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PRELIMINARY STUDY OF SOIL COMPOSITION FROM BALKAN ENDEMIC NEPHROPATHY AREAS, USING A GC-MS METHOD

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Abstract

Balkan endemic nephropathy (BEN) is a chronic kidney disease occurring predominantly in certain rural villages from Balkan Peninsula, including Romania. Peculiar environmental factors, such as drinking water, organic contaminants derived from coal, plant toxins, mycotoxins, heavy metals have been proposed to be causing the disease. Still, one of these etiological factors stands out: *Aristolochia clematitis L.*, a plant that contains aristolochic acids (AAs) known as carcinogenic and nephrotoxic compounds. These phytotoxins are geochemically stable in BEN areas' soils and could enter the human food chain after being accumulated by crop plants. The aim of the present study was to examine the composition of soil and soil organic matter (SOM) samples from BEN endemic and nonendemic areas, using a simple and rapid microwave methanolic extraction method followed by gas-chromatography coupled with mass spectrometry (GC-MS) analysis. The results indicated similarities across all samples, both from endemic and nonendemic areas. A common compound (aristolone) was detected in samples where *Aristolochia* plants were present nearby the collecting area or collected simultaneously with the soils, therefore we can assume that this compound was transferred from plant parts into the soil.

Key words: aristolone, Balkan endemic nephropathy, gas chromatography-mass spectrometry, soil organic matter

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1. Introduction

The characterization of soil composition is a method of environmental screening, which could identify compounds with possible toxic effects, originating from natural or anthropogenic sources (Shang et al., 2015). The study of soil composition represents a requirement for environmental assessment and a possibility of its effective management (Haleyur et al., 2016). Soil content analysis has become a standard evaluation of pollution level, of plants' compounds pathways into the human food chain (Legind and Trapp, 2011; Baran et al., 2019).

BEN (Balkan endemic nephropathy) is a multifactorial environmentally induced chronic kidney disease, geographically limited to the Balkans; in Romania BEN areas are mainly in small villages within Mehedinti County (Craciun and Rosculescu, 1970; Gluhovschi et al., 2010; Tatu et al., 1998). Sixty years of research have passed and yet its etiology is only partially unknown. BEN environmental

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etiological factors described in the literature data are water, coal, plant alkaloids, mycotoxins, etc. (Craciun and Rosculescu, 1970; Gluhovschi et al., 2010; Stefanović et al., 2006; Stiborová et al., 2016; Tatu et al., 1998). Until recently, soil was never described as possible cofactor (Chan et al., 2016; Gruia et al., 2018; Li et al., 2016).

One of the cofactors involved in the disease, present in both endemic and nonendemic areas is *Aristolochia* sp., a plant with worldwide distribution (Debelle et al., 2008). Regarding the chemical structure, *Aristolochia* species contain many groups of compounds: aristolochic acid derivatives, aporphines, amides, benzylisoquinolines, terpenoids, biphenyl ethers, flavonoids, benzenoids, steroids and others (Wu et al., 2004, 2005). The toxic activity (mostly nephrotoxic) is predominantly attributed to aristolochic acids due to the phenanthrenic nucleus and the nitro functional group (Hranjec et al., 2005; Long and Voice, 2007). Phenanthrene-derived compounds have a significant role in the aristolochic acid derivatives formation: sodium salts, alkyl esters, sesquiterpenes and diterpenes, aristolactams and aristolactones (Wu et al, 2005).

There are multiple routes of human exposure to aristolochic acids, and soil contamination could be a significant one (Hranjec et al., 2005; Long and Voice, 2007). *Aristolochia clematitis L.* plants are particularly found near crop fields (cultivable soils) and gardens, so the *Aristolochia* seeds commingle with culture plants when harvested, as Ivić first suggested in 1969 (Ivić, 1969). Crop field contamination with aristolochic acids and therefore their uptake from soil through the ingested plants could be a way to enter the human food chain, as it was demonstrated in previous studies (Chan et al., 2016; Li et al., 2016; Pavlović et al., 2013).

In Romania, BEN is present in villages from the South-Western part of the country, mostly in Mehedinti County (Craciun and Rosculescu, 1970; Gluhovschi et al., 2010; Tatu et al., 1998); years ago BEN patients were reported in small villages from Caras-Severin County, but during the last few years the disease was not reported to be present in this location anymore. In this study we also included nonendemic villages located outside the BEN areas, either where *Aristolochia* plant is widely spread and the disease was not reported (Plugova, Caras-Severin County) or where the plant has never been encountered and the disease is not present (Timisoara, Timis County).

Our objective was to identify, by a simple and fast extraction and analysis method the organic composition of the first two layers of soil (soil organic matter and soil samples) collected from both BEN endemic and nonendemic areas, with or without the presence of *A. clematitis.* We used a microwave methanolic extraction method followed by a GC-MS analysis method. This is a preliminary new approach, and yet unreported in the literature, for analysis of these types of samples with a BEN and non-BEN provenience.

2. Material and methods

2.1. Reagents

All chemicals and solvents used were of analytical grade and purchased from Sigma-Aldrich.

2.2. Sample collection

A fieldtrip was carried out in May 2016, during a warm and dry period. Both soil (depth of 5-20 cm) and surface soil (also called soil organic matter (SOM), first 5 cm surface layer) with and without *Aristolochia* (A) plant parts were collected. Some soil samples, one from endemic and two from nonendemic areas, were collected from the corn root plants (Table 1). Also voucher specimens of *A. clematitis* L. plants were collected alongside the soil and SOM samples and were deposited in the herbarium of the Botanical Department of the University of Medicine and Pharmacy "Victor Babes" Timisoara. Based on these samples, the plant was indeed identified as belonging to the species *Clematitis*.

Table 1. Sample details about type, location and presence or absence of *Aristolochia* plant

Area type	***Sample code***	***Sample type***	***Presence/Absence of A. clematitis plant/seeds***	***Sample location***
Endemic rural	SOM1-A	SOM	yes	Erghevita village, cultivable garden 1, Mehedinti County; 44°36'25.5"N 22°46'50.2"E
	SOM2	SOM	no	
	SOM3	SOM	no	
	S1-A	soil	yes	
	S2-A	soil	yes	
	S3-A	soil (corn)	yes	Erghevita village, cultivable garden 2, Mehedinti County; 44°36'24.1"N 22°46'49.0"E
	S4	soil	no	
	S5	soil	no	
Non-endemic rural	S6-A	soil (corn)	yes	Plugova village, crop field, Caras-Severin County; 44.9626° N, 22.3565° E
	S7	soil (corn)	no	
Non-endemic urban	S8	soil	no	Timisoara city, cultivable garden, Timis County; 45.736948, 21.240782

2.3. Sample preparation

In order to perform sample extraction, a ratio of 1:1 (w/v) of soil and soil organic matter (1 g) (dried previously at 60ºC) and methanol (1 mL) was added, then the mixture was heated at 350 Watts (medium power) in a microwave oven (MOULINEX, Canada) for short periods (twice for 30 seconds each). Samples were centrifuged at 2000 rpm for 10 min, the supernatant was collected and evaporated under nitrogen. Samples were resuspended in 20 µL methanol and vortexed before GC-MS injection. 3 µL of each sample were manually injected.

2.4. Instrumentation and chromatographic conditions

The sample analysis was performed on a gas chromatograph (Scion 436-GC, Bruker Daltonik GmbH, Hamburg, Germany) coupled with a triple quadrupole tandem mass spectrometer (Scion TQ, Bruker Daltonik GmbH, Hamburg, Germany). Compounds separation was performed on a BR-5MS WCOT fused-silica capillary column (30 m×0.25 mm×0.25 µm) column at constant helium flow of 1 mL/min. The column oven temperature was set at a rate of 6ºC/min, starting from a temperature of 50ºC with 1 min hold up to 300ºC with 5 min final hold. Samples were injected in split mode (1:20), and the inlet temperature was set at 230ºC. The MS scan parameters included full scan with a mass range of m/z 50-500 Da, and the ionization energy was set at 70eV. Identification of compounds was performed with NIST11 mass spectral library. The mass spectra of the unknown compounds found in the extracted soils were identified by comparison to the mass spectra from the database library (Orem et al., 2007; Orem et al., 2014).

3. Results and discussions

The use of methanol, as a solvent for extractions, could provide a useful general overview of molecular classes contained in soil and soil organic matter. In order to see the organic composition of soil, a GC-MS analysis was performed. Compounds are presented as the summed intensities of mass spectral peaks plotted against the retention time (RT) expressed as Total Ion Chromatogram (TIC) with an output of corresponding chemical compounds (Fig. 1).

The organic compounds from soil and SOM samples were separated and identified. Based on chemical similarities, GC-MS analysis provided 103 complex compounds, derived from aliphatic and aromatic hydrocarbons. The organic compounds identified in the samples are presented in Table 2 along with their corresponding retention time (RT) and peak area.

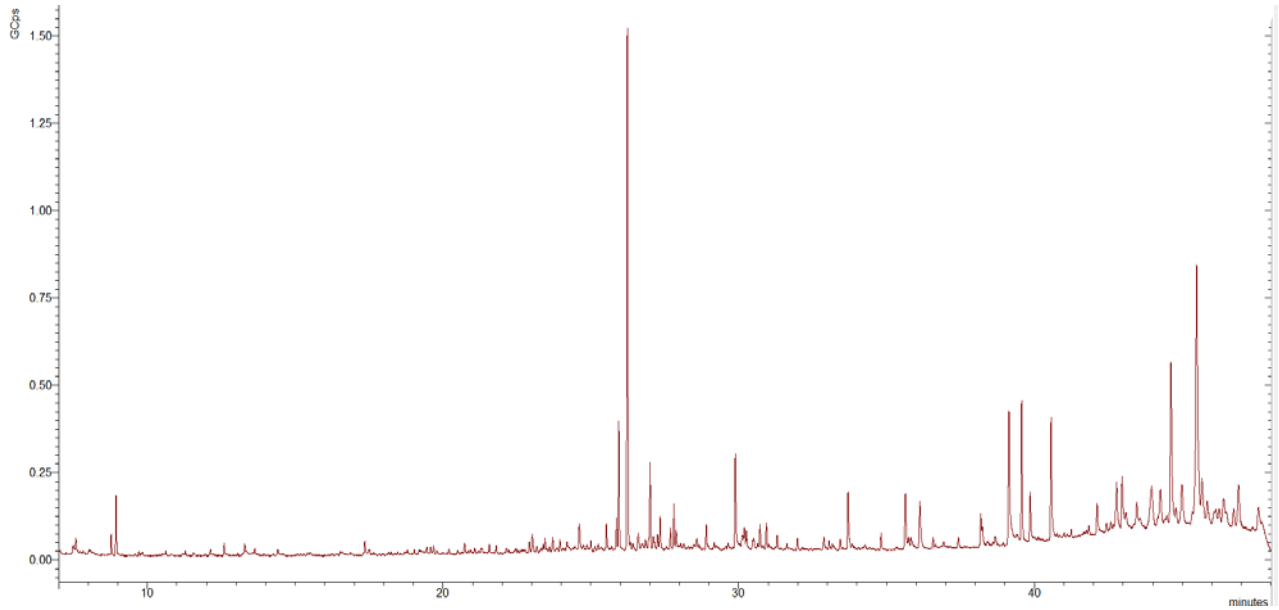

Fig. 1. TIC of SOM1-A, from a rural cultivable garden in the BEN endemic area with *Aristolochia* plants growing in the proximity (Erghevita village, Mehedinti County)

Table 2. List of compounds identified by GC-MS in soil and SOM samples

				Endemic									***Nonendemic***	
No.	***Rt***	***Compounds***	***Class***	***SOM1-A***	***SOM2***	***SOM3***	***S1-A***	***S2-A***	***S3-A***	***S4***	***S5***	***S6-A***	***S7***	***S8***
1	7.25	benzaldehyde	aldehyde		3.59E+07			8.51E+07						5.25E+08
2	7.42	hexanoic acid	fatty acid			3.02E+08								

3	8.02	decane,2,6,7-trimethyl	alkane	2.17E+07		5.76E+07								
4	8.78	1-hexanol 2-ethyl	alcohol	4.13E+07	1.39E+08		1.09E+08	1.30E+07			3.88E+07			
5	8.94	benzyl alcohol	alcohol	7.53E+07	7.73E+07		1.16E+08	3.55E+08	4.54E+07		7.82E+07	6.01E+07		4.37E+08
6	9.71	acetophenone	ketone	4.12E+06	4.38E+07		1.32E+08	6.29E+07						
7	9.78	butanoic acid, 2-cyano-3-methyl, ethyl ester	ester			9.13E+07		3.00E+08			8.18E+07			
8	9.83	fumaric acid,3-methylbutyl-3-enyl undecyl ester	ester	8.32E+06								4.73E+07		
9	10.62	10-methyl-E-11-tridecen-1-ol proprionate	alcohol	3.75E+06	2.77E+07			1.23E+08	3.09E+07					
10	12.14	octanoic acid	fatty acid	5.56E+06	3.94E+07	4.76E+07		1.38E+08	8.38E+07	1.13E+08		4.78E+07		2.96E+08
11	13.29	benzofuran,2,3-dihydro	heterocyclic compound	7.95E+06		1.93E+07			2.83E+07				1.61E+08	
12	13.64	1H-pyrrole,2,5-dione,3-ethyl,4-methyl	heterocyclic compound	4.49E+06	2.44E+07	1.41E+07			3.36E+07					
13	14.41	nonanoic acid	fatty acid	3.91E+06		3.14E+07		1.08E+08	7.88E+07	6.66E+07		5.06E+07		
14	16.52	benzaldehyde, 3-hydroxy-	aldehyde				1.46E+08			7.19E+07		4.19E+07	2.36E+08	
15	17.36	vanillin	aldehyde	8.21E+06	8.91E+07	5.92E+07	1.01E+08	2.63E+08	1.10E+08		6.44E+07	5.04E+07	3.11E+08	
16	18.17	beta-vatirenene	sesquiterpene				6.24E+07	6.51E+07	2.56E+07			3.89E+07	7.45E+07	
17	18.48	phenol,2-methoxy-4-(1-propenyl)	phenol			3.24E+07	3.17E+07	8.16E+07			2.76E+07			
18	19.03	cyclododecane methyl	cycloalkane		2.61E+07	1.35E+07	1.92E+08	9.19E+07	5.30E+07	4.12E+07	2.76E+07			
19	19.68	phenol-2,4-bis(1,1-dimethylethyl)-	phenol	6.29E+06	5.01E+07	3.28E+07	1.42E+08	3.13E+07	9.69E+07	7.74E+07	6.00E+07	6.09E+07	1.34E+08	4.88E+07
20	20.2	2(4H)-benzofuranone,5,6,7,7a-tetrahydro-4,4,7a-trimethyl-	ketone	1.57E+06	3.99E+07	1.35E+07	3.48E+07	4.53E+07	3.42E+07					
21	20.72	dodecanoic acid	fatty acid	4.47E+06	3.74E+07	6.69E+07		7.01E+07	1.01E+08	7.36E+07	3.72E+07	9.87E+07	3.66E+08	
22	21.07	tetradecanal	aldehyde	5.23E+06	3.61E+07			8.66E+07	7.17E+07	4.80E+07	3.07E+07			
23	23.01	naphthalene,1,6-dimethyl-4-(1-methylethyl)-	PAH											2.58E+08
24	23.17	(R)-(-)-(Z)-14-Methyl-8-hexadecen-1-ol	alcohol	4.38E+06	6.65E+07	1.84E+07	7.47E+07	1.32E+08	1.38E+08	1.14E+08	4.32E+07			
25	23.45	nonadecane	alkane	1.65E+07	4.02E+07	1.47E+08	1.30E+08		4.47E+07	4.72E+07	2.99E+07			7.48E+07
26	23.71	E-2-tetradecen-1-ol	alcohol	1.08E+07	8.03E+07	5.22E+07	2.32E+08	1.61E+08	1.71E+08	1.10E+08	1.24E+08	6.71E+07	1.30E+08	
27	23.95	1-dodecanol,3,7,11-trimethyl	alcohol	6.96E+06	4.46E+07		4.64E+07	2.10E+08	1.80E+08	1.11E+08				
28	24.19	1-hexadecanol	alcohol	6.80E+06	7.61E+07	3.86E+07	2.33E+08	3.29E+08	4.33E+08	1.72E+08	1.02E+08	1.48E+08	9.29E+07	
29	24.6	aristolone	sesquiterpene	2.02E+08			1.66E+09	1.60E+09	1.48E+08			5.20E+08	1.65E+09	
30	24.87	Z,E-3,13-octadecadien-1-ol	alcohol	3.84E+06	2.89E+07	1.17E+07		1.34E+08	1.17E+08	7.50E+07	2.80E+07	3.75E+07		1.80E+07
31	24.99	phenanthrene	PAH											2.76E+08
32	25	methyl 8-methyl nonanoate	FAME					1.29E+08	1.40E+08				8.55E+07	
33	25.14	methyl 12-methyltridecanoate	FAME	3.35E+07	6.60E+07	1.78E+07	9.50E+07	1.06E+08	1.01E+07	7.30E+07	6.65E+07	5.82E+07		1.00E+08
34	25.53	pentadecanal	aldehyde	1.65E+07	1.36E+08	2.88E+07	6.49E+07	1.67E+08	3.37E+08	2.38E+08	1.00E+08	7.41E+07	1.06E+08	1.23E+08
35	25.62	pentadecanoic acid	fatty acid						1.69E+08				5.81E+08	
36	25.73	oleic acid	fatty acid					1.11E+08	9.58E+07			7.46E+07	4.83E+08	
37	25.86	3,7,11,15-tetramethyl,2-hexadecen-1-ol	alcohol	1.22E+07	8.34E+07	2.07E+08	1.68E+08	6.17E+08	9.67E+08	4.19E+08	1.48E+08	1.95E+08	5.90E+08	
38	25.94	2-pentadecanone, 6,10,14-trimethyl	ketone	1.41E+08	5.22E+08	2.87E+08	7.60E+08	1.39E+09	1.63E+09	1.02E+09	3.98E+08	4.85E+08	1.08E+09	4.40E+08
39	26.23	1,2-benzenedicarboxylic acid bis (2-methylpropyl) ester	aromatic ester	1.23E+09	5.12E+08	5.40E+08				4.70E+08	5.96E+07			1.49E+08
40	26.6	9-eicosyne	alkene		1.07E+08	7.30E+07	2.13E+08	4.31E+08	8.73E+08		1.57E+08	1.78E+08	2.81E+08	

41	27	7,9-di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	alkadiene	5.46E+07	1.75E+08	1.08E+08						1.68E+08	3.12E+08	
42	27.34	methyl 11-methyl dodecanoate	FAME	3.28E+07	3.27E+08	1.25E+08	3.93E+08	3.78E+08	4.29E+08	2.67E+08	5.33E+08	1.24E+08	6.25E+08	
43	27.57	(Z)-11-hexadecenoic acid	fatty acid				3.50E+08	2.94E+08	1.83E+08			1.32E+08	1.59E+09	
44	27.8	Di-N-butylphthalate	aromatic ester	1.10E+08	1.28E+08	4.49E+07	7.81E+07					5.57E+07		1.73E+09
45	27.88	palmitic acid	fatty acid	1.01E+07	9.56E+08	1.34E+08	3.93E+08	1.33E+09	7.39E+08	1.41E+08	1.24E+08	3.57E+08	4.06E+09	
46	28.58	7-hexadecenal	aldehyde	1.04E+07	7.97E+07	2.71E+07	1.99E+08	1.02E+08	1.86E+08	2.03E+08	9.46E+07	8.32E+07		1.25E+08
47	28.66	1-phenantrenecarboxylic acid, 7-ethyltetradecahydro-1,4a,7-trimethyl, methyl ester-	PAH											7.75E+08
48	28.9	oxirane tetradecyl	epoxid	1.60E+07	1.85E+08	5.35E+07	1.13E+08	2.59E+08	3.08E+08	3.22E+08	1.17E+08	1.18E+08	2.80E+08	2.32E+08
49	29.13	methyl 7,11,14-eicosatrienoate	FAME											2.04E+08
50	29.32	4,14-dimethyl-11-isopropyltricyclo[7.5.0.0.(10,14)]tetradec-4-en-8-one	ketone				2.44E+08					7.90E+07		1.13E+09
51	29.64	fluoranthene	PAH	6.37E+07	5.58E+07	8.59E+6	4.10E+07	4.61E+07	3.82E+07	4.93E+07	1.21E+07	1.86E+07	2.87E+07	5.39E+08
52	29.88	1-eicosanol	fatty alcohol	8.52E+07	1.18E+08	3.27E+07	1.98E+08	4.80E+08	2.36E+08	1.97E+08	5.79E+07	1.09E+08	1.78E+08	
53	29.99	9,12-hexadecadienoic acid,methyl ester	FAME				1.94E+08	2.26E+08	7.96E+07		4.67E+07			
54	30.11	9-octadecenoic acid (Z), methyl ester	FAME	1.77E+07			6.85E+08	4.36E+08		3.02E+08	1.28E+08		3.62E+08	
55	30.19	cyclopentadecanone,4-methyl	cycloketone	1.86E+07	1.71E+08	6.47E+07		3.81E+08	3.19E+08	2.41E+08	1.06E+08	1.64E+08	3.89E+08	
56	30.25	phytol	diterpene alcohol	1.11E+07	3.60E+08	7.51E+07	1.80E+08	4.61E+08	4.15E+08	3.09E+08	5.16E+07	1.50E+08	3.20E+08	
57	30.47	pyrene	PAH											5.83E+08
58	30.51	hexadecanoic acid, 15-methyl, methyl ester	FAME	9.72E+06	2.04E+08	9.48E+07	7.80E+07		1.12E+08	1.08E+08	2.17E+08		1.72E+08	
59	30.64	9,12-octadecadienoic acid,methyl ester	FAME			8.64E+07		3.32E+08	1.60E+08	3.20E+07	3.36E+07	1.32E+08	2.46E+09	
60	30.72	1-hexadecyn-3-ol,3,7,11,15-tetramethyl	alcohol	1.38E+07	9.17E+07	7.30E+07	1.69E+09	6.32E+08	3.56E+08	1.95E+08	7.11E+07	1.81E+08		
61	31.3	2,2-(2-Chlorophenyl-4'-chlorophenyl)-1,1-dichloroethene	aromatic halogenures		2.94E+07				1.43E+08					2.48E+07
62	31.88	retene	PAH											7.43E+08
63	32.11	benzene-1,3-dimethoxy-5-[(1E)-2-phenylethenyl]	aromatic hydrocarbure											2.64E+08
64	32.88	1-heneicosanol	alcohol	1.35E+07	1.00E+08	6.14E+07	3.28E+08	9.92E+08	4.43E+08	4.75E+08	1.36E+08	1.47E+08	3.71E+08	2.07E+08
65	33.05	hentriacontane	alkane	1.29E+07	1.05E+08	9.99E+07	3.87E+08	3.35E+08	2.13E+08	3.09E+08	1.60E+08	1.79E+08	2.08E+08	3.31E+08
66	33.21	(1,2,3-trimethyl-cyclopent-2-enyl) methanol	alcohol											2.28E+08
67	33.69	4,8,12,16-tetramethylheptadecan-4-olide	acid	7.83E+07	1.95E+08	1.38E+08	3.38E+08	9.09E+08	7.66E+08	5.53E+08	1.32E+08	2.22E+08	6.89E+08	2.34E+08
68	34.14	8-isopropyl-1,3-dimethylphenanthrene	PAH											1.02E+08
69	34.82	Z-2-octadecen-1-ol	alcohol	1.32E+07	1.13E+08	9.97E+07	7.55E+07	4.70E+08	1.49E+08	4.11E+08	2.43E+08	1.86E+08	6.27E+08	1.90E+08
70	35.22	benz[a]anthracene	PAH											2.44E+08
71	35.35	chrysene	PAH											4.25E+08
72	35.63	behenic alcohol	fatty alcohol	5.14E+07	4.64E+08	2.96E+08	1.71E+09	1.88E+09	1.44E+09	2.66E+09	5.49E+08	6.03E+08	1.60E+09	9.85E+08
73	35.75	tritetracontane	alkane		2.71E+08	1.39E+08	7.63E+08	1.15E+09	2.81E+08	6.29E+08	3.43E+08	2.28E+08	4.12E+08	5.11E+08
74	36.12	diisooctyl phthalate	aromatic ester	6.68E+07	3.24E+08	2.98E+08	4.60E+08		1.51E+09	5.79E+08	3.59E+08	3.37E+08		3.63E+09
75	36.93	1-heneicosyl formate	ester			3.16E+07	2.42E+08	1.70E+08	1.19E+08	1.95E+08	5.82E+07	5.61E+07	2.13E+08	
76	38.18	1-heptacosanol	alcohol		3.98E+08	2.66E+08	1.17E+09	3.21E+09	1.61E+09	2.25E+09	2.53E+08	2.75E+08	1.87E+09	9.72E+08
77	38.67	cis-9,10-epoxyoctadecan-1-ol	alcohol			8.48E+07		3.67E+08	2.19E+08	3.24E+08	1.62E+08	1.04E+08	3.18E+08	

78	39.14	9-octadecenamide-(Z)	fatty amide	1.88E +08	3.19E +08	6.97E +07	1.74E +09	4.24E +08	2.76E +08	6.32E +08		4.40E +08	1.05E+ 09	
79	39.15	benzo[b]fluoranthene	PAH											3.94E+ 08
80	39.57	squalene	triterpenes	2.63E +08	1.04E +09	4.36E +08	2.05E +09		1.91E +09	3.00E +09	5.00E +08		2.29E+ 09	7.04E+ 08
81	40.04	perylene	PAH											1.44E+ 08
82	40.19	benzo[k]fluoranthene	PAH											1.19E+ 08
83	40.56	octacosanol	fatty alcohol	1.48E +08	2.34E +09	1.28E +09	6.57E +09	1.42E +10	3.83E +09	5.77E +09	1.42E +09	2.22E +09	6.28E+ 09	1.99E+ 09
84	41.85	methyl-6,9,12,15,18-heneicosapentaenoate	FAME	6.17E +07	6.29E +07	4.31E +07	1.84E +08	8.88E +07	5.05E +07	1.47E +8	4.25E +07	4.21E +07	1.78E+ 08	
85	42.44	cholesta-4,6-dien-3-ol (3.beta)-	phytosterol					1.75E +08	2.10E +08					
86	42.78	hexatriacontyl pentafluoroproprionate	alkane	3.94E +07	1.65E +09	4.33E +08	1.51E +09	8.66E +09	1.13E +09	1.54E +09	6.90E +08	5.65E +08	1.42E+ 09	1.23E+ 09
87	42.96	butyl 4,7,10,13,16,19-docosahexaenoate	FAME	6.03E +06	1.04E +09	3.24E +08	1.71E +09	1.13E +09	1.03E +09	3.18E +09	4.75E +08	3.64E +08	1.01E+ 09	5.78E+ 08
88	43.09	cholestanol	phytosterol		8.12E +08				2.43E +08	5.20E +08	1.41E +08			
89	43.46	cholesta-8,24-dien,3-ol,4-methyl-3beta,4alpha	phytosterol		2.10E +08	1.12E +08	7.49E +08	5.61E +08	4.37E +08	6.21E +08	1.45E +08		1.04E+ 09	
90	43.69	benzo[ghi]perylene	PAH											7.85E+ 07
91	43.95	retinol acetate	phytosterol	1.89E +07	6.43E +08	6.24E +08	1.90E +09	2.40E +09	1.02E +09	1.06E +09	5.59E +08	6.65E +08	2.66E+ 09	
92	44.26	retinal	phytosterol		4.41E +08	2.92E +08	1.83E +09	1.23E +09	6.14E +08	6.18E +08	3.30E +08	2.55E +08	1.69E+ 09	
93	44.61	stigmasta-5,22-diene,3-methoxy	phytosterol	1.45E +08	9.35E +08	1.12E +09	2.06E +09	1.71E +09	1.65E +09	2.11E +09	9.72E +08	5.01E +08	2.94E+ 09	
94	44.78	cholestan-3-ol,2-methylene-(3 beta,5 alpha)-	phytosterol	2.11E +08	1.18E +08	3.58E +08			1.68E +08	4.81E +08	3.44E +08			
95	44.98	lithocholic acid	phytosterol	6.60E +06	3.19E +08		7.56E +08		1.00E +09	9.06E +08				
96	45.48	beta-sitosterol	phytosterol	1.76E +08	2.95E +09	2.28E +09	4.45E +09	1.24E +10	4.78E +09	4.01E +09	2.06E +09	1.31E +09	6.65E+ 09	2.04E+ 09
97	45.66	stigmastanol	phytosterol	1.09E +07	7.65E +08	3.00E +08	6.07E +08	8.02E +08	7.46E +08	7.15E +08	4.43E +08	1.45E +08	7.07E+ 08	4.51E+ 08
98	45.86	tricyclo[20.8.0.0(7,16)] triacontane-1(22),7(14)-diepoxy	cycloalkane			8.26E +07	3.58E +08	1.73E +08	3.29E +08	4.88E +08	1.29E +08	1.98E +08	7.67E+ 08	
99	46.25	pregnan-3-one	phytosterol			1.14E +08		3.99E +08	2.37E +08	3.15E +08				
100	46.39	9,19-cycloergost-24(28)-en-3-ol,4-,14-dimethyl-acetate	phytosterol		2.33E +08		4.26E +08	3.98E +08	5.04E +08	5.50E +08		2.60E +08	6.37E+ 08	
101	46.73	9,19-cyclolanostan-3-ol,acetate	phytosterol				1.38E +08	1.99E +08	1.38E +08	1.74E +08	6.14E +07			
102	46.9	lupeol	triterpene		2.57E +08	2.39E +08	3.35E +08	1.12E +09	9.42E +08	7.51E +08	2.41E +08	1.69E +08	6.73E+ 08	
103	47.57	cholest-4-en-3-one	phytosterol			2.17E +08	5.03E +08	1.78E +09	4.24E +08	3.67E +08	2.14E +08	1.36E +08	1.19E+ 09	

(FAME= Fatty acid methyl ester; PAH= Polycyclic aromatic hydrocarbon)

Summarizing the proportion in which the components were found the one detected in the highest proportion (59.2%) could be considered soil lipid fractions, mainly derived from aliphatic hydrocarbons (18.44% fatty acids and fatty acids methyl esters, 16.5% alcohols, 13.59% phytosterols, 5.82% aldehydes and 4.85% ketones) (Vancampenhout et al., 2009). Organic compounds identified most frequently with the peak area ranging from 10E+08 to 10E+09 include: aristolone; 2-pentadecanone, 6,10,14-trimethyl; methyl 11-methyl dodecanoate; palmitic acid; behenic alcohol; 1-heptacosanol; 9-octadecenamide-(Z); squalene; octacosanol; hexatriacontyl pentafluoroproprionate; butyl 4,7,10,13,16,19-docosahexaenoate; retinol acetate; retinal; stigmasta-5,22-diene,3-methoxy; beta-sitosterol; cholest-4-en-3-one. Aristolone occurs commonly in *Aristolochia* species, while the other compounds could be found in other types of soils and plants as well (Vancampenhout et al., 2009).

One explanation regarding the composition could be that soil and SOM samples result from a complex biomass, plant degradation with microbial and physicochemical transformations; this composition predominantly consists of organic compounds having an important contribution to the ecological dynamics (Abelenda et al., 2011; Derenne and Quénéa, 2014; Vancampenhout et al., 2009). The sources of aliphatic hydrocarbons are plant waxes and suberin released from the root and the bark of the trees (Lorenz et al., 2007). While long-chain aliphatics are found in soils with low microbial activity, short-chain aliphatics originate from microbial degradation, as well as fatty acids; the latter are primarily derived from plant waxes or lipid biomacromolecules (Abelenda et al., 2011).

The most noticeable dissimilarity is related to the occurrence of PAHs in the soil sample from the urban garden, S8, (from Timisoara city) in comparison to the other 10 rural soils (from Erghevita and Plugova villages). It is well known that PAHs are toxic, environmentally persistent pollutants, mainly generated by the incomplete combustion of organic materials. They have the tendency to remain in soils for a long time due to their hydrophobicity (Doick et al., 2005; Marquez-Bravo et al., 2016; Rabideau et al., 2007; Von Lau et al., 2018).

Taking into consideration that PAH sources (pyrogenic and petrogenic) occur more frequently in the urban areas than in the rural areas, the probability of accumulating greater PAH concentrations in the urban soils is higher than in rural areas (Marquez-Bravo et al., 2016; Rabideau et al., 2007). Modern urban gardening practices require automatic special machines for the soil preparation and cultivation, which could extend PAH contaminations through the soil from spills of oil or from incompletely combusted motor fuel (Abdel-Shafy and Mansour, 2016; Jiang et al., 2009; Marquez-Bravo et al., 2016). While sample S8 comprises 13 PAHs -naphthalene; 1,6-dimethyl-4-(1-methylethyl)-, phenanthrene; 1-phenantrenecarboxylic acid, 7-ethyltetradecahydro-1,4a,7-trimethyl, methyl ester-; fluoranthene; pyrene; retene; 8-isopropyl-1,3-dimethylphenanthrene; benz[a]anthracene; chrysene; benzo[b]fluoranthene; perylene; benzo[k]fluoranthene; benzo[ghi]perylene)-, all the other 10 samples from the rural areas contain only one detectable PAH (fluoranthene) at a lower abundance.

The presence of fluoranthene in the soils could be attributed to the usage of pesticides for the cultivable gardens, as fluoranthene is known to be an intermediate in the manufacturing process of agrochemicals (Abdel-Shafy and Mansour, 2016). Based also on our field observations, the villagers use pesticides on field crops and gardens in order to remove the *Aristolochia* plants, which basically invade the areas with food cultures.

Another interesting aspect found from our analysis was the detection of a terpenoid compound, aristolone, in six samples (SOM1-A, S1-A, S2-A, S3-A, S6-A and S7). The first 5 samples (SOM1-A, S1-A, S2-A, S3-A, S6-A) were collected simultaneously with *A. clematitis* (A) plant parts or seeds unlike the later nonendemic soil, where *Aristolochia* plants are present in the vicinity. *Aristolochia* plants grow nearby cultivable gardens as weeds and thus we can assume the soil assimilated aristolone derived from plant parts or seeds that were carried by the wind or have fallen on the ground. This sesquiterpene is a specific component found in the composition of *Aristolochiaceae* family, but not exclusively to it (Benmehdi et al., 2017; Kostalova et al., 1991; Lajide et al., 1993; Sulyman et al., 2016; Wu et al, 2004, 2005).

4. Conclusions

The proposed GC-MS method succeeded in detecting, with a considerable sensitivity, over 100 organic compounds in soil and SOM samples collected from BEN endemic and nonendemic areas.

BEN endemic and nonendemic character, as well as the presence or absence of *Aristolochia* plants does not considerably influence the soil composition as much as the rural or urban location of sampling. Soil lipid fractions mainly derived from aliphatic hydrocarbons were detected in the highest proportion throughout the samples collected from rural endemic and nonendemic areas, while in the urban soil we found a higher occurrence of PAHs, which are of concern, because accumulated in the urban soil they can be a contributing route of exposure for individuals within cities.

Aristolone was detected in the samples that contained *Aristolochia* plant parts or seeds taken together with the soil or SOM, with one exception of a nonendemic sample, where the plant grew in the vicinity of the collecting area. Furthermore we can undertake the hypothesis that the soil assimilated aristolone, because *Aristolochia* plants grow nearby the cultivable garden. Aristolone was not detectable in the endemic samples without the plant and was absent in the samples where this plant was never present. In this regard, aristolone could be considered like a molecular biomarker for the presence of *Aristolochia* plants in a certain environmental setting.

This preliminary study holds great impact for the field of environmental health providing a feasible soil analysis procedure with an easy solvent extraction method and a complex chromatographic method of analysis. However, further investigations are required for additional clarification of the role of soil contamination with phytotoxins in the etiology of Balkan endemic nephropathy. In this regard, microwave-assisted extraction and a solid phase microextraction methods coupled with a sensitive GC/MS analysis could be employed in order to identify a larger spectrum of potentially toxic organic contaminants in soil, with public health implications..

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