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Steroids from poison hemlock (*Conium maculatum* L.): a GC–MS analysis

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Abstract: The steroid content of *Conium maculatum* L. (poison hemlock), Apiaceae, a well-known weed plant species, was studied herein for the first time. This was achieved by detailed GC–MS analyses of twenty two samples (dichloromethane extracts of different plant organs of *C. maculatum* at three or four different stages of phenological development, collected from three locations). In total, twenty four different steroids were identified. Six steroids had an ergostane nucleus while the other ones possessed a stigmastane carbon framework. The identity of these compounds was determined by spectral means (MS fragmentation), GC co-injections with authentic standards and chemical transformation (silylation). Steroid compounds were noted to be the main chemical constituents of root extracts (up to 70 %) of this plant species in the last phase of development. The predominant ones were stigmasta-5,22-dien-3β-ol (stigmasterol) and stigmast-5-en-3β-ol (β-sitosterol). In an attempt to classify the samples, principal component analysis (PCA) and agglomerative hierarchical clustering (AHC) were performed using steroid percentages as variables.

Keywords: *Conium maculatum* L.; Apiaceae; GC–MS analyses; steroids; stigmasterol; β-sitosterol.

INTRODUCTION

Poison hemlock, *Conium maculatum* L. (Apiaceae), is an herbaceous weed of European origin found throughout many parts of the world. Every part of this plant, especially the fresh leaves and fruits, contains a volatile, oily alkaloid, which is so poisonous that a few drops prove fatal to small animals.[1] As a medicine, hemlock is a sedative and antispasmodic, and in sufficient doses acts as a paralyser to the centers of motion. Greek and Arabian physicians were in the practice of using it to cure indolent tumors, relieve swellings and pains of the

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joints, as well as for affections of the skin.[1] Recently, the chemical composition of the leaf and flower essential oils of *C. maculatum* from Serbia as well as the essential oil of Iranian hemlock were published.[2,3] Birkett reported only the major volatile antifeedant constituents from *C. maculatum*.[4] There was a great deal of work performed on the biologically active compounds, such as alkaloids, flavonoids, coumarins, polyacetylenes, vitamins and non-volatile oils, of *C. maculatum*[1,5,6] but there have been no reports on the steroid content of this plant species. During a routine GC–MS screening of Apiaceae taxa from Serbia, steroid compounds were found to be present in high percentages in the dichloromethane extracts of *C. maculatum*. Hence, it was decided to study the steroid profile of hemlock in more detail: 1) to identify the major and minor steroid constituents, 2) to ascertain the sequestration of these compounds (*i.e.*, to determine their plant part distribution pattern) and 3) to track changes in this profile during different phenophases.

EXPERIMENTAL

Plant material

Plant material (roots, preanthesis aerial parts and in full anthesis, and umbels with ripe schizocarps) was collected from three different locations (Crveni krst, Ledena stena and Palilula) in the city Niš (SE Serbia), from March to August 2009 (three or four collections per location). In total 22 samples were subjected to analyses. The plants were identified by Niko Radulović and voucher specimens (numbers from 200905 to 200916) were deposited at the Herbarium of the Faculty of Science and Mathematics, Niš.

Extraction of steroids

The mentioned above fresh plant samples were macerated with an appropriate volume of freshly distilled dichloromethane (50 ml per 10 g of plant material) at room temperature in the dark for one month. The obtained extracts were gravity filtered through small columns packed with 1 g of Celite® (Merck, Germany) in order to remove all insoluble material.

The extracts were dried over anhydrous magnesium sulfate (Aldrich, USA). The solvent was evaporated under a gentle stream of nitrogen at room temperature, in order to exclude any loss of extract volatiles, and analyzed immediately upon isolation. In order to determine the extract yields, the solvent was removed completely by exposing the residues obtained after removal of the bulk of the CH_2Cl_2 under a stream of nitrogen to *vacuum* at room temperature for a short period to eliminate the solvent completely. The pure extracts were then measured on an analytical balance and multiple gravimetric measurements were taken during 24 h to ensure that all of the solvent had evaporated.

Silylation of the extracts

Typical procedure:[7] trimethylchlorosilane (trimethylsilyl chloride, TMSCl, 0.2 mmol) was added with stirring to a mixture of the dry extract (*ca.* 250 mg), dry triethylamine (0.3 mmol) and dimethyl sulfoxide (0.02 mmol) in dry diethyl ether (10 ml). Temperature of the mixture was kept at 10 °C by occasional cooling. After one hour, the reaction mixture was poured into ice-water (10 ml). After washing the ethereal solution with water, the extract was dried over $MgSO_4$ and evaporated *in vacuo*. The obtained residue was completely dissolved in dry diethyl ether (final concentration 10 mg ml^{-1}) and subjected to GC–MS analysis as described below.

Gas chromatography–mass spectrometry (GC–MS) and gas chromatography (GC)

The GC–MS analyses (three repetitions of each sample) were performed using a Hewlett-Packard 6890N gas chromatograph equipped with a fused silica capillary column DB-5MS (5 % phenylmethylsiloxane, 30 m×0.25 mm, film thickness 0.25 μm, Agilent Technologies, USA) and coupled with a 5975B mass selective detector from the same company. The injector and interface were operated at 250 and 320 °C, respectively. The oven temperature was raised from 70 to 315 °C at a heating rate of 5 °C min^{-1} and then held isothermally for 10 min. Helium at a flow rate of 1.0 ml min^{-1} was used as the carrier gas. The samples, 1 μl of the solutions prepared as mentioned above, were injected in a pulsed split mode (the flow was 1.5 ml min^{-1} for the first 0.5 min and then set to 1.0 ml min^{-1} throughout the remainder of the analysis; split ratio 40:1). The MS conditions were as follows: ionization voltage 70 eV, acquisition mass range 35–500 and scan time 0.32 s. The extract constituents were identified by comparison of their linear retention indices (relative to C_7–C_{37} alkanes on the DB-5MS column)[8] with literature values[9-11] and their mass spectra with those of authentic standards (ergost-5-en-3β-ol, stigmasta-5,22-dien-3β-ol, stigmast-5-en-3β-ol, purchased from Sigma-Aldrich, USA), as well as those from the Wiley 6, NIST02, MassFinder 2.3 and a self-made MS library with the spectra corresponding to pure substances and literature data.[12-14] GC (FID) analysis was performed under the same experimental conditions using the same column as described for the GC–MS. The percentage composition of the extracts was computed from the GC peak areas without any corrections**.**

Data analysis

Principal component analysis (PCA) and agglomerative hierarchical clustering (AHC) were performed using the Excel program plug-in XLSTAT version 2009.4.05. Both methods were applied utilizing the original variables (GC (FID) percentages of the identified extract constituents). The AHC was determined using the Pearson dissimilarity (as aggregation criterion: simple linkage, unweighted pair-group average and complete linkage) and Euclidean distance (aggregation criterion: weighted pair-group average, unweighted pair-group average and the Ward method). The definition of the groups was based on Pearson correlation, using complete linkage and the unweighted pair-group average method.

RESULTS AND DISCUSSION

The chemical composition of CH_2Cl_2 extracts (yields ranged from 0.05 to 2.70 % (w/w) based on fresh plant material weight) of different plant organs of *C. maculatum* was investigated (by GC–MS) at different stages of phenological development, from three locations (22 samples in total). The total ion chromatograms of the CH_2Cl_2 extracts were primarily screened for the presence of steroids (mass spectral comparison with Wiley and NIST data bases). The extracts were additionally submitted to a derivatization procedure (trimethylsilyl chloride) to verify the identification of steroid alcohols through the shift in their RI values. Moreover, the trimethylsilyl ethers yielded informative mass spectra, and, in certain instances, they afforded highly characteristic modes of fragmentation from which structural details were inferred. The identity of the compounds was corroborated whenever possible by GC co-injections of authentic samples. Thus, the identities of the steroids were established by at least three (at best four) independent means.

In three cases, the identification was performed in a less straightforward way and these will be given in more detail. Root samples, after anthesis, contained four different 3,6-diones. Two of them closely eluting were identified as: stigmasta-4,22-diene-3,6-dione (**1**) and stigmast-4-ene-3,6-dione (**2**). Compound **2** possessing an M^+ at *m*/*z* 426 in its MS was identified by mass spectral comparison using in-house MS libraries and with literature data.[12] However compound **1**, having a very similar MS fragmentation pattern and an additional unsaturation, as inferred from its M^+ (*m*/*z* 424), gave no match in the library search. The difference in the amu values of the fragments between compounds **1** and **2** was lost when the *m*/*z* value dropped below the intense *m*/*z* 285 fragment ion that in compound **2** corresponds to a cation formed by the cleavage of the C-17 side-chain (SC). This suggested that the additional double bond (unsaturation) is situated in the side chain of the stigmast-4-ene-3,6-dione framework and that compounds **1** and **2** have identical nuclei (M^+–SC, *m*/*z* 285). The main MS fragmentation pathways for compounds **1** and **2** are shown in Fig. 1. The position between C-22 and 23 of the unsaturation was initially deduced from the ion $(M–112)^+$.[13] Its position was further ascertained according to two additional facts: 1) the retention properties of closely related 5α-stigmasta-3,6-dione and 5α-stigmast-22-ene-3,6--dione (the other two 3,6-diones in the root extracts, identified by MS comparison with literature data)[13] show the same RI increment as the one for **2** and **1**; 2) the difference in the MS caused by the introduction of the double bond within the mentioned analogous pair is the same as the one observed in the case of compounds **1** and **2**.

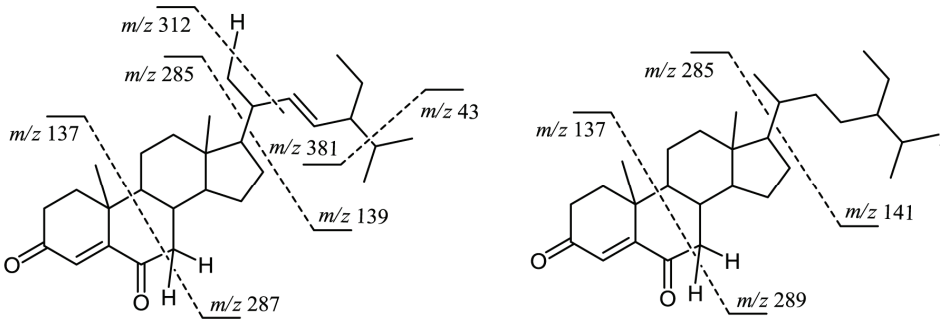


Fig. 1. The main MS fragmentation pathways for stigmasta-4,22-diene-3,6-dione (**1**) and stigmast-4-ene-3,6-dione (**2**).

A similar discussion stands for the identification of 5α-stigmast-22-en-3β-ol (**3**), detected in II (4r) (1.5 %) and III (4s) (0.8 %). 5α-Ergost-22-en-3β-ol possesses an analogous and almost identical MS (NIST07) to that of compound **3** differing in the mass increment of 14 amu and this difference is readily obser-

vable in the parent ion at *m*/*z* 414 of the homologous **3**. Stigmasta-4,6-dien-3-one was identified in the same way, by comparing its mass spectrum with that of cholesta-4,6-dien-3-one. The molecular ion of cholesta-4,6-dien-3-one was at *m*/*z* 382 while the molecular ion for the "unknown" compound was at *m*/*z* 410. Subtraction of these molecular ions gives a difference of 28 amu, suggesting that they are members of a homologous series, two methylene units apart.

Silylation provided additional evidence for the proposed structures of other identified compounds (Table I). A non-allylic 3-hydroxy-Δ^5-steroid structure of three detected steroids (ergost-5-en-3β-ol, stigmasta-5,22-dien-3β-ol, stigmast-5--en-3β-ol, Table I) was readily confirmed by their conversion to ethers (sample II (4r) was subjected to TMSCl/Et_3N due to the high content of sterols and the presence of a large number of different components of interest). The trimethylsilyl ether strongly promoted the fragmentations to yield characteristic complementary pair of ions at *m*/*z* 129 and $[M–129]^+$.[15] This allowed the discernment of Δ^5-steroids from other unsaturated 3-ol steroids. Additionally, the silylation permitted the positioning of the double bonds through the retro-Diels–Alder reaction on ring B (together with the corresponding loss of H_2O or TMSOH), which gives *m*/*z* 95 (in both, the free alcohol and TMS ether) for 5α-stigmast-7-en-3β-ol and *m*/*z* 119 for Δ^5-steroids. In general all steroids and their TMS derivatives had the following feature: a prominent ion at *m*/*z* 255 that is ascribed to a fragment derived by loss of molecules of water plus the side-chain in the alcohols or the side--chain plus $HOSiMe_3$ in the ethers.[16]

TABLE I. Identified steroids of dichloromethane extracts of *C. maculatum*; *RI* – retention indices on a DB-5 column relative to C_{31}–C_{37} *n*-alkanes;[8] the experimental values were in good agreement with the literature ones;[9–11] TMS – sterols converted to trimethylsilyl ethers

Steroids	Designation	*RI*	*RI* (Lit.)	*RI* (TMS)	*RI* (Lit.) (TMS)	*m*/*z* (M^+)
Ergosta-5,7,9(11),22-tetraen-3β-ol	E1	3150	–	–	31619	394
Ergosta-5,8,22-trien-3β-ol	E2	3158	3113±5[10]	–	–	396
Ergosta-5,7,22-trien-3β-ol	E3	3187	3152±6[10]	–	3232±2[10]	396
5α-Ergosta-7,22-dien-3β-ol	E4	3202	–	–	–	398
Ergost-5-en-3β-ol[a]	E5	3211	–	3231	3249[11]	400
Stigmasta-5,22-dien-3β-ol[a]	S1	3244	3248[9,10]	3262	3274[9,11]	412
5α-Stigmast-22-en-3β-ol	S2	3253	–	–	–	414
5α-Stigmasta-7,22-dien-3β-ol	S3	3295	3295[9]	–	–	412
Stigmast-5-en-3β-ol[a]	S4	3311	3290±10[10]	3329	3342±4[10]	414
5α-Stigmastan-3β-ol[a]	S5	3325	3317[9]	3343	3349[9]	416
Ergost-4-en-3-one	E6	3354	–	–	–	398
5α-Stigmastan-3-one	S6	3370	–	–	–	414
5α-Stigmast-7-en-3β-ol[a]	S7	3382	–	3401	3404[9]	414
5α-Stigmasta-7,22-dien-3-one	S8	3393	–	–	–	410
Stigmasta-4,22-dien-3-one[a]	S9	3399	–	–	–	410
5α-Stigmasta-7,16-dien-3β-ol[a]	S10	3401	–	–	–	412

TABLE I. Continued

Steroids	Designation	*RI*	*RI* (Lit.)	*RI* (TMS)	*RI* (Lit.) (TMS)	*m/z*(M^+)
5α-Stigmast-7-en-3-one	S11	3420	–	–	–	412
Stigmast-4-en-3-one	S12	3458	3435±4[10]	–	–	412
Stigmasta-4,6-dien-3-one[a]	S13	3518	–	–	–	410
Stigmasta-4,22-diene-3,6-dione	S14	3538	–	–	–	424
Stigmast-4-ene-3,6-dione[a]	S15	3541	–	–	–	426
5α-Stigmasta-22-ene-3,6-dione[a]	S16	3598	–	–	–	426
5α-Stigmastane-3,6-dione[a]	S17	3601	–	–	–	428
3β-Hydroxystigmast-5-en-7-one[a]	S18	3609	–	–	–	428

[a]EIMS, 70 eV, *m/z* (rel. int. %): S10 – 314 (20), 271 (100), 131 (35), 121 (28), 107 (75), 95 (50), 91 (47), 55 (46); S7 – 414 (100), 255 (95), 107 (52), 105 (45), 95 (33), 91 (37), 55 (48), 43 (69); TMS ether of S7 – 486 (32), 471 (18), 381 (10), 255 (60), 229 (18), 213 (14), 147 (10); S13 – 410 (84), 174 (45), 161 (48), 160 (57), 136 (100), 95 (49), 57 (50), 43 (76); S18 – 428 (100), 395 (29), 287 (37), 205 (14), 187 (35), 161 (36), 135 (34), 43 (52); mass spectra of S9, S15, S16 and S17 were identical to those reported previously;[12,13] mass spectra of trimethylsilyl ether derivatives of S5, S4, S1 and E5 were identical to those reported in a previous study[14]

The steroids (Table I) accounted for 5.3–68.3 % of the total extracts (area percentage of the GC chromatograms, Tables II and III). In total, twenty four different steroids were identified in the investigated samples (Tables I–III; out of 22, only one sample did not contain any detectable amount of steroid compounds, II (1a)). The structures of the identified steroids are presented in Fig. 2.

Steroid compounds were noted to be the main chemical constituents of the root extracts (Table II) of this plant species in the late summer when the plant was almost dry (the highest amount of sterols per gram of fresh plant material was 61.0–68.3 %). Only six steroids had an ergostane nucleus (Table I) while the other ones possessed a stigmastane carbon framework. The predominant were stigmasta-5,22-dien-3β-ol (stigmasterol, 14.4–15.5%) and stigmast-5-en-3β-ol (β-sitosterol, 12.5–18.9 %), the former one is the most common phytosterol in terrestrial plants. The two sterols were most abundant in the last phase of development and were present in all investigated samples of *C. maculatum*. Together with β-sitosterol and stigmasterol, other minor identified steroids possessed a C-3-oxygenation pattern. The occurrence of C-3-oxygenated steroids was previously reported in higher plants from very diverse plant families. The following *C. maculatum* steroids can serve as examples: 5α-stigmast-22-en-3β-ol (*Kirganelia reticulate* – Euphorbiaceae, *Allamanda cathartica* – Apocynaceae),[17,18] 5α-stigmasta-7,16-dien-3β-ol (found only in *Clinopodium vulgare* – Lamiaceae),[19] 5α-stigmasta-7,22-dien-3-one (*Gustavia augusta* – Lecythidaceae, *Clinopodium umbrosum* – Lamiaceae),[20,21] 5α-stigmasta-7,22-dien-3β-ol (*Camellia japonica* – Theaceae, *Bupleurum aureum* – Apiaceae)[22,23] and 5α-stigmast-7-en-3β-ol (*Cucurbita pepo* – Cucurbitaceae, *Tricholepis glaberrima* – Asteraceae).[24,25] Stigmast-4-ene-3,6-dione was often found to be present with other 4-en-3-ones or 3,6-diones (5α-stigmastane-3,6-dione, stigmast-4-en-3-one, stigmasta-4,22-dien-3-one

TABLE II. Percentage of steroids ± standard error from the GC (FID) chromatograms and yields (%, w/w) of dichloromethane extracts of roots of *C. maculatum* at three/four different stages of phenological development, from three locations; I, II, III – three collection locations (Crveni krst, Ledena stena, Palilula, respectively); 1, 2, 3, 4 – different stages of development (preanthesis – 1,2, anthesis – 3, ripe fruits – 4); r – roots; tr – trace amount (<0.05 %)

Steroid[a]	I (1r)	I (2r)	I (3r)	I (4r)	II (1r)	II (2r)	II (3r)	II (4r)	III (2r)	III (3r)	III (4r)
E1[b]	–	–	–	0.3±0.02	–	–	–	0.5±0.04	–	–	0.3±0.01
E2	–	–	–	0.4±0.03	–	–	–	0.5±0.03	–	–	0.3±0.02
E3	–	–	–	6.5±0.35	–	–	–	6.0±0.33	–	–	5.3±0.29
E4	–	–	–	–	–	–	–	0.3±0.01	–	–	0.2±0.01
E5	0.1±0.04	–	0.2±0.01	1.0±0.15	–	0.2±0.01	0.2±0.01	1.4±0.23	–	0.1±0.01	1.0±0.15
S1	3.0±0.21	5.0±0.27	5.0±0.29	15.2±0.93	1.8±0.23	4.6±0.67	4.9±0.23	14.4±0.89	1.1±0.13	3.1±0.26	15.5±0.89
S2	–	–	–	–	–	–	–	1.5±0.19	–	–	–
S3	–	–	–	–	–	–	–	0.1±0.01	–	–	1.0±0.17
S4	2.0±0.19	4.7±0.24	4.1±0.17	12.5±0.85	1.0±0.11	3.5±0.27	4.8±0.20	18.9±0.97	0.7±0.03	2.1±0.16	13.6±0.76
S5	–	–	0.4±0.02	1.9±0.28	–	–	0.5±0.04	2.5±0.14	–	–	–
E6	–	–	–	1.0±0.13	–	–	–	–	–	–	–
S6	–	–	–	1.4±0.19	–	–	–	1.3±0.15	–	–	1.1±0.15
S7	0.2±0.01	0.4±0.06	0.2±0.01	0.9±0.04	–	0.3±0.04	0.2±0.01	1.7±0.18	–	–	1.4±0.23
S8	–	–	–	–	–	–	–	4.9±0.31	–	–	–
S9	–	–	0.2±0.01	9.0±0.54	–	0.1±0.01	0.2±0.01	–	–	–	9.6±0.61
S11	–	–	–	–	–	–	–	0.8±0.03	–	–	1.0±0.12
S12	–	0.3±0.02	0.4±0.02	8.5±0.41	–	0.2±0.02	0.4±0.03	9.7±0.42	–	0.1±0.01	10.1±0.69
S13	–	–	–	0.8±0.23	–	–	–	0.8±0.02	–	–	0.6±0.04
S14	–	–	–	0.6±0.04	–	–	–	0.4±0.02	–	–	tr
S15	–	–	–	1.2±0.15	–	–	–	0.9±0.05	–	–	tr
S16	–	–	–	0.2±0.01	–	–	–	0.6±0.02	–	–	tr
S17	–	–	–	0.3±0.02	–	–	–	1.1±0.23	–	–	tr
S18	–	–	–	0.1±0.01	–	–	–	–	–	–	–
Total	5.1–5.5	10.1–10.5	10.2–10.7	61.5–61.9	2.3–2.7	8.6–8.9	11.0–11.3	68.1–68.4	1.4–1.9	5.3–5.7	59.9–60.2
Yield	0.19	0.17	0.40	0.44	0.21	0.18	0.22	0.40	0.16	0.30	0.35

[a]Compounds listed in order of elution from a DB-5 column; [b]for steroid designation see Table I

TABLE III. Percentage of steroids ± standard error from the GC (FID) chromatograms and yields (%, w/w) of dichloromethane extracts of the aerial parts (a) and fruits (f) of *C. maculatum* at three/four different stages of phenological development, from three locations: I, II, III – three collection locations (Crveni krst, Ledena stena, Palilula, respectively); 1, 2, 3, 4 – different stages of development (preanthesis – 1,2, anthesis – 3, ripe fruits – 4); a - aerial parts; f – fruits; tr – trace amount (<0.05 %)

Steroid[a]	I (1a)	I (2a)	I (3a)	I (4f)	II (2a)	II (3a)	II (4f)	III (2a)	III (3a)	III (4f)
E2[b]	–	–	–	–	–	–	0.2±0.02	–	–	0.4±0.02
E3	–	–	–	1.4±0.16	–	–	2.4±0.27	–	–	2.4±0.29
E5	–	–	0.1±0.01	–	–	–	0.4±0.03	–	0.2±0.01	0.5±0.04
S1	3.3±0.28	4.2±0.36	3.2±0.29	2.3±0.22	5.1±0.37	3.4±0.28	4.5±0.32	1.2±0.11	2.2±0.24	4.0±0.37
S2	–	–	–	–	–	–	–	–	–	0.8±0.07
S3	tr	2.1±0.16	0.9±0.07	–	tr	–	–	–	tr	tr
S4	3.0±0.31	3.0±0.24	1.8±0.19	1.6±0.17	4.1±0.32	2.1±0.25	2.9±0.31	0.8±0.09	2.3±0.20	2.4±0.18
S5	–	–	–	0.3±0.02	–	–	0.8±0.04	–	–	–
S7	1.3±0.11	2.8±0.28	1.2±0.09	0.6±0.04	1.5±0.13	0.8±0.09	1.1±0.12	–	1.5±0.14	1.2±0.11
S9	–	–	–	–	–	–	0.3±0.01	–	–	0.3±0.02
S10	–	0.8±0.05	0.2±0.01	–	–	–	–	–	0.3±0.02	0.2±0.01
S12	–	–	–	–	–	–	–	–	–	0.4±0.02
Total	7.3–7.8	12.7–13.0	7.1–7.6	6.0–6.4	10.5–10.8	6.2–6.5	12.4–12.7	1.9–2.2	6.2–6.6	12.2–12.7
Yield	0.16	0.05	0.31%	2.70%	0.05%	0.16%	1.70%	0.17%	0.60%	1.45%

[a]Compounds listed in order of elution from a DB-5 column; [b]for steroid designation see Table I

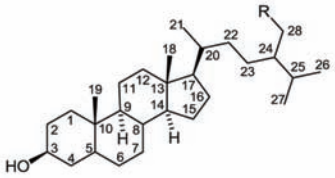
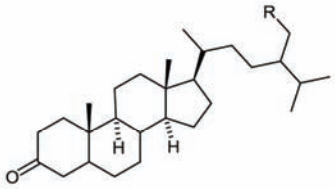
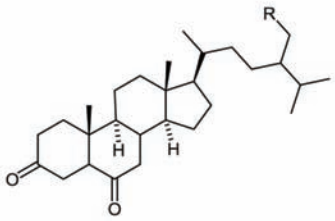
Steroid structure	Designation	Δ	R
	E1	5,7,9(11),22	H
	E2	5,8,22	H
	E3	5,7,22	H
	E4	7,22	H
	E5	5	H
	S1	5,22	CH_3
	S2	22	CH_3
	S3	7,22	CH_3
	S4	5	CH_3
	S5	/	CH_3
	S7	7	CH_3
	S10	7,16	CH_3
	S18*	5	CH_3
	E6	4	H
	S6	/	CH_3
	S8	7,22	CH_3
	S9	4,22	CH_3
	S11	7	CH_3
	S12	4	CH_3
	S13	4,6	CH_3
	S14	4,22	CH_3
	S15	4	CH_3
	S16	22	CH_3
	S17	/	CH_3

Fig. 2. Structures of the identified steroids from *C. maculatum* (*steroid S18 (3β-hydroxy-stigmast-5-en-7-one) has an additional carbonyl group at C-7).

or/and stigmasta-4,22-diene-3,6-dione), for example in *Aristolochia triangularis*, *Aristolochia tubiflora* (Aristolochiaceae), *Polygonum chinensis* (Polygonaceae) and *Ptychopetalum olacoides* (Olacaceae).[26–29] These are interesting due to the noted anti-inflammatory and anti-allergic properties for these sterols (from *P. chinensis*).[28] Stigmasta-4,22-diene-3,6-dione, an allelochemical substance, found also in *Pistia stratiotes* (Araceae) was established to inhibit the growth of some microalgae.[30] Previously, 5α-stigmast-7-en-3-one was reported only from three plant species: *Gypsophila trichotoma* (Caryophyllaceae),[31] *Centratherum anthelminticum* (Asteraceae)[32] and *Coccinia indica* (Cucurbitaceae),[33] while stigmasta--4,6-dien-3-one was only found to occur naturally in *Senecio crassiflorus* (Asteraceae)[34] and *Prosopis alpataco* (Fabaceae).[35] Several of the steroids listed in Table I are quite rare plant constituents. Stigmast-22-ene-3,6-dione, detected only

in the last stages of development from all three locations, was previously reported only for the stem extracts of *Phoenix dactylifera* (Arecaceae).[36] 3β-Hydroxystigmast-5-en-7-one was detected only in the sample I (4r). Previously, it was reported in *Oryza sativa* (Poaceae).[37]

In order to try to classify the samples, principal component analysis (PCA) and agglomerative hierarchical clustering (AHC) were performed with the GC (FID) percentages of the steroid compounds as variables. The dendrogram obtained as the result of AHC is depicted in Figs. 3 and 4. The AHC analysis performed using the identified extract constituents indicated five groups (classes) of extracts under study.

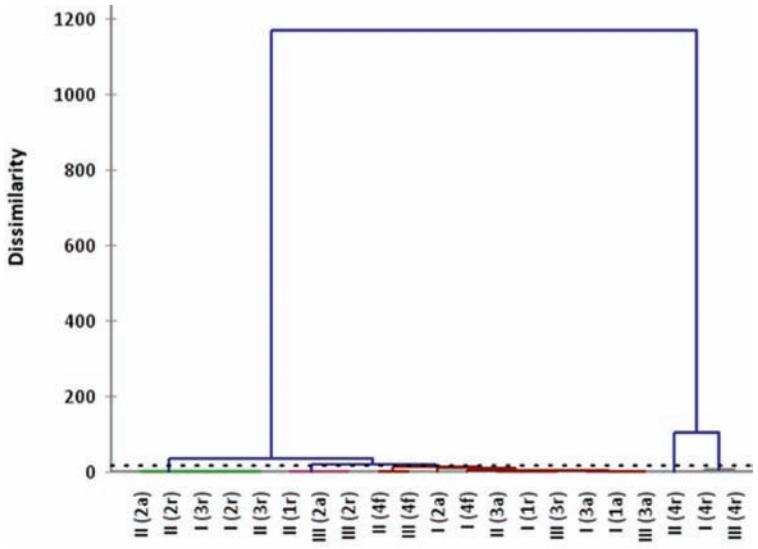


Fig. 3. Dendrogram (AHC analysis) obtained using the percentage of the identified steroid constituents (root and aerial parts extracts) as the original variables obtained by Euclidean distance dissimilarity (dissimilarity within the interval [0,1200]), using the aggregation criterion – the Ward method.

Generally, all samples were statistically very similar (visible from the low dissimilarity values). AHC analysis grouped the root extracts of *C. maculatum* (the last stage of phenological development) from locations I and III together in the dendrogram. They were also closely related to the sample 4r from location II but it formed a separate class. The presence of numerous minor steroids was a mutual feature of samples I, III and II (4r), but they differed in the content of stigmasta-7,22-dien-3-one and stigmasta-4,22-dien-3-one (Table II). The samples II (1r), III (2a) and III (2r) were placed within the same class due to the fact that they contained only two sterols (stigmasterol and β-sitosterol) in lower relative amount (0.7–1.8 %) compared to the rest of the samples. Assuming that the in-

tensity of steroid biosynthesis follows the phenological development, the plants from location III seem to have matured slower than those from location II. Since all of the plants eventually reached the same level of steroid production in the last phase, this could be attributed to a whole array of environmental factors (temperature, illumination period variations, *etc*. for the different locations).

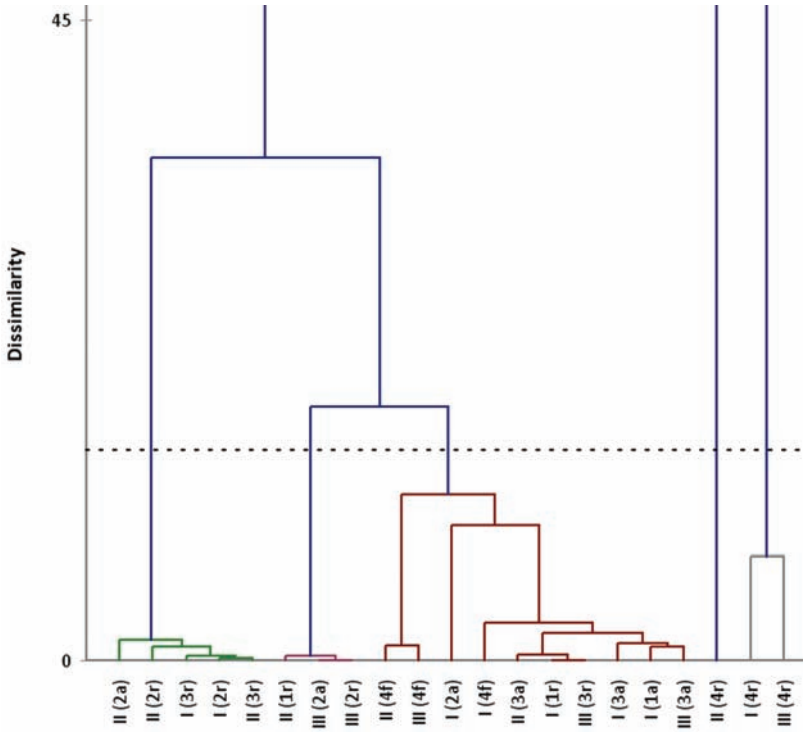


Fig. 4. Enlargement of a part (dissimilarity interval [0,45]) of Fig. 3..

The PCA analysis utilizing the original variables (relative content of the identified compounds) revealed a number of strong dependences between the extract constituents. High coefficients were noted between certain steroids and their dehydro derivatives: ergosta-5,7,9(11),22-tetraen-3β-ol and ergosta-5,7,22-trien--3β-ol (0.901); stigmasta-5,22-dien-3β-ol and stigmast-5-en-3β-ol (0.958); 5α--stigmastan-3-one and stigmast-4-en-3-one (0.983); stigmast-4-en-3-one and stigmasta-4,6-dien-3-one (0.979); 5α-stigmast-22-en-3,6-dione and 5α-stigmastane--3,6-dione (0.998); stigmasta-4,22-diene-3,6-dione and stigmast-4-ene-3,6-dione (0.998), possibly because the biosynthesis of these steroids is mutually interdependent (*i.e*., substrates of the same dehydrogenase).

CONCLUSIONS

Steroid compounds were noted to be the main chemical constituents of the root extracts (up to 70 %) of *C. maculatum* in the last stage of phenological development. The predominant were stigmasta-5,22-dien-3β-ol (stigmasterol) and stigmast-5-en-3β-ol (β-sitosterol). The identified steroid compounds (and their possible biological/pharmacological properties) contribute to knowledge on *C. maculatum* and add interest to this plant species. These results demonstrate the pronounced variability of the steroid constituents found in *C. maculatum* and stresses the importance of investigations dealing with the chemistry of separate plant organs and different plant harvesting periods and locations.

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И З В О Д

СТЕРОИДИ ИЗ КУКУТЕ (*Conium maculatum* L.): GC–MS АНАЛИЗА

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У овом раду су изнети резултати испитивања стероида биљне врсте *Conium maculatum* L. (кукута), из породице Apiaceae, добро познате отровне коровске биљке. Урађене су детаљне GC–MS анализе двадесет и два узорка (дихлорметански екстракти биљних органа врсте *C. maculatum*; материјал је сакупљан са три локације, у току три или четири фенофазе). Двадесет и четири стероида су идентификована, од којих шест има ергостанско језгро, док остали поседују стигмастански скелет. Идентификација ових једињења је вршена на основу њихове MS фрагментације, као и GC коинјекцијом чистих једињења и хемијском трансформацијом (силиловањем). Уочено је да су стероиди главни састојци екстраката корена (до 70 %) ове биљне врсте у последњој фази фенолошког развића. Најзаступљенији су били стигмаста-5,22-диен-3β-ол (стигмастерол) и стигмаст-5-ен-3β-ол (β-ситостерол). Да бисмо покушали да класификујемо наше узорке, урађена је статистичка обрада добијених резултата коришћењем методе анализе главне компоненте (PCA) и агломеративне хијерархијске кластер анализе (AHC), са релативним садржајем стероида као варијаблама.

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