*Chemistry of Natural Compounds, Vol. 43, No. 4, 2007*

# FATTY ACIDS OF *Stachys milanii* SEEDS

**G. Stojanovic,[1] A. P. Ligon,[2] A. Smelcerovic,[2] J. Lazarevic,[3] M. Spiteller,[2]* and R. Palic[1]**

UDC 665.127:582.4

*For the first time the total acid composition of the seeds of* Stachys milanii *Petrovic, a species endemic to the central and eastern Balkan Peninsula and growing spontaneously at two localities, was examined by GC and GC/MS. The major fatty acid was linoleic or oleic acid. Besides fatty acids that represent the usual seed oil constituents, 6-octadecynoic and 9-oxononanoic acid were identified in considerable amounts. The high content of 6-octadecynoic acid makes* S. milanii *seed oil a good potential source for the active substance in antifungal preparations. From the chemotaxonomical point of view, small amounts of octadecatrienoic acid detected in the seeds suggested classification of* S. milanii *as an archaic taxon.*

**Key words:** *Stachys milanii*, Lamiaceae, seed fatty acids, 6-octadecynoic acid, 9-oxononanoic acid.

The investigation of fatty acid composition of plant seeds is of interest for the isolation of uncommon fatty acids, but it has also other implications because the fatty acids play a role in taxonomy, phylogeny, and in the food industry.

*S. milanii* is endemic to central and eastern regions of the Balkan Peninsula that occurs rarely within small spots of diversity. Exclusively literature data concerning the chemical composition and antimicrobial activity of the essential oil and ethereal extracts isolated from the aerial plant parts are published by our research group [1]. Fatty acids were not identified as the essential oil constituents, while the free fatty acids or fatty acid esters are one of the most abundant components found in the ethereal extract with a prevalence of 34.7%. 2-Ethyl substituted fatty acids that represent the rare characteristic of the plant kingdom were identified in the ethereal extract in an abundance of 10.2%.

According to our best knowledge, the composition of the seed fatty acids has been analyzed for eight *Stachys* species so far [2, 3]. The isolated compounds represent the common seed fatty acids. On the other hand, seed oil isolated from certain plant species belonging to the subfamily Stachyoideae is a source of unusual allenic fatty acids such as laballenic (5,6-octadecadienoic), lamenallenic ((*E*) 5,6,16-octadecatrienoic) [4], and pholmic (7,8-eicosadienoic) [5].

The requirement of chemotaxonomy for inclusive data classification for this rare and endangered plant species, as well as the assumption that the plant may synthesize some of the unusual and rare fatty acids in detectable amounts, made us determined to isolate and analyze the seed fatty acid composition of *S. milanii*. The results obtained are presented in this paper.

The yield of the chloroform-methanol extract obtained by complete evaporation of the solvents *in vacuo* was 22.2% (Lalinacka Slatina sample) and 26.5% (Probistip sample) (w/w seed). The content of total fatty acids in the *S. milanii* seeds from Lalinacka Slatina was 10.8% (108 mg/g of seed) and from Probistip 16.9% (169 mg/g of seed). The total fatty acid content of the Probistip sample is comparable with those previously reported (from 16.2 to 26.9%) for seven *Stachys* species [2], while the content of the Lalinacka Slatina sample was smaller. The composition of fatty acid methyl esters is summarized in Table 1.

The major fatty acid was linoleic or oleic acid. The content of oleic acid was similar in both examined samples (35.9 and 35.1%). Similarities between *S. milanii* and the previously investigated *Stachys* species [2, 3] are observed only in the content of palmitic and stearic acids (Table 2). *S. milanii* seeds contain a smaller quantity of linoleic acid than previously published for other *Stachys* species. However, oleic and linolenic acids were more abundant than in other examined species. The linolenic/linoleic acid ratio (0.07 for the Lalinacka Slatina sample and 0.09 for the Probistip sample) was similar to this index for *S. silvatica* (0.07) and higher than those for other examined species (0.001–0.03).

1) Department of Chemistry, Faculty of Science and Mathematics, University of Nis, Visegradska 33, 18000 Nis, Serbia and Montenegro; 2) Institute of Environmental Research, University of Dortmund, Otto-Hahn-Str. 6, 44221 Dortmund, Germany, fax +49 (0)231 755 4085, e-mail: m.spiteller@infu.uni-dortmund.de; 3) Department of Pharmacy, Faculty of Medicine, B. Taskovic 81, 18000 Nis, Serbia and Montenegro. Published in Khimiya Prirodnykh Soedinenii, No. 4, pp. 315-317, July-August, 2007. Original article submitted March 27, 2006.

 0009-3130/07/4304-0380 

TABLE 1. Fatty Acids from *Stachys milanii* Seeds (% weight fraction)

| Rt, min | Acid | Identified by | Quantified by | $S_1$ | $S_2$ |
|---|---|---|---|---|---|
| 29.3 | Octanoic | a, b | c, d | 3.8 | N.d. |
| 34.5 | Nonanoic | b | d | 1.4 | N.d. |
| 39.5 | Decanoic | a, b | c, d | 0.5 | N.d. |
| 48.4 | 4-Oxooctanoic | b | d | 1.4 | N.d. |
| 52.9 | 9-Oxononanoic | b | d | 10.0 | 2.0 |
| 65.1 | Hexadecanoic | a, b | c, d | 3.0 | 4.1 |
| 72.4 | (*Z*)-9-Octadecenoic | a, b | c, d | 34.9 | 35.1 |
| 72.6 | (*E*)-9-Octadecenoic | a, b | c, d | 1.0 | 1.3 |
| 72.7 | Octadecanoic | a, b | c, d | 0.6 | 0.8 |
| 73.1 | (*Z,Z*)-9,12-Octadecadienoic | a, b | c, d | 29.3 | 39.8 |
| 74.4 | 6-Octadecynoic | b | d | 9.6 | 12.3 |
| 74.5 | (*Z,Z,Z*)-9,12,15-Octadecatrienoic | a, b | c, d | 2.0 | 3.6 |
| 82.8 | *cis*-9-Eicosenoic | b | d | 0.4 | N.d. |
| 83.0 | *cis*-11-Eicosenoic | a, b | c, d | 0.4 | N.d. |

_____

a = retention time identical to standard compounds; b = identification based on comparison of mass spectra; c = quantification based on standard compounds; d = quantification based on 100% method [14]; $S_1$ = sample from Lalinacka Slatina; $S_2$ = sample from Probistip; N.d. = not detected (<0.05%).

TABLE 2. Fatty Acid Content (mg/g seed) and Composition (% weight fraction) of the Seeds of Examined Species of the Genus *Stachys*

| Section [15] | Species | Content, mg/g | 16:0 | 18:0 | 18:1 | 18:2 | 18:3 | 18:3/18:2 |
|---|---|---|---|---|---|---|---|---|
| *Betonica* | *S. alopecuros* (L.) Benth. [2] | 171 | 7.6 | 2.5 | 24.7 | 64.4 | 0.8 | 0.01 |
| | *S. betonicaeflora* [3] | 241[c] | 1.2 | - | 25.9 | 68.4 | 1.7 | 0.02 |
| | *S. monieri* (Gouan) P.W. Ball [2] | 162 | 3.8 | 1.5 | 21.8 | 72.4 | 0.5 | 0.01 |
| *Creticae* | *S. byzantina* K. Koch. [2] | 198 | 5.6 | 2.0 | 22.5 | 69.4 | 0.5 | 0.01 |
| | *S. lanata* Jacq. [2] | 192 | 4.8 | 1.7 | 16.2 | 77.2 | 0.1 | 0.001 |
| *Eriostomum* | *S. balansae* Boiss. et Koschy [2] | 176 | 3.7 | 0.8 | 18.5 | 77.0 | - | - |
| *Olisia* | *S. annua* L. [2] | 269 | 4.4 | 1.5 | 19.1 | 72.9 | 2.2 | 0.03 |
| | *S. milanii* Petrovic[a] | 108 | 3.0 | 0.6 | 35.9 | 29.3 | 2.0 | 0.07 |
| | *S. milanii* Petrovic[b] | 168 | 4.1 | 0.8 | 36.4 | 39.8 | 3.6 | 0.09 |
| *Stachys* | *S. silvatica* L. [2] | 180 | 4.4 | 1.4 | 22.1 | 67.0 | 5.1 | 0.07 |

_____

a = sample from Lalinacka Slatina; b = sample from Probistip; c = oil content (mg/g seed).

Beside fatty acids that represent the usual seed oil constituents, 6-octadecynoic (tariric) and 9-oxononanoic (azelaaldehydic) acids were identified in considerable amounts. Tariric acid was present in high percentage (9.6% for the Lalinacka Slatina sample and 12.3% for the Probistip sample). This acid has been found in *Alvaradoa amorphoides* seeds [6], two *Picramnia* species [7], some *Sideritis* species [8], as well as in some moss and liverwort species [9]. Tariric acid inhibits the growth of fluconazole-susceptible and fluconazole–resistant *Candida albicans* strains [10]. The antimicrobial investigation of extracts was performed in three replicates using the disk diffusion technique with a dose of 50 µL of solution prepared by dissolving the dry extract in DMSO to a concentration of 1 mg/mL. The width of the fungicidal activity (including the 6 mm disk in diameter) was 12±1 mm for Lalinacka Slatina and 10±1mm for Probistip. The fungistatic activity of the extracts could not be negligible bearing in mind the concentration of tariric acid in the extracts, which was 46.5 and 78.3 mg/g for the Lalinacka slatina and Probistip samples, respectively. Therefore, *S. milanii* seed oil could be a good source for anticandidal pharmaceutical preparations.

Azelaaldehydic acid was detected in both samples but in higher percentage in seeds from Lalinacka Slatina. It is known that this acid is a secondary autoxidation product of linoleic acid [11]. Also, hydroperoxide lyase cleaves linoleic acid to *cis*-nonenal and azelaaldehydic acid [12]. The fact that the sum of the content of azelaaldehydic acid and linoleic acid in both samples is similar confirms the origin of the azealaldehydic acid in *S. milanii* seed extracts.

The 2-ethyl substituted fatty acids that characterize the ether extract of the above-ground parts of *S. milanii* [1] were not found in the examined seeds. From the chemotaxonomic point of view, the small amounts of octadecatrienoic acid detected in the seeds suggested classification of *S. milanii* as an archaic taxon.

The main conclusion of this report is that the composition of *S. milanii* seed fatty acids from two localities is similar, which confirms the conservative nature of the composition of seed oils and their usefulness as taxonomic markers. The presented and previously published results [1] show the ability of *S. milanii* to synthesize unusual fatty acids such as 2-ethyl substituted fatty acids, azelaaldehydic, and tariric acid.

## EXPERIMENTAL

**Plant Material.** *S. milanii* seeds were collected on 14$^{th}$ August 2003 in the region of the Lalinacka Slatina Salt Marsh (near Nis, Southern Serbia) and on 20$^{th}$ August of the same year on the location Probistip, East Former Yugoslav Republic of Macedonia. Voucher specimens (16029 and 16016, respectively) have been deposited in the Herbarium of the Department of Botany, Faculty of Biology, University of Belgrade, Serbia and Montenegro. All solvents employed were of analytical grade (obtained from Merck, Germany) and were redistilled before use.

**Isolation.** Isolation and esterification of total fatty acids were performed according to a previously published procedure [2].

The methyl esters of fatty acids were analyzed by GC/MS and GC/FID. Constituents were identified by comparison of their retention times with standards (a certified standard mixture of 19 FAME, Supelco 18920–1AMP) and/or their mass spectra with those from the NIST MS library (Version 2.0a) and Wiley MS library (Version 6).

**GC/FID.** A Thermo Finnigan trace gas chromatograph equipped with a fused silica capillary column (DB-5, 30 m × 0.25 mm × 0.25 μm) and FID was used. The operating conditions were: temperature program, 60–320°C at 10°C/min and 320°C (4 min); injector temperature 310°C; detector temperature 320°C; carrier gas helium (1 mL/min); split mode (1:10).

**GC/MS.** Analyses were performed on a Thermo Finnigan trace gas chromatograph and trace MS$^{PLUS}$ detector, equipped with a fused silica column (DB-5, 30 m × 0.25 mm × 0.25 μm) with the same temperature program as for the GC/FID and a joined series of three fused silica columns (HP-FFAP, 30 m × 0.25 mm × 0.25 μm; HP-1701, 30 m × 0.25 mm × 0.25 μm; and HP-5, 30 m × 0.25 mm × 0.25 μm). These series of three fused silica columns are comparable with Supelco SP-2560 columns which are used to separate FAMEs of native oils. Temperature program was 60°C to 230°C at 2.5°C/min; carrier gas helium with pressure program, 158 kPa to 487 kPa at 2 kPa/min. Ionization was performed at 70 eV. Solutions (1 μL) were injected in the splitless mode.

The quantification was performed by the known FAME concentration of the standard mixture and the 100% method over the peak area [13] (see Table 1).

The *in vitro* antimicrobial activity of the *S. milanii* lypophilic extract was tested against *Candida albicans* (ATCC 10231).

**Evaluation of Antimicrobial Activity**. The disc diffusion method was employed for the determination of antimicrobial activity of the samples [15]. Briefly, a suspension of the tested microorganisms (0.1 mL of $10^8$ cells per mL) was spread on the solid media plates. Trypton soy agar (TSA-Torlak, Belgrade) was used as the nutritive media for growing *C. albicans*. Agar plates were prepared in 90 mm Petri dishes with 22 mL of agar, giving a final depth of 4 mm. Sterile filter paper disks ("Antibiotica Test Blattchen", Schleicher and Schuell, Dassel, Germany, 6.0 mm in diameter) were impregnated with 50 μL of sample solutions of DMSO (1 mg per 1 mL of solvent) and placed on inoculated plates. These plates, after standing at 4°C for 2 h, were incubated at 30°C for 48 h. The disk imbued with 50 μL of DMSO was used as a negative control. The diameters of the inhibition zones were measured in millimeters using a "Fisher-Lilly antibiotic zone reader" (Fisher Scientific Co. USA). Each test was performed in triplicate and repeated three times and the results analyzed for statistical significance. Mean values were selected.

**ACKNOWLEDGMENT**

The authors from Serbia acknowledge the Ministry of Science and Environment Protection of the Republic of Serbia for financial support (Project 142054B). We thank the Alexander von Humboldt Foundation, Bonn, Germany, for supporting the work in this paper through a fellowship to A. Smelcerovic.

**REFERENCES**

1. N. Radulovic, J. Lazarevic, G. Stojanovic, and R. Palic, *Biochem. Syst. Ecol.*, **34**, 341 (2006).
2. P. D. Marin, V. Sajdl, S. Kapor, B. Tatic, B. Petkovic, and S. Duletic, *Biochem. Syst. Ecol.*, **20**, 389 (1992).
3. A. S. Akramova, A. U. Umarov, and A. L. Markman, *Khim. Prir. Soedin*., 244 (1968).
4. D. T. Asibekova, S. D. Gusakova, and A. U. Umarov, *Khim. Prir. Soedin*., 561 (1983).
5. K. Aitzetmuller, N. Tsevegsuren, and K. Vosmann, *Fett/Lipid.*, **99**, 74 (1997).
6. M. B. Pearl, R. Kleiman, and F. R. Earle, *Lipids*, **8,** 627 (1973).
7. G. F. Spencer, R. Kleiman, F. Earle, and I. Wolff, *Lipids*, **5,** 285 (1970).
8. A. Ertan, N. Azcan, B. Demirci, and K. H. C. Baser, *Khim. Prir. Soedin.,* 301 (2002).
9. V. Dembitsky and T. Rezanka, *Biochem. Syst. Ecol.*, **23,** 71 (1995).
10. X. C. Li, M. Jacob, H. El Sohly, D. Nagle, T. Smillie, L. Walker, and A. Clark, *J. Nat. Prod.*, **66,** 1132 (2003).
11. K. Kanazawa and M. Natake, *Agric. Biol. Chem.*, **50**, 115 (1986).
12. I. S. Kim and W. Grosch, *J. Agric. Food Chem*., **29**, 1220 (1981).
13. DFG-Einheitsmethoden, *Gaschromatographie: Analyse der Fettsauren und Fettsaureverteilung. In: Deutsche Einheitsmethoden zur Untersuchung von Fetten, Fettprodukten, Tensiden und Verwandten Stoffen.* Wiss. Verl.-Ges., Stuttgart. C-VI 10a (00) (2000).
14. NCCLS (National Committee for Clinical Laboratory Standards) *Performance Standards for Antimicrobial Disk Susceptibility Testing,* 6th International Supplement, Wayne Pa. M100-S9 (1997).
15. R. Bhattacharjee, *Not. Roy. Bot. Gard. Edinb*., **38**, 65 (1980).