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FLAVONOID COMPOSITION OF *Equisetum arvense* AND *E. x litorale* STUDIED BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY

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6-Chloroapigenin was isolated from the ether-soluble part of the MeOH extract from *Equisetum arvense* L. in the 1980s by Syrchina et al. [1]. This became the first instance of the discovery of such compounds in higher plants. The structure of the isolated compound was elucidated using chromatographic and spectral characteristics (EI-MS, UV, PMR) and qualitative reactions [1].

We performed a comprehensive chemical and pharmacological investigation of *Equisetum* spp. under the auspices of a program to study Siberian medicinal plants and to create new drugs based on them. We studied the aerial part of 10 *Equisetum* species (*E. arvense* L., *E. sylvaticum* L., *E. pratense* Ehrh., *E. palustre* L., *E. fluviatile* L., *E. x litorale* Kuehlew, *E. hyemale* L., *E. scirpoides* Michx., *E. variegatum* Schleichee ex F. Weber & D. Mohr., and *E. ramosissimum* Desf.).

Total polyphenolic compounds were obtained from ground air-dried raw material (100 g) by exhaustive extraction with EtOH (80%, 2×) for 60 min with refluxing on a water bath and a 1:5 raw material:extractant ratio. EtOH was vacuum distilled at 50°C from the combined extract. The resulting aqueous residue was worked up successively with $CHCl_3$, EtOAc, and *n*-BuOH. The resulting total fractions were used to isolate pure compounds and to perform high-performance liquid chromatography–mass spectrometry (HPLC–MS).

The EtOAc fractions of the EtOH extract obtained from *E. arvense* and *E. x litorale* were chromatographed over columns of silica gel (Chemapol, L 40/100 μm) using benzene:acetone (100:0→90:10), $CHCl_3$:acetone (100:0→70:30), and hexane:$CHCl_3$ (100:0→50:50). Subfractions were separated over columns of Woelm polyamide (Germany) (EtOH:H_2O, 0:100→95:5, acetone) with subsequent preparative TLC on Merck KGaA Kieselgel 60 F_{254} plates (Germany) using toluene:EtOAc:HCOOH (5:4:1), EtOAc:MeOH:H_2O (9.6:1.9:1), $CHCl_3$:acetone:MeOH (16:4:1), and $CHCl_3$:acetone (4:1). Chromatographic separation of the EtOAc fraction isolated nine compounds labeled **1** (12 mg), **2** (15), **3** (3), **4** (4), **5** (5), **6** (8), **7** (1), **8** (0.8), and **9** (10).

Compounds **1**–**6** and **9** were identified as kaempferol-3-glucoside (**1**), genkwanin-5-glucoside (**2**), apigenin-5-glucoside (**3**), luteolin-5-glucoside (**4**), kapmpferol-3-sophoroside (**5**), apigenin-4′-glucoside (**6**), and kaempferol-3,7-diglucoside (**9**) and were reported previously in the literature for several *Equisetum* species.

Elution of the columns by the benzene:acetone mixture isolated dichlorokaempferol (**7**) and dichloroapigenin (**8**), which were purified over polyamide columns and recrystallized from MeOH. The chromatography was monitored by TLC using $CHCl_3$:acetone:MeOH (16:4:1) and $CHCl_3$:acetone (4:1). HPLC–MS was used to establish the proposed structures of these compounds.

Retention times, UV and mass spectra of the corresponding peaks, and comparison with standards, known samples, and the literature were the principal criteria used to solve the HPLC and mass spectral data and to choose a proposed structure. The classical studies of Harborne et al. [2, 3] and reviews and original studies of other researchers [4, 5] were consulted in order to identify the flavonoids.

Our results showed the presence in the EtOAc fractions of *E. arvense* and *E. x litorale* of two new and previously unreported dihalogenated flavonoids. This was a very interesting development because halogenated phenolic compounds are known to be encountered very rarely in nature. The majority of known halogenated phenolic compounds were isolated from microorganisms and lower plants. Therefore, the observation of such compounds in *Equisetum* spp. on one hand confirmed their ancient origin and, on the other, was a link between lower and higher plants.

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The UV spectrum of **7** exhibited absorption maxima at 267, 327, and 357 nm that were characteristic of flavonols. The UV spectrum of **8** was similar to those of flavones with absorption maxima at 275 and 340 nm. The mass spectra of these compounds suggested the presence in them of two halogen atoms according to the number and intensity ratios of fragment ions. Mass spectra of the compounds recorded in Neg.Scan. and Pos.Scan. modes exhibited near the molecular ion three fragment ions $[M]^+/[M + 2]^+/[M + 4]^+$ with separations of 2 amu between the components. Thus, in APCI and Pos.Scan. mode, the first compound gave a peak with retention time 8.230 min and *m*/*z* pattern 355/357/359, and fragment-ion intensity ratio 100:67:23; the second compound, a peak with retention time in the range 11.862–11.920 min, *m*/*z* pattern 339/341/343, and fragment-ion intensity ratio 100:63:15. In APCI and Neg.Scan. mode, the fragment-ion intensity ratio for **7**, which had retention time 8.220 min, was 100:72:31; for **8**, with retention time in the range 11.851–11.886 min, 100:54:11. This was characteristic of Cl-containing compounds with two Cl atoms. Obviously the molecular weight of the main component of **7** was 354.0; of **8**, 338.0. Calculations showed that **7** (354 – 70 + 2) = 286 could be kaempferol (*m*/*z* 286.0). With respect to the location of the halogen atoms, it could be proposed that they were located in the 6- and 8-positions of ring A or the 3′- and 5′-positions of ring B.

Our hypotheses were based on literature data for known natural halogenated structures containing up to four halogen atoms. The halogen atoms were always situated in *ortho*- or *para*-positions relative to the phenol hydroxyl. This allowed us to propose that **7** was dichlorokaempferol, the Cl atoms in which could be located either in the 6- and 8-positions of ring A or in the 3′- and 5′-positions of ring B.

The second chloro-substituted compound was apigenin (338 – 70 + 2) = 270, the Cl atoms in which, like in the first compound, could be located in the 6- and 8-positions of ring A or in the 3′- and 5′-positions of ring B. Thus, **8** was presumed to be dichloroapigenin.

Thus, we observed two new and previously unreported Cl-containing compounds, dichloroapigenin and dichlorokaempferol, in the EtOAc fraction of *E. arvense* and *E. x litorale*. These are described for the first time not only from *Equisetum* spp. but also in general from higher plants.

Chromatographic analysis was performed by chemical ionization in a liquid chromatograph with diode-matrix UV and mass selective detectors (Agilent Technologies, 1100 Series LS/MSD). The diode-matrix detector (model G1315B) enabled absorption spectra to be detected and recorded in the UV range 230–500 nm; the mass-selective detector, molecular weights of components to be determined to an accuracy of 0.1 amu. The separation was conducted over a column with reversed-phase Zorbax XDB-C8 Rx-C18 (4.6 × 150 mm) with 5-μm particle diameter and pore size 80 Å using HCOOH (2.3%):MeOH (gradient from 40 to 90% MeOH from 5 to 10 min) at flow rate 1 mL/min and 30°C (thermostatted). The diode-matrix detector performed simultaneous detection at wavelengths 280 and 340 nm with band width 40 nm and control wavelength 560 nm of width 100 nm. The mass-selective detector (model G1946C) was used in chemical-ionization mode at atmospheric pressure (APCI). Negative and positive ions were scanned in the *m*/*z* range from 100 to 1000. Combination of the mass-selective detector in two modes (APCI with Pos.Scan. and APCI with Neg.Scan.) with the UV detector gave the most complete information about the components in the studied plants. The potential on the fragmenter for positive ions was 40 V; for negative, 70 V. The drying gas (N_2) flow rate was 7 L/min at 340°C. The sprayer pressure was 60 psi (lb/in^2). The vaporizer temperature was 400°C.

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