SUPPLEMENTARY MATERIAL

A comparative study on chemical composition and antimicrobial activity of essential oils from three Phlomis species from Uzbekistan

Nilufar Z. Mamadalieva*a, Fadia S. Youssefb, Mohamed L. Ashourb, Davlat Kh. Akramova, Sobirdjan A. Sasmakovb, Nurmurod Sh. Ramazonova, and Shahnoz S. Azimovaa

ABSTRACT

Essential oils obtained from the aerial parts of Phlomis bucharica, P. salicifolia and P. sewerzowii were determined using GC-FID and GC-MS methods. A total of 76 components were identified in the three species representing 97.12, 88.34, and 96.41% of the whole oil, respectively. High percentages of thymol (20.41%) and camphor (14.46%) exist in P. bucharica oil. Methyl palmitate predominates in P. salicifolia oil representing 51.15% whereas thymol (35.76%) is the major constituent in P. sewerzowii essential oil. GC-MS analyses showed that P. bucharica and P. sewerzowii are more closely related comparable to P. salicifolia. The antimicrobial activity of the essential oils was assessed against different microorganisms using agar-disc diffusion and broth microdilution assay. Among the three tested species, the essential oil of P. salicifolia showed the highest antibacterial activity.

Keywords: Lamiaceae; Phlomis; Essential oils; GC-MS; GC-FID; Antimicrobial activity

*Corresponding author: E-mail: nmamadalieva@yahoo.com

Experimental part

Plant material

The aerial parts of Phlomis bucharica Regel, P. salicifolia Regel and P. sewerzowii Regel were collected from Surkhan-Darya and Tashkent regions of Uzbekistan in the summer of 2015. The plants were identified at the Department of Herbal Plants, Institute of the Chemistry of Plant Substances (ICPS, Uzbekistan) by Dr. O.A. Nigmatullaev. The voucher specimens of P. bucharica (accession number (A.N.) N20101022), P. salicifolia (A.N. N201010112) and P. sewerzowii (A.N. N20131351) have been deposited at the Department of Herbal Plants (ICPS, Uzbekistan).

Essential oil isolation

The essential oils were obtained by hydro-distillation of 150 g of the air-dried aerial parts (moisture content 10-12%) of Phlomis bucharica, P. salicifolia and P. sewerzowii, separately for 4 h using a Clevenger type apparatus to give a yield of 0.11, 0.21 and 0.19% w/w, respectively. The yield was calculated as an average of triplicate experiments. The oils were collected in dichloromethane, dried over anhydrous sodium sulphate and stored at −8°C until use.

GC-FID analysis

GC-FID analysis was performed using a Shimadzu GC-2010 plus gas chromatograph (Shimadzu Corporation, Kyoto, Japan) supplied with an FID detector and a RTX-5 fused-bonded cap
column (Restek; 30 m x 0.25 mm i.d., film thickness 0.25 µm, USA). The oven temperature was programmed isothermal at 50°C for 2 min, then raised from 50 to 300 °C at 7 °C/min, and finally held isothermal at 300°C for 10 min; injector temp., 250°C; detector temp., 300°C; carrier gas, He (1.5 mL/min); with split mode (split ratio, 1:20). The sample 0.1 µL was injected automatically to the chromatograph using AOC-20i auto sampler. GC solution® software ver. 2.4 (Shimadzu Corporation, Kyoto, Japan) was used for recording and integrating the chromatograms. Average areas under the peaks of three independent chromatographic runs of each sample were used for calculating the % composition of each component (total peak area = 100%).

**GC-MS analysis**

Mass spectrum was recorded using Shimadzu GC-2010 plus gas chromatograph (Shimadzu Corporation, Kyoto, Japan) coupled to a quadrupole mass spectrometer Shimadzu QP-2010 equipped with Rtx-5MS fused bonded column (30 m x 0.25 mm i.d. x 0.25 µm film thickness) (Restek, USA) equipped with a split–splitless injector. The capillary column was directly coupled to a quadrupole mass spectrometer. The initial column temperature was kept at 45°C for 2 min (isothermal) and programmed to 300°C at a rate of 5°C/min, and kept constant at 300°C for 5 min (isothermal). Detector and injector temperatures were 300 and 250°C, respectively. Helium carrier gas flow rate was 2 mL/min. Mass spectra were recorded applying the following condition: (equipment current) filament emission current, 60 mA; ionization voltage, 70 eV; ion source, 200°C. Diluted samples (0.5%, v/v) were injected with split mode (split ratio 1:15). The sample (1 µL) was injected automatically to the chromatograph using AOC-20i auto sampler. GC solution® software ver. 2.4 (Shimadzu Corporation, Kyoto, Japan) was used for recording and integrating the chromatograms. The compounds were identified by comparison of their mass-spectral data and retention indices (RIs) with those of the Wiley Registry of Mass Spectral Data (9th Ed.), NIST Mass Spectral Library (2011), references (Adams 2007) and with our own Laboratory database (Labib et al. 2017; Mamadalieva et al. 2017; Mamadalieva et al. 2018 ). In addition, the major identified compounds in the essential oil samples were further confirmed by co-injection of some of the available authentic references. The Retention Index (RI) was calculated by relating the retention time of the tested compound “t” to the retention time of co-injected series of straight chain aliphatic hydrocarbons (C₈-C₂₈) using the following equation:

$$RI = 100N + 100 \times \frac{(Rt)_t - (Rt)_N}{(Rt)_{N+1} - (Rt)_N}$$

Where; RI: retention index. N, N+1: Number of carbon atoms in the smaller and larger n-paraffins bracketing the substance. Rt: adjusted retention time of either the substance or the hydrocarbons

**The antimicrobial activity**

**Microbial strains**

The antimicrobial activity was evaluated using standard microbial strains: Gram-positive bacteria *Staphylococcus aureus* (ATCC 25923) and *Bacillus subtilis* (RKMUz 5); Gram-negative bacteria *Pseudomonas aeruginosa* (ATCC 27879) and *Escherichia coli* (RKMUz 221); and the fungus *Candida albicans* (RKMUz 247). The RKMUz microorganism cultures were obtained from the strain collection of the Institute of Microbiology, Academy of Sciences Uzbekistan.

**Evaluation of the antimicrobial activity using the disc diffusion method**
The antibacterial activity of the essential oils was determined using the modified agar-disc diffusion method. Sterile nutrient agar (25 g agar/L distilled water) was inoculated with bacterial cells (200 µL of bacterial cell in 2 mL 0.9% NaCl suspension and 20 mL medium) and poured into Petri dishes to give a solid medium. Candida albicans (1×10^6 colony forming units per mL) was inoculated into sterile Mueller-Hinton-agar. Forty microliters of test material (5 mg/mL of the essential oils) dissolved in the same solvent used for extraction, was applied on sterile paper discs (Whatman No.1, 6 mm diameter). Ampicillin, ceftriaxone and fluconazole (Himedia Laboratories Pvt. Limited) were used as positive controls and the solvents as negative controls. The solvents were allowed to evaporate in a stream of air. The discs were deposited on the surface of inoculated agar plates. Plates were kept for 3 h in refrigerator to enable the diffusion of the substances into the agar. Plates with bacteria were incubated for 24 h at 37°C and plates with yeasts for 48 h at 26°C. The inhibition zone (including the disc diameter) was measured and recorded after the incubation time. An average zone of inhibition was calculated for the three replicates in independent assays (Wayne 2009a).

**Determination of minimum inhibitory concentration (MIC) and minimum microbicidal concentration (MBC)**

The broth micro-dilution method was used to determine MIC and MBC as described by Clinical and Laboratory Standards Institute (CLSI) (Wayne 2009b). Firstly, 5% (v/v) dimethyl sulfoxide (DMSO) (Merck, Darmstadt, Germany) as an emulsifier and 0.05% (w/v) agar-agar (Merck, Darmstadt, Germany) as a stabilizer of the essential oil were added in 10 mL Mueller-Hinton Broth (MHB, Merck, Darmstadt, Germany) medium. After autoclaving of medium, different concentrations of the essential oil (0.017, 0.035, 0.07, 0.156, 0.312, 0.625, 1.25, 5, 10, 20, and 40 µg/mL) were set up in Mueller-Hinton broth (MHB) medium. The 96-well plastic microdilution tray with round bottom wells were prepared by dispensing into each well 90 µL of MHB containing different concentrations of the essential oil and 10 µL of the bacterial inoculum, which was approximately 1×10^8 CFU/mL. Wells containing DMSO and inoculums were used as negative control, whereas ampicillin, ceftriaxone and nystatin consist of positive control. The lowest concentration of the essential oil showing visually no growth (by comparing with the first growth control) was taken as MIC. Determination of the minimum concentration of the essential oil that reduces 99.99% of population bacteria (MBC) was done by culturing of 10 µL of each well without any invisible growth. The culturing was performed on Mueller-Hinton agar medium and incubated at 37°C for 24 h.

**References**

Adams RP. 2007. Identification of essential oil components by gas chromatography/mass spectrometry. 4th ed. Carol Stream, IL: Allured Publishing Co.

Labib RM, Youssef FS, Ashour ML, Abdel-Daim MM, Ross SA. 2017. Chemical composition of Pinus roxburghii bark volatile oil and validation of its anti-inflammatory activity using molecular modelling and bleomycin-induced inflammation in albino mice. Molecules. 2017. 22: 1384.

Mamadalieva NZ, Youssef FS, Ashour ML, Sasmakov SA, Tiezzi A, Azimova SS. 2018. Chemical composition, antimicrobial and antioxidant activities of the essential oils of three Uzbek Lamiaceae species. Nat Prod Res. doi: 10.1080/14786419.2018.1443088. [Epub ahead of print]
Mamadalieva NZ, Sharopov F, Satyal P, Azimova SS, Wink M. 2017. Composition of the essential oils of three Uzbek Scutellaria species (Lamiaceae) and their antioxidant activities. Natural Product Research 31(10): 1172-1176.

Wayne P. 2009. Clinical and Laboratory Standards Institute (CLSI) performance standards for antimicrobial disk diffusion susceptibility tests 19th ed. approved standard. CLSI document M100-S19, 29.

Wayne P. 2009a. Clinical and Laboratory Standards Institute (CLSI) Method for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically. Approved standard 8th ed., CLSI document M07-A8, USA.
Table S1. Volatile oil compositions of *P. bucharica*, *P. salicifolia* and *P. sewerzowii*

| Compound            | RI   | Calculated | RI    | Reported | Content (%)  | Identification method |
|---------------------|------|------------|-------|----------|--------------|-----------------------|
|                     |      |            |       |          | *P. bucharica* | *P. salicifolia* | *P. sewerzowii* |
| 1. *n*-Nonane       | 889  | 900        | 1.36  | 0.22     | 2.95         | MS, RI               |
| 2. Methyl caproate  | 917  | 916        | 1.49  |          |              | MS, RI               |
| 3. *α*-Pinene       | 925  | 926        | 1.56  | 0.22     | 2.95         | MS, RI, AU           |
| 4. Camphene         | 942  | 942        | tr.   |          |              | MS, RI, AU           |
| 5. Benzaldehyde     | 956  | 956        | 1.23  | 0.63     |              | MS, RI, AU           |
| 6. 2-Methylnonane   | 958  | 962        | tr.   |          |              | MS, RI               |
| 7. 3-Methylnonane   | 965  | 968        | tr.   | 0.63     |              | MS, RI               |
| 8. 1-Octen-3-ol     | 978  | 978        | 1.21  |          |              | MS, RI               |
| 9. *n*-Decane       | 997  | 1000       | 1.78  | 0.37     | 4.83         | MS, RI               |
| 10. *α*-Cymene      | 1024 | 1023       | tr.   |          |              | MS, RI               |
| 11. Limonene        | 1028 | 1028       | tr.   |          |              | MS, RI               |
| 12. 1,8-Cineole     | 1031 | 1031       | 13.69 | 0.39     |              | MS, RI, AU           |
| 13. 2-Methyldecane  | 1063 | 1063       | tr.   |          |              | MS, RI               |
| 14. Linalool oxide  | 1074 | 1074       | 0.37  |          |              | MS, RI, AU           |
| 15. *n*-Undecane    | 1099 | 1100       | 0.89  | 0.58     | 2.98         | MS, RI               |
| 16. *β*-Linalool    | 1101 | 1101       |      |          | 2.46         | MS, RI               |
| 17. Methyl caprylate| 1125 | 1125       |      | 0.72     |              | MS, RI               |
| 18. Camphor         | 1147 | 1144       | 14.46 |          |              | MS, RI, AU           |
| 19. Borneol         | 1169 | 1169       | 9.82  |          |              | MS, RI, AU           |
| 20. *n*-Caprylic acid | 1177 | 1177 | tr. |          |              | MS, RI               |
| 21. 4-Terpineol     | 1179 | 1179       | tr.   |          |              | MS, RI               |
| 22. *α*-Terpineol   | 1193 | 1193       | 1.23  | 0.30     | tr.          | MS, RI, AU           |
| 23. *n*-Dodecane    | 1197 | 1200       | tr.   | tr.      | tr.          | MS, RI               |
| 24. Verbenone       | 1212 | 1213       |      |          |              | MS, RI               |
| 25. Methyl nonanoate | 1225 | 1226       | 1.13  |          |              | MS, RI               |
| 26. Isobornyl formate | 1231 | 1233 | tr. |          |              | MS, RI               |
| 27. Pulegone         | 1239 | 1244       |      |          |              | MS, RI               |
| 28. Nonanoic acid   | 1276 | 1276       | tr.   |          |              | MS, RI               |
| 29. Bornyl acetate  | 1290 | 1290       | 1.04  |          |              | MS, RI               |
| 30. Thymol          | 1298 | 1297       | 20.41 |          | 35.76        | MS, RI, AU           |
| 31. Carvacrol       | 1307 | 1307       | 5.74  |          | 8.90         | MS, RI, AU           |
| 32. Methyl caprate   | 1326 | 1326       | 0.46  |          |              | MS, RI               |
| 33. *n*-Capric acid | 1370 | 1370       | tr.   |          |              | MS, RI               |
| 34. *α*-Cubebene    | 1380 | 1372       |      |          |              | MS, RI               |
| 35. Methyl undecanoate | 1387 | 1389 | tr. |          |              | MS, RI               |
| Compound                        | RI       | Content (%) | Identification method |
|--------------------------------|----------|-------------|-----------------------|
|                                | Calculated | Reported | P. bucharica | P. salicifolia | P. sewerzowii |                      |
| 36. β-Damascenone               | 1388      | 1388       | tr.        | 2.92          |               | MS, RI               |
| 37. β -Elemene                  | 1395      | 1395       | tr.        |               |               | MS, RI               |
| 38. Methyl undecanoate          | 1424      | 1426       | tr.        |               |               | MS, RI               |
| 39. β -Caryophyllene            | 1425      | 1425       | tr.        | 8.43          |               | MS, RI, AU           |
| 40. Geranyl acetone             | 1455      | 1455       | tr.        |               |               |                      |
| 41. (E)-β -Farnesene            | 1459      | 1459       | 1.22       |               |               | MS, RI               |
| 42. α -Humulene                 | 1461      | 1461       | tr.        |               |               | MS, RI               |
| 43. α-Muurolene                 | 1484      | 1484       | tr.        |               |               | MS, RI               |
| 44. Germacrene D                | 1484      | 1484       | tr.        |               |               | MS, RI               |
| 45. α-Selinene                  | 1492      | 1492       | 1.22       |               | 4.19          | MS, RI, AU           |
| 46. β -Ionomone                 | 1493      | 1493       | tr.        |               |               | MS, RI               |
| 47. α-Muurolene                 | 1508      | 1508       | tr.        |               |               | MS, RI               |
| 48. r-Cadinene                  | 1522      | 1522       | tr.        |               |               | MS, RI               |
| 49. Methyl dodecanoate          | 1526      | 1524       | 1.22       |               |               | MS, RI               |
| 50. Myristicin                  | 1529      | 1528       | tr.        |               |               | MS, RI               |
| 51. δ-Cadinene                  | 1531      | 1531       | tr.        |               |               | MS, RI               |
| 52.Elemol                       | 1557      | 1557       | 0.32       |               |               | MS, RI               |
| 53. n-Dodecanoic acid           | 1566      | 1566       | tr.        |               |               | MS, RI               |
| 54. Caryophyllene oxide         | 1593      | 1593       | 0.82       | tr.           | 8.32          | MS, RI, AU           |
| 55. Methyl tridecanoate         | 1624      | 1625       | tr.        |               |               | MS, RI               |
| 56. r-Eudesmol                  | 1643      | 1642       | 3.84       |               |               | MS, RI               |
| 57. r-Cadinol                   | 1651      | 1652       | tr.        | tr.           |               | MS, RI               |
| 58. β -Eudesmol                 | 1663      | 1658       | 3.39       | tr.           |               | MS, RI               |
| 59. δ-Cadinol                   | 1665      | 1665       | tr.        |               |               | MS, RI               |
| 60. α-Eudesmol                  | 1666      | 1662       | 3.56       | tr.           |               | MS, RI               |
| 61. Cedr-8-en-13-ol             | 1669      | 1668       | 3.88       |               |               | MS, RI               |
| 62. α-Bisabolol                 | 1679      | 1672       | 0.88       |               |               | MS, RI               |
| 63. Methyl myristate            | 1726      | 1726       | 3.17       |               |               | MS, RI               |
| 64. Methyl pentadecanoate       | 1824      | 1826       | 0.74       |               |               | MS, RI               |
| 65. Hexahydrofarnesyl acetone   | 1844      | 1844       | 7.54       | 7.69          | 8.25          | MS, RI, AU           |
| 66. Methyl palmitate            | 1926      | 1926       | tr.        | 51.15         |               | MS, RI, AU           |
| 67. Methyl heptadecanoate       | 2026      | 2028       | 0.51       |               |               | MS, RI               |
| 68. Methyl linoleate            | 2101      | 2098       | 3.79       |               |               | MS, RI               |
| 69. Methyl linolenate           | 2108      | 2099       | 5.99       |               |               | MS, RI, AU           |
| 70. r-Palmitolactone            | 2115      | 2120       | 0.5        |               |               | MS, RI               |
Table S1 (Cont’d). Volatile oil compositions of *P. bucharica*, *P. salicifolia* and *P. sewerzowii*

| Compound | RI | Content (%) | Identification method |
|----------|----|-------------|-----------------------|
|          | Calculated a) | Reported b) | *P. bucharica* | *P. salicifolia* | *P. sewerzowii* |
| 71. Phytol | 2120 | 2122 | 0.66 | tr. | MS, RI |
| 72. Methyl stearate | 2132 | 2133 | 3.73 | | MS, RI |
| 73. n-Tricosane | 2300 | 2300 | 0.37 | | MS, RI |
| 74. n-Tetracosane | 2393 | 2400 | tr. | | MS, RI |
| 75. n-Hexacosane | 2599 | 2600 | tr. | | MS, RI |
| 76. n-Hepatcosane | 2698 | 2700 | tr. | | MS, RI |

|       |         |             |                   |
|-------|---------|-------------|-------------------|
|       |         | *P. bucharica* | *P. salicifolia* | *P. sewerzowii* |
|       |         | 1.56        | 1.06              | 2.57             |
| Monoterpene hydrocarbons | | | | |
| Oxygen containing monoterpene | | | | |
| Sesquiterpene hydrocarbons | | | | |
| Oxygen containing sesquiterpene | | | | |
| Others | | | | |
|       |         | 66.39       | 1.06              | 47.12            |
|       |         | tr.         | tr.               | 16.47            |
|       |         | 23.91       | 8.01              | 19.49            |
|       | 5.26 | 79.27       |                    | 10.76            |
| Total identified components | | 97.12 | 88.34 | 96.41 |

a) *RI*<sub>calculated</sub>: Retention index determined experimentally on a Rtx-5MS capillary column. 
b) *RI*<sub>reported</sub>: Published Kovats retention indices.
Table S2. Antibacterial effect evaluated by mean diameter of inhibition zone (mm) for *P. bucharica*, *P. salicifolia* and *P. sewerzowii* essential oil using agar disk diffusion assay

| Sample              | Gram-positive bacteria | Gram-negative bacteria | Fungi         |
|---------------------|------------------------|------------------------|---------------|
|                     | *B. subtilis*<br>(RKMUz 5) | *S. aureus*<br>(ATCC 25923) | *P. aeruginosa*<br>(ATCC 27879) | *E. coli*<br>(RKMUz 221) | *C. albicans*<br>(RKMUz 247) |
| *P. bucharica*      | 7.08 ± 0.12            | na                     | na            | na            | na             |
| *P. salicifolia*    | 8.12 ± 0.13            | 8.08 ± 0.20            | na            | 7.12 ± 0.13   | na             |
| *P. sewerzowii*     | 7.04 ± 0.10            | na                     | na            | na            | na             |
| Ampicillin (20µg/disc) | 26.04 ± 0.10         | 26.08 ± 0.12           | nt            | 26.08 ± 0.12 | nt             |
| Ceftriaxone (25 µg/disc) | nt                     | nt                     | 25.04 ± 0.10  | nt            | nt             |
| Fluconazole (30 µg /disc) | nt                     | nt                     | nt            | nt            | 27.12 ± 0.13   |

na: not active; nt: not tested. Data were presented as the mean ± standard deviation (SD), *P*≤0.05
Table S3. Antibacterial effect evaluated by minimum inhibitory concentration (MIC) and minimum microbicidal concentration (MBC) in (µg/mL) for *P. bucharica*, *P. salicifolia* and *P. sewerzowii* essential oil

| Sample          | *B. subtilis* (RKMUz 5) | *S. aureus* (ATCC 25923) | *P. aeruginosa* (ATCC 27879) | *E. coli* (RKMUz 221) | *C. albicans* (RKMUz 247) |
|-----------------|-------------------------|---------------------------|------------------------------|-----------------------|----------------------------|
|                 | MIC        | MBC        | MIC        | MBC        | MIC        | MBC        | MIC        | MBC        | MIC        | MBC        |
| *P. bucharica*  | 20         | 25         | >40        | >40        | >40        | >40        | >40        | >40        | >40        | >40        |
| *P. salicifolia*| 18         | 20         | 18         | 18         | >40        | >40        | 20         | 20         | >40        | >40        |
| *P. sewerzowii*| >40        | >40        | >40        | >40        | >40        | >40        | >40        | >40        | >40        | >40        |