolic acids ranged from 96.3% to 98.9%. Rosmarinic acid was the most frequent phenolic acid in the ethanolic extract of roots, leaves and calli of* S. leriifolia *(0.33, 4.65 and 6.39 mg/g dry extract, respectively) followed by salvianolic acid B (0.11, 0.13 and 0.10 mg/g) and caffeic acid (0.15, 0.20 and 0.07 mg/g). The highest dry weight (0.226 g) of callus was achieved in medium containing 2 mg/L 2,4-D and 1 mg/L KIN. To conclude, the present study indicated that rosmarinic acid is the major phenolic*

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acid in the roots, leaves and calli of S. leriifolia*, using a simple, rapid, efficient and reproducible HPLC method for the separation and simultaneous determination of phenolic acids. Moreover, the content of evaluated phenolics was found to be higher in the leaf extract vs. root and callus extracts of the plant.*

Keywords Callus culture, Chromatographic separation, Lamiaceae, Phenolic acid, *Salvia*

INTRODUCTION

The genus *Salvia*, commonly known as sage, comprises about 900 species worldwide.[1] Out of the identified species, 58 are found in Iran[2] from which 17 are endemic.[3] This genus belongs to the Lamiaceae (formerly called Labiatae) family. *Salvia leriifolia* Benth. is a perennial herbaceous plant that is endemic to Khorasan and Semnan provinces. Two of the most commonly used vernacular names of the plant are Nouroozak and Jobleh.[2]

S. leriifolia has several medicinal applications and commonly used to relieve pain, decrease blood sugar and treat inflammation and insomnia.[4, 5] Phenolic compounds occur frequently in *Salvia* species and are responsible for many of the reported biological and pharmacological activities of this genus.[6] Antimutagenic,[7] antioxidant, antiplatelet, antitumor and antiviral activities are amongst these biological activities.[8] A number of phenolic compounds such as salvianolic acids A-K are unique to the *Salvia* genus. Salvianolic acid B has been reported to possess many interesting biological effects such as radical scavenging,[9] prevention of infarct-induced left ventricular remodeling,[10], neuroprotective[11] and cancer chemoprotective activities.[12] Rosmarinic acid as a phenolic compound also possesses antiviral, antibacterial, anti-inflammatory and antioxidant activities.[13]

The increasing trend in the application of biotechnologic and tissue culture methods in the field of pharmacognosy has enabled the scientists to produce standardized materials independent of environmental variables [14]. Callus cultures have been successfully established for the purpose of producing caffeic acid derivatives from several *salvia* species including *S. officinalis, S. fruticosa*[15] and *S. miltiorrhiza*[16].

Unlike other *Salvia* species,[17–21] no study has yet been conducted on chemical composition and phenolic acid determination in *S. leriifolia*. In addition, there has been no previous literature describing the establishment of culture conditions and production of phenolic acids in callus of *S. leriifolia*. Hence, the present study set out to investigate the phenolic composition of leaves, roots and dried calli of *S. leriifolia* using high-performance liquid chromatography with diode-array detection

(HPLC-DAD). Moreover, establishment of a callus culture from shoot apical meristem explants of the plant was carried out and the best concentration of growth inducers was determined.

MATERIALS AND METHODS

Plant materials

The roots and leaves of *S. leriifolia* were collected from Koohsangi region in Mashhad (Khorasan-Razavi province, Iran) and a voucher specimen (accession no: 11293; identifier: Mr. M. R. Joharchi) was deposited at the Herbarium of the School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran. Samples were protected from humidity and light and were air dried at room temperature.

Callus culture

Seeds of *S. leriifolia* (collected on May 2011) were dipped in 70% ethanol for 30 seconds, then in 3% sodium hypochlorite for 5 min, and finally rinsed in sterile distilled water for three times. Afterwards, seed coats were aseptically removed and the embryos were transferred to half-strength Murashing and Skoog (MS) medium.[22] The cultures were maintained at 24 ± 1°C temperature and 16 hrs light (40–45 $\mu mol^{-2}S^{-1}$)/8 hrs dark condition. The basic medium used for experimentation was MS medium supplemented with 100 mg/L myoinositol and 30 mg/L sucrose, and solidified with 7 g/L agar. The pH of the medium was adjusted to 5.8 before sterilization by autoclaving at 121°C for 20 min. All materials and growth regulators were sourced from Sigma-Aldrich Co (USA). Shoot apical meristems, obtained from aseptic 2 weeks old seedling were used as primary explants in the establishment of callus cultures on MS basal medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) (0, 1, 2, 3 mg/L) combined with kinetin (KIN) (0, 0.3, 1 mg/L) in a total of 6 hormonal supplementations tested. Plant tissue culture vessels containing 20 mL MS medium were inoculated with one primary explant, in a total of 20 vessels per each hormonal MS medium variant tested. Calli were stabilized through subculturing on the same medium conditions for four times with intervals of 30 days. At the end of the fourth subculture, the efficacy of each tested medium variant on calli growth was determined by recording their fresh and dry weight after freeze drying for 36 hours. Calli maintained with 2,4-D (2 mg/L) and KIN (1 mg/L) were selected for phenolic acid assays. The treatments were arranged in a completely randomized design with five replicates per treatment.

Extraction procedure

Extractions were carried out in a manner similar to those reported by Dong J. *et al.*[23] In brief, 0.5 g of dried leaves, roots and freeze dried callus culture were separately macerated in 20 mL of ethanol (60% w/w) and then sonicated for 30 min. After filtration, the solvent was removed on a rotary evaporator, then distilled water (20 mL) was added to the remaining residues and pH of the solution was adjusted at 2.0. Phenolic constituents were extracted by ethyl acetate as an organic solvent. Ethyl acetate was then separated from the aqueous phase and removed on a rotary evaporator. The remaining residues were dissolved in 10 mL of methanol and filtered through a 0.25 µm filter for further studies.

Instrumentation and chromatographic conditions

Standard preparation

Stock solutions of 2 mg/mL rosmarinic acid, salvianolic acid B and caffeic acid (Figure 1) (all purchased from Sigma-Aldrich Co, UK) were prepared in ethanol (70% v/v). Subsequently, concentrations of 10, 20 and 40 µg/mL for salvianolic acid B and caffeic acid, and 10, 20, 40 and 200 µg/mL for rosmarinic acid were prepared from the stock.

Chromatographic conditions

HPLC was conducted on Knauer HPLC system equipped with Knauer Smartline 1000 pump (flow rate 1 mL/min) and Knauer Smartline PDA 2800 UV detector. The extract solutions were separated and analyzed using a 150×4.6 mm Macherey-Nagel C18 (5 µm) column. Injection volume was 20 µL. Separation was performed with a gradient elution. The mobile phase consisted of phosphoric acid 1% and methanol (min, water:methanol; 0, 60:40; 17, 50:50; 18, 40:60; 25–30, 60:40). Full UV (200–400 nm) and spectral evaluation of three phenolic acids was performed on the Diode-array detector. Identification of phenolic

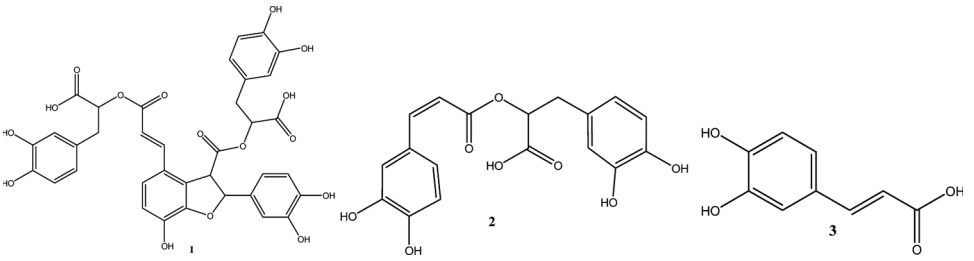

FIGURE 1 Structures of salvianolic acid B (1), rosmarinic acid (2) and caffeic acid (3).

acids peaks in chromatogram was carried out through comparison of respective retention times and spectra against those of known standards. Quantification was performed by setting the detection wavelength as 330 nm for these three phenolic acids.

Validation parameters

The applied method was validated for linearity, limit of detection (LOD), limit of quantification (LOQ), recovery, precision and repeatability.

Linearity

Integrated peak areas of increasing concentrations of these three phenolic acids (10, 20, 40 and 200 µg/mL) were plotted against their concentrations and linear regression was performed by the least squares methods.

Sensitivity

LOD and LOQ were calculated as a signal to noise ratio of 3 and 10, respectively.

Recovery

The recovery rates were determined by spiking sample solutions with authentic standards. The quantity of each component was measured from the corresponding calibration curve.

Precision and repeatability

The inter-day and intra-day variations were determined by analyzing three different concentrations (10, 20 and 40 µg/mL) of each phenolic compound solution. The inter-day variation was measured by injecting three times in three different days and the intra-day variation was measured by injecting three times in a single day.

RESULTS AND DISCUSSION

As shown in Figure 2, phenolic compounds were well separated using the applied HPLC method and no interaction between phenolic acid peaks was observed.

The calibration curves for rosmarinic acid ($y=0.0451x-25.285$; $r^2=0.9953$), caffeic acid ($y=0.0104x-1.0648$; $r^2=0.9962$) and salvianolic acid B ($y=0.0514x+1.4266$; $r^2=0.999$) gave a linear response. LOD values

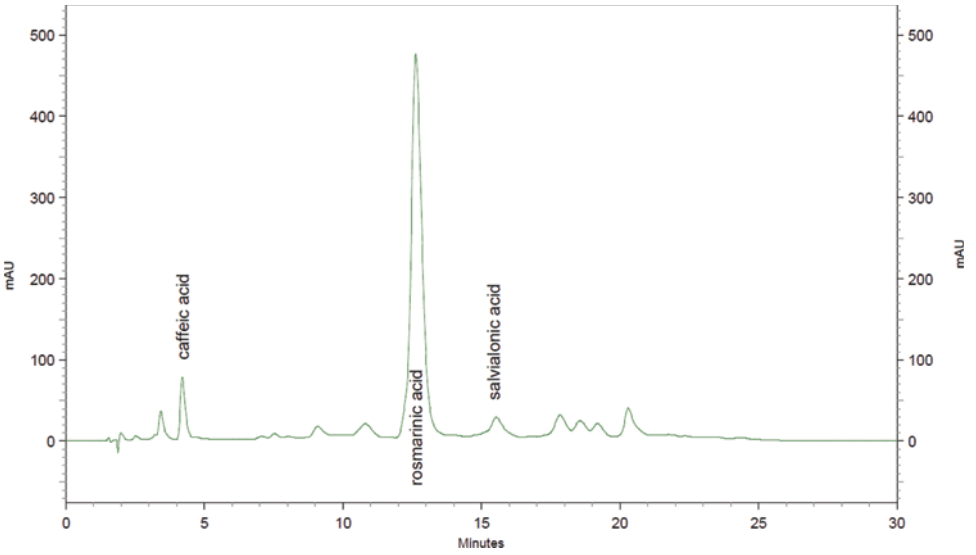

FIGURE 2 Sample HPLC chromatogram of phenolic compounds of the dried leaves of *S. leriifolia*. Identification of phenolic acids peaks in chromatogram was carried out through comparison of respective retention times and spectra against those of known standards.

for rosmarinic acid, caffeic acid and salvianolic acid B were 0.2, 1.0 and 0.5 µg/mL, respectively. LOQ values for these three phenolic acids were 0.5, 2.0 and 1.0 µg/mL, respectively.

Our HPLC analysis showed reliable recovery values (96.3%~98.9%) for all three phenolic compounds. As shown in Table 1, recovery values were in an acceptable range. Relative standard deviations (RSD) of inter- and intra-day variations were below 5%.

As another part of study, the effect of growth regulators on *S. leriifolia* callus induction was evaluated. The effects of combinations of different concentrations of growth regulators on callus induction as well as fresh and dry callus weights are summarized in Table 2.

A white yellowish, friable callus was induced on shoot apical meristem explants 10 days after culture initiation. Four weeks after incubation, the

TABLE 1 Recovery values and inter- and the intra-day variations for phenolic compounds

	Recovery%±SD		Precision and repeatability	
Phenolic compound	Leaves	Roots	Inter-day (RSD%)	Intra-day (RSD%)
Caffeic acid	97.19±1.34	97.53±1.36	2.4	3.5
Rosmarinic acid	98.84±1.74	98.97±1.45	2.8	3.4
Salvianolic acid B	96.54±1.03	96.33±1.15	4.8	4.7

TABLE 2 Effect of growth regulators o S. leriifolia callus induction, fresh and dry weight after 4 weeks of culture.

dry weight (g)	Fresh weight (g)	Callus induction (%)	PGR combination (mg/L)	
			KIN	2,4-D
0	0	0	0	0
0.048	0.49	88.88	0.3	1
0.0262	0.251	70.00	0.3	2
0.0197	0.192	55.25	0.3	3
0.030	0.323	89.56	1	1
0.226	2.281	94.71	1	2
0.0101	1.443	78.57	1	3

Note: Data are from five indented experiments. Mains are not significantly different using Duncan's multiple range tests ($p \leq 0.05$). 2,4-D: 2,4-dichlorophenoxyacetic acid; KIN: kinetin.

best callus induction rate (94.71%) and highest dry weight (0.226 g) were achieved in MS medium containing 2,4-D (2 mg/L) and KIN (1 mg/L). No callus induction was observed during the culture period in control media. The results of simultaneous determination of phenolic acids are shown in Table 3.

In the present study, a gradient elution procedure for simultaneous determination of phenolic constituent of the leaves, roots and callus of *S. leriifolia* was used. According to the results of validation parameters, we used a suitable, reliable and reproducible HPLC method for simultaneous determination of the phenolic constituents of *S. leriifolia*. Given the considerable difference in the polarity of phenolic acids, it is not possible to achieve an ideal resolution using an isocratic system.[17] The present findings indicated that rosmarinic acid is the major phenolic constituent of the roots, leaves and callus of *S. leriifolia*. In previous studies, rosmarinic acid, salvianolic acid A, salvianolic acid B (lithospermic acid B), caffeic acid, protocatechualdehyde, protocatechuic acid, and danshensu were reported as major phenolic constituents of other *Salvia* species.[17] In an

TABLE 3 Results of determination of three phenolic compounds in the dried leaves, roots and freeze dried callus

	Dried roots	Dried leaves	Freeze dried callus
Phenolic compound	Content (mg/g) (%Total extract)	Content (mg/g) (%Total extract)	Content (mg/g) (%Total extract)
Rosmarinic acid	0.33 0.43	4.65 5.51	6.39 7.99
Caffeic acid	0.15 0.020	0.20 0.2	0.07 0.09
Salvianolic acid B	0.11 0.14	0.13 0.152	0.10 0.13

investigation on *S. miltiorrhiza,* rosmarinic acid and salvianolic acid B were found to be the predominant phenolic compounds present in the dried roots and transformed cells of the plant, respectively. However, the contents of rosmarinic acid in the dried roots and cultured cells were much lower than those obtained in the present study.[17] In this latter study, a reverse-phase gradient HPLC method was applied using methanol/formic acid/water as the mobile phase. The detection limits for rosmarinic acid, salvianolic acid B and caffeic acid were 0.02, 0.05 and 0.004 mg/L, and recovery rates were 95.5–96.7%, 94.4–96.0% and 96.3–98.2%, respectively. In another study, Liu and colleagues used an HPLC-DAD method for quantification of six major phenolic acids (including rosmarinic acid and salvianolic acid B) in *S. miltiorrhiza* roots and some other related herbal preparations. The recovery rate for rosmarinic acid was found to be 97.61–103.74% and for salvianolic acid B 101.85–104.78%. These rates are comparable with those obtained in the present study. However, LOD as well as inter- and intra-day variation values found in this latter study were smaller than those found in the present study, which indicates higher sensitivity and precision using a gradient elution system containing phosphoric acid (0.026% v/v) and acetonitrile as the mobile phase.[17] In consistence, Fiore and coworkers successfully employed a gradient HPLC system with acetonitrile/water/formic acid combination as the mobile phase for the determination of phenolic compounds in different parts of *S. miltiorrhiza*.[20] Findings of a recent study by Kosar et al. also demonstrated that rosmarinic acid is the leading phenolic acid present in the ethyl acetate, methanol, 50% methanol and water extracts of *S. halophila*. In the aforementioned study, methanol/water/acetic acid constituted the mobile phase of the applied gradient HPLC system.[19] Chen et al. used HPLC-DAD with 0.5% (v/v) aqueous formic acid and acetonitrile for the separation and quantification of phenolic acids in the aerial parts of *S. miltiorrhiza*. Reported LOD (0.12, 0.20 and 0.30 µg/mL for rosmarinic acid, caffeic acid and salvianolic acid B, respectively), LOQ (0.32, 0.58 and 0.69 µg/mL) and recovery% (101.58%, 96.86% and 95.64%) values were close to those obtained in the current study. Overall, the content of salvianolic acid B in the aerial parts and, in particular, roots of the plant was greater than that of rosmarinic and caffeic acids.[18] In another survey using the same isolation system, the amount of 12 phenolic acids (incuding rosmarinic acid and caffeic acid) was determined in methanol and ethanol extracts of 16 *Salvia* species not inclusive of *S. leriifolia*. Based on the findings, methanolic extracts contained higher contents of rosmarinic acid compared to ethanolic extracts and the amount of this phenolic acid peaked in the *S. ekimiana* (153.5 mg/100g plant), *S. potentillifolia* (110.1 mg), *S. ericeo-tomentosa* var. *hatayica* (101.5 mg) and *S. aytachii* (83.1 mg). However, caffeic acid was present only in some species and at

much lower amounts (0.18–2.04 mg/100g plant) compared to rosmarinic acid.[21] The same as the current study, all HPLC eluting mobile phases in the previously published investigations contained an acid component. According to Yuan et al., acidification of mobile phase helps more successful separation of phenolics and eliminates the problems with tailing of peaks in the chromatogram.[17]

To sum up, the present study introduced an efficient, simple, rapid and reproducible HPLC method for separation and simultaneous determination of phenolic acids in the roots, leaves and calli of *S. leriifolia*. The findings also showed that ethanol extract of callus and, in particular, leaves of *S. leriifolia* are a good source of phenolic acids and thus could be used for the isolation of this bioactive phytochemical.

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