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Dedicated to Professor Costel Sârbu on the Occasion of His 65th Anniversary

ANALYSIS OF PHYTOCONSTITUENT PROFILE OF FENUGREEK –*TRIGONELLA FOENUEM-GRAECUM* L. - SEED EXTRACTS

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ABSTRACT. Fenugreek (*Trigonella foenum-graecum* L.) is a well-known herb for its efficiency in the prevention/treatment of diabetes among other chronic diseases. The aim of present study was to analyse the phytoconstituent profile of aqueous and hydro-alcoholic extracts of fenugreek seeds produced in Hungary. The aqueous and hydro-aqueous extracts were analysed using a UHPLC-ESI-MS approach, and in the first 54, while in the second extract 67 phytoconstituents were identified that mostly corroborate the previously described health promoting effects of fenugreek. However, it remains a huge challenge to correlate the phytoconstituent composition of the two extracts with the generated dose dependent hormetic response and cytotoxic effects that were reported by us in case of some human breast cancerous cell lines.

Keywords: *fenugreek, Trigonella foenum-graecum, phytoconstituents, UHPLC-ESI-MS*

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INTRODUCTION

The fenugreek (*Trigonella foenum-graecum* L.) has been grown in Asia, Africa and Europe from ancient times being utilized as a food (fresh shoots), spice (seed) and herbal remedy. Its popularity has ever been increasing so that recently, it is cultivated in countries like India, Pakistan, China, Russia, Greece, Turkey, Israel, Egypt, Sudan, Morocco, Tunisia, Germany, Austria, United Kingdom, Spain, Portugal, USA and Argentina. Due to its large cultivation areal, several fenugreek ecotypes and/or varieties were described upon taxonomical characters comprising morphological features like seed types. Furry (1950) was proposing six fenugreek seed types like Yemenese, Transcaucasian, African, Afghan, Chinese-Persian and Indian, while Petropoulus (1973) had been suggesting categories like the Flourescent, Ethiopian, Indian and Mediterranean seed types [1,2].

Several beneficial biological and pharmacological properties are attributed to the fenugreek seeds such as anti-diabetic, hypocholesterolaemic, contraceptive and anti-fertility, gastric ulcer and wound healing, anti-cancer, anti-microbial, anthelmintic and anti-nociceptive effects, respectively [3].

The fenugreek seeds contain (per 100g of edible portion): 369 calories, 7.8% moisture, 28.2 g protein, 5.9 g fat, 54.5 g total carbohydrate, 8g fibre, 3.6 g ash [4]. Fenugreek seeds containing diosgenin are considered one of the few natural sources of steroid sapogenins that is used for the synthesis of sex hormones, oral contraceptives and medicinally useful steroids [5]. Several furostanol saponins called trigoneosides Ia, Ib, IIa, IIb, IIIa, IIIb, IVa, Va, Vb, VI, VIIb, VIIIb, IX were isolated form fenugreek seeds originating from India [6,7]. Trigoneosides like Xa, Xb, XIb, XIIa, XIIb and XIIIa were identified from fenugreek seeds of the Egyptian origin [8]. Graecunins H-N are glycosides of diosgenin have also been isolated from fenugreek seeds, and belong to the spirostanol saponins [9]. Fenugrin B is another saponin that was also identified in fenugreek seeds [10]. Among sterols campesterol, stigmasterol, β-sitosterol and cholesterol were shown in different parts of the plant including seeds [11]. The fenugreek saponins exhibited hypocholesterolemic activity in rats [12]. Triterpenoids like lupeol, botulin, betulinic acid and soyasaponin were also isolated from fenugreek seeds [13]. Another important compound found in fenugreek seeds is the trigonelline which is the methylbetaine derivate of nicotinic acid, and its hypoglycemic and antipellagra effects have been demonstrated [14-16]. The flavonoid content of fenugreek seeds had been intensively analysed, and it was suggested to confer antibacterial activity to seed extracts [17]. Quercetin, luteolin, vitexin, orientin, isoorientin, vicenin-1, vicenin-2, naringenin, kaempherol, 7,4'-dimethoxyflavanone were identified among flavonoids. Other phenolic compounds were detected in different parts of plants (root, shoot, and pod) like

scopoleptin, trigocoumarin, chlorogenic, caffeic and coumaric acids [18]. Studies and estimations have shown that the 4-hydroxyisoleucine represents up to 30-80 percent of free amino acid pool in fenugreek seeds [19,20]. A non-protein amino acid (S)-canavanine, and other amino acids like lysine and tryptophane were identified in fenugreek seeds [21,22]. The protein content of fenugreek leaves and seeds reaches 25-30 percent, so that approximately equals to that of soybeans [20]. It was suggested that the hypocholesterolemic effects of fenugreek seeds could be related to the amino acid content or to the relatively high fibre content (54 percent) and saponins (5 percent), [23]. Among vitamins in fenugreek seeds had been identified thiamine, riboflavin, pyridoxine, cyanocobalamine, niacin, Ca-pantothenate and biotin, while vitamin C was present mostly in the vegetative organs of the plant [24,25]. The lipid content of dried fenugreek seeds had been shown to reach approximately 7.5 percent, and the lipid profile consisted of neutral lipids (triacylglycerol, diacylglycerols, monoacylglycerols, free fatty acids, and sterols), glycolipids and phospholipids [26].

In the current paper we are reporting the UHPLC-ESI-MS chemomapping of aqueous and hydro-alcoholic fenugreek seed extracts that were found by us to induce dose dependent hormetic response and cytotoxicity in case of human breast cancerous cell lines [27]. We were able to detect 54 and 67 phytoconstituents in the aqueous and hydro-alcoholic artichoke extracts, respectively. Some of the newly identified compounds were confirmed by standards, while other have been already described by others [27-33].

RESULTS AND DISCUSSION

The aqueous and the hydro-alcoholic extracts of fenugreek seeds were investigated with the reversed phase UHPLC-ESI-MS in positive and negative ionisation modes as described in Materials and Methods. The gradient mobile phase was based on acetonitrile and water. There have been 54 phytoconstituents identified in the aqueous fenugreek seed extract as shown on Figure 1-2 and in Table 1.

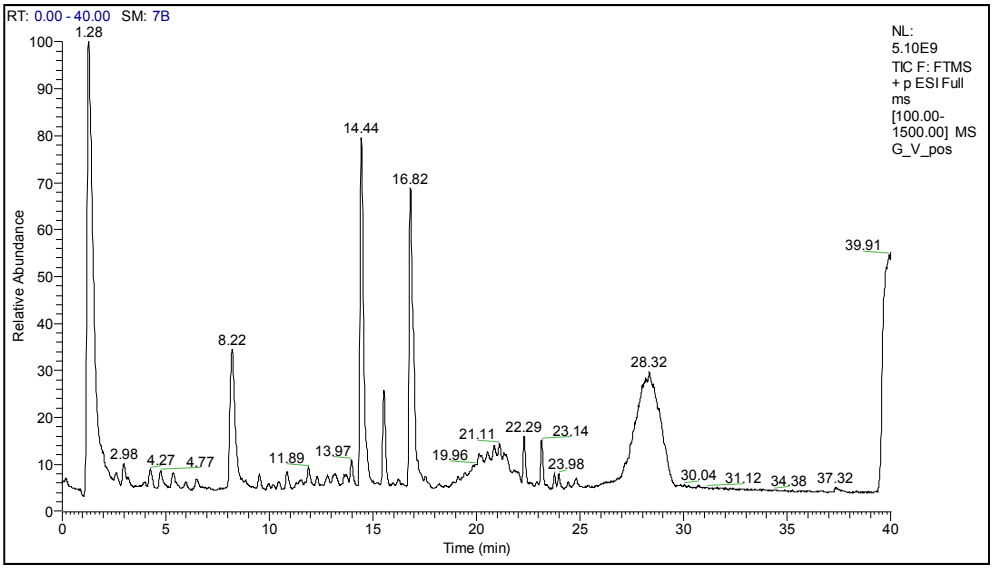

Figure 1. Total ion chromatogram of aqueous extract of fenugreek in positive ionisation mode.

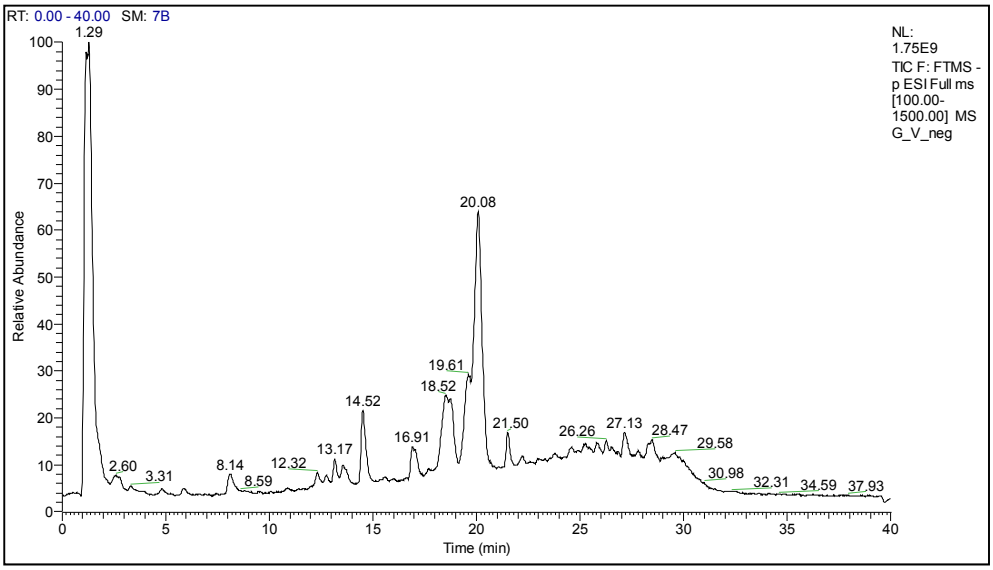

Figure 2. Total ion chromatogram of aqueous extract of fenugreek in negative ionisation mode.

Table 1. Phytoconstituents identified in the aqueous fenugreek seed extract. Rt –retention time; [M+H]+ - molecular ion masses; [M+H]- - the found fragment ion mass; Ref- references; (*) [M]+; (**) confirmed by standards.

Peak	R_t	$[M+H]^+$	$[M-H]^-$	Formula	Fragments found	Assignment	Ref.
1	1.27	104.10754*		$C_5H_{14}NO$	60.0814, 59.0735	Choline	27
2	1.30	138.05550*		$C_7H_8NO_2$	110.0601, 96.0450	Trigonelline	27
3	1.31	175.11951		$C_6H_{14}N_4O_2$	158.0922, 130.0975	Arginine**	27
4	1.31	148.06099		$C_5H_9NO_4$	130.0863, 102.0553	Glutamic acid	27
5	1.31	118.08681		$C_5H_{11}NO_2$	59.0736, 58.0657	Betaine	27
6	1.32	133.06132		$C_4H_8N_2O_3$	116.0342, 88.0397	Asparagine**	27
7	1.43	189.12392		$C_8H_{16}N_2O_3$	172.0961, 130.0863	N-α-Acetyl-lysine	
8	1.46	148.09737		$C_6H_{13}NO_3$	130.0862, 113.0598	4-Hydroxyiso-leucine	27
9	1.49	324.05968		$C_9H_{13}N_3O_5$	112.0507, 95.0243	Cytidine**	
10	1.51	146.09296		$C_5H_{11}N_3O_2$	128.0821, 111.0555	4-Guanidino-butyric acid	
11	1.52	130.08681		$C_6H_{11}NO_2$	84.0812, 67.0548	Pipecolic acid	
12	1.56	136.06233		$C_5H_5N_5$	119.0352, 94.0406	Adenine	
13	1.66		283.06786	$C_{10}H_{12}N_4O_6$	151.0248, 108.0188	Xanthosine	
14	1.73		243.06171	$C_9H_{12}N_2O_6$	200.0558, 153.0293	Uridine	
15	1.75	170.08172		$C_8H_{11}NO_3$	152.0704, 134.0600	Pyridoxine**	27
16	1.82	182.08172		$C_9H_{11}NO_3$	165.0545, 147.0439	2-Hydroxyphenyl-alanine	
17	1.96	123.05584		$C_6H_6N_2O$	106.0287, 96.0447	Nicotinamide**	27
18	2.01	330.06035		$C_{10}H_{12}N_5O_6P$	232.0828, 136.0617	Adenosine 3',5'-cyclic monophosphate	
19	2.10	277.13997		$C_{11}H_{20}N_2O_6$	259.1286, 213.1231	Saccharopine	
20	2.23	385.12942		$C_{14}H_{20}N_6O_5S$	136.0618, 134.0271	S-Adenosyl-homocysteine	
21	2.60	152.05724		$C_5H_5N_5O$	135.0301, 128.0455	Guanine	

Peak	R_t	$[M+H]^+$	$[M-H]^-$	Formula	Fragments found	Assignment	Ref.
22	2.63		282.08385	$C_{10}H_{13}N_5O_5$	150.0407, 133.0142	Guanosine	
23	2.74		163.03952	$C_9H_8O_3$	119.0487, 93.0329	p-Coumaric acid	
24	2.95	268.10458		$C_{10}H_{13}N_5O_4$	136.0617, 119.0358	Adenosine**	
25	3.10	252.10967		$C_{10}H_{13}N_5O_3$	136.0618, 117.0548	2'-Deoxyadenosine	
26	3.21	166.08681		$C_9H_{11}NO_2$	149.0598, 131.0493	Phenylalanine**	27
27	4.86	220.11850		$C_9H_{17}NO_5$	202.1073, 184.0967	Pantothenic acid**	27
28	6.49	205.09771		$C_{11}H_{12}N_2O_2$	188.0706, 170.0599	Tryptophan**	27
29	6.75	129.05517		$C_6H_8O_3$	111.0443, 101.0600	Sotolone	27
30	8.31	190.05042		$C_{10}H_7NO_3$	162.0547, 144.0435	Kynurenic acid	
31	9.53	295.12940		$C_{14}H_{18}N_2O_5$	278.1017, 232.0965	γ-Glutamylphenyl-alanine	
32	11.56	186.11302		$C_9H_{15}NO_3$	168.1017, 150.0909	Ecgonine	
33	12.32		593.15065	$C_{27}H_{30}O_{15}$	503.1202, 473.1087	Vicenin-2	28
34	12.75		593.15065	$C_{27}H_{30}O_{15}$	503.1215, 473.1088	Apigenin-di-C-hexoside (Vicenin-2-isomer)	28
35	13.17		563.14009	$C_{26}H_{28}O_{14}$	503.1187, 473.1091	Vicenin-3	28
36	13.74	449.10839		$C_{21}H_{20}O_{11}$	395.0760, 377.0658	Isoorientin	27
37	13.75		563.14009	$C_{26}H_{28}O_{14}$	503.1194, 473.1096	Vicenin-1	28
38	13.90	200.12867		$C_{10}H_{17}NO_3$	182.1174, 100.0759	Ecgonine methyl ester	
39	14.43		577.15574	$C_{27}H_{30}O_{14}$	503.1193, 473.1097	Apigenin-6-C-glucoside-8-C-rhamnoside	28
40	14.65	433.11348		$C_{21}H_{20}O_{10}$	379.0805, 361.0709	Isovitexin	27
41	18.25		1195.57478	$C_{56}H_{92}O_{27}$	705.3873, 609.3632	Trigofoenoside G	31
42	18.49		905.47461	$C_{44}H_{74}O_{19}$	773.4326, 611.3798	Trigoneoside Ia	29
43	18.62		1063.53252	$C_{51}H_{84}O_{23}$	609.3646, 447.3091	Protoyuccagenin-S4	31
44	18.83		919.49026	$C_{45}H_{76}O_{19}$	773.4315, 611.3812	Trigoneoside Xa	30

Peak	R_t	$[M+H]^+$	$[M-H]^-$	Formula	Fragments found	Assignment	Ref.
45	18.84		905.47461	$C_{44}H_{74}O_{19}$	773.4336, 611.3795	Trigoneoside Ib	29
46	19.61		919.49026	$C_{45}H_{76}O_{19}$	773.4322, 611.3808	Trigoneoside Xb	30
47	19.73		887.46405	$C_{44}H_{72}O_{18}$	593.3680, 431.3171	Trigoneoside VIII	30
48	19.78		1225.58534	$C_{57}H_{94}O_{28}$	1077.2218 901.4799	Trigoneoside XIIIa	30
49	19.88		889.47970	$C_{44}H_{74}O_{18}$	757.4387, 595.3850	Trigoneoside IIa	29
50	19.98		1063.53252	$C_{51}H_{84}O_{23}$	755.4216, 593.3688	Trigoneoside IVa	29
51	20.00		1065.54817	$C_{51}H_{86}O_{23}$	757.4368, 595.3844	Trigofoenoside C	29
52	20.10		1047.53760	$C_{51}H_{84}O_{22}$	755.4216, 575.3581	Asparasaponin I (Protodioscin, Trigonelloside C)	31
53	20.30		901.47970	$C_{45}H_{74}O_{18}$	755.4237, 593.3704	Trigoneoside XIIa	30
54	20.37		903.49535	$C_{45}H_{76}O_{18}$	757.4390, 595.3836	Trigoneoside IIIa	29

There have been 67 phytoconstituents identified in the hydro-alcoholic fenugreek seed extract as shown in Table 2.

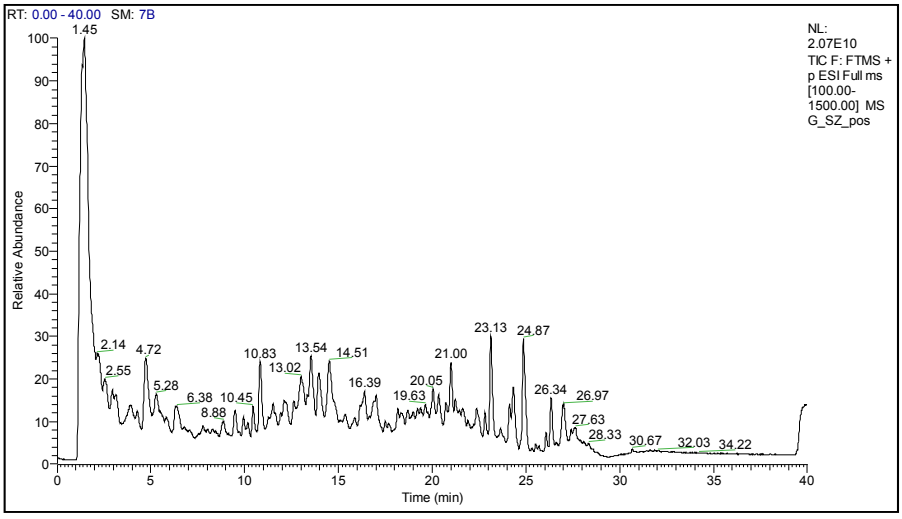

Figure 3. Total ion chromatogram of hydro-alcoholic extract of fenugreek in positive ionisation mode.

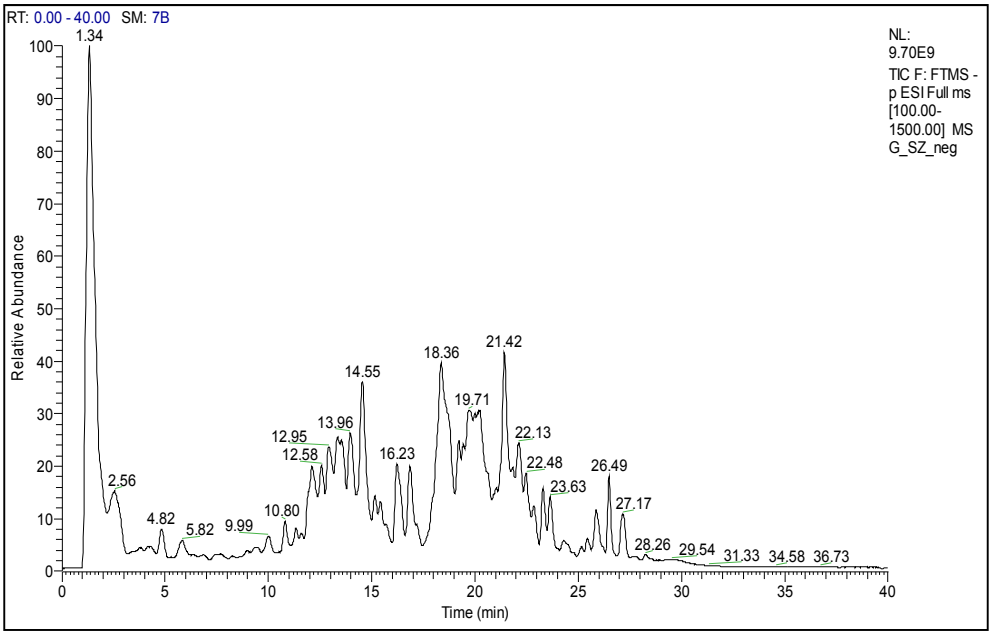

Figure 4. Total ion chromatogram of hydro-alcoholic extract of fenugreek in negative ionisation mode.

Table 2. Phytoconstituents identified in the hydro-alcoholic fenugreek seed extract. Rt –retention time; [M+H]+ - molecular ion masses; [M+H]- - the found fragment ion mass; Ref- references; (*) [M]+; (**) confirmed by standards.

Peak	R_t	$[M+H]^+$	$[M-H]^-$	Formula	Fragments found	Assignment	Ref.
1	1.22	104.10754*		$C_5H_{14}NO$	60.0814, 59.0736	Choline	27
2	1.32	138.05550*		$C_7H_8NO_2$	110.0604, 96.0447	Trigonelline	27
3	1.31	175.11951		$C_6H_{14}N_4O_2$	158.0923, 130.0975	Arginine**	27
4	1.31	148.06099		$C_5H_9NO_4$	130.0863, 102.0553	Glutamic acid	27
5	1.31	118.08681		$C_5H_{11}NO_2$	59.0736, 58.0657	Betaine	27
6	1.40	189.12392		$C_8H_{16}N_2O_3$	172.0962, 130.0862	N-α-Acetyl-lysine	
7	1.42	146.09296		$C_5H_{11}N_3O_2$	128.0810, 111.0556	4-Guanidinobutyric acid	

Peak	R_t	$[M+H]^+$	$[M-H]^-$	Formula	Fragments found	Assignment	Ref.
8	1.46	130.08681		$C_6H_{11}NO_2$	84.0812, 67.0547	Pipecolic acid	
9	1.63	124.03986		$C_6H_5NO_2$	96.0448, 80.0500	Nicotinic acid**	27
10	1.72		283.06786	$C_{10}H_{12}N_4O_6$	151.0248, 108.0188	Xanthosine	
11	1.73		243.06171	$C_9H_{12}N_2O_6$	200.0556, 153.0292	Uridine	
12	1.72	170.08172		$C_8H_{11}NO_3$	152.0705, 134.0601	Pyridoxine**	27
13	1.79	182.08172		$C_9H_{11}NO_3$	165.0545, 147.0440	2-Hydroxyphenyl-alanine	
14	1.93	123.05584		$C_6H_6N_2O$	106.0288, 96.0448	Nicotinamide**	27
15	2.09	277.13997		$C_{11}H_{20}N_2O_6$	259.1282, 213.1233	Saccharopine	
16	2.20	385.12942		$C_{14}H_{20}N_6O_5S$	136.0617, 134.0270	S-Adenosyl-homocysteine	
17	2.62		282.08385	$C_{10}H_{13}N_5O_5$	150.0408, 133.0142	Guanosine	
18	2.66		163.03952	$C_9H_8O_3$	119.0487, 93.0331	p-Coumaric acid	
19	2.95	268.10458		$C_{10}H_{13}N_5O_4$	136.0617, 119.0358	Adenosine**	
20	3.10	252.10967		$C_{10}H_{13}N_5O_3$	136.0617, 117.0547	2'-Deoxyadenosine	
21	3.17	166.08681		$C_9H_{11}NO_2$	149.0600, 131.0492	Phenylalanine**	27
22	3.37	153.04126		$C_5H_4N_4O_2$	136.0142, 110.0351	Xanthine	
23	4.80	220.11850		$C_9H_{17}NO_5$	202.1071, 184.0967	Pantothenic acid**	27
24	6.33	205.09771		$C_{11}H_{12}N_2O_2$	188.0705, 170.0597	Tryptophan**	27
25	6.75	129.05517		$C_6H_8O_3$	111.0443, 101.0601	Sotolone	27
26	8.35	190.05042		$C_{10}H_7NO_3$	162.0547, 144.0444	Kynurenic acid	
27	9.50	295.12940		$C_{14}H_{18}N_2O_5$	278.1019, 232.0964	γ-Glutamylphenyl-alanine	
28	9.94	134.04534		$C_4H_7NO_4$	116.0344, 88.0397	Aspartic acid	
29	9.94	298.09739		$C_{11}H_{15}N_5O_3S$	163.0423, 145.0318	5'-S-Methyl-5'-thioadenosine	
30	10.92		455.09680	$C_{17}H_{21}N_4O_9P$	255.0886, 241.0725	Flavin mononucleotide	

Peak	R_t	$[M+H]^+$	$[M-H]^-$	Formula	Fragments found	Assignment	Ref.
31	11.53	186.11302		$C_9H_{15}NO_3$	168.1018, 150.0914	Ecgonine	
32	11.81	271.06065		$C_{15}H_{10}O_5$	253.0483, 243.0648	Genistein	56
33	12.10		593.15065	$C_{27}H_{30}O_{15}$	503.1193, 473.1087	Vicenin-2	28
34	12.58		593.15065	$C_{27}H_{30}O_{15}$	503.1192, 473.1088	Apigenin-di-C-hexoside	28
35	12.81	193.05009		$C_{10}H_8O_4$	178.0259, 165.0544	Scopoletin	27
36	13.10		563.14009	$C_{26}H_{28}O_{14}$	503.1177, 473.1092	Vicenin-3	28
37	13.17	229.08647		$C_{14}H_{12}O_3$	211.0754, 183.0804	Resveratrol	
38	13.52	449.10839		$C_{21}H_{20}O_{11}$	395.0754, 377.0649	Isoorientin	27
39	13.70		563.14009	$C_{26}H_{28}O_{14}$	503.1196, 473.1077	Vicenin-1	28
40	13.82	200.12867		$C_{10}H_{17}NO_3$	182.1176, 100.0602	Ecgonine methyl ester	
41	13.96	433.11348		$C_{21}H_{20}O_{10}$	415.1003, 397.0918	Vitexin	27
42	14.33		577.15574	$C_{27}H_{30}O_{14}$	503.1193, 473.1084	Apigenin-6-C-glucoside-8-C-rhamnoside	28
43	14.51	433.11348		$C_{21}H_{20}O_{10}$	379.0811, 361.0699	Isovitexin	27
44	14.77		461.10839	$C_{22}H_{22}O_{11}$	371.0772, 353.0667	Scoparin	
45	15.69	493.13461		$C_{23}H_{25}O_{12}$	331.0806, 316.0572	Tricin-7-O-glucoside	57
46	16.18	595.14517		$C_{30}H_{26}O_{13}$	431.0971, 413.0861	Luteolin-8-C-(2"-O-(E)-p-coumaroyl-glycoside)	58
47	17.53		271.06065	$C_{15}H_{12}O_5$	227.0709, 177.0181	Naringenin	
48	18.12		1195.57478	$C_{56}H_{92}O_{27}$	705.3867, 609.3640	Trigofoenoside G	31
49	18.36		905.47461	$C_{44}H_{74}O_{19}$	773.4312, 611.3799	Trigoneoside Ia	29
50	18.49		1063.53252	$C_{51}H_{84}O_{23}$	609.3642, 447.3113	Protoyuccagenin-S4	31
51	18.75		919.49026	$C_{45}H_{76}O_{19}$	773.4318, 611.3799	Trigoneoside Xa	30
52	18.91		905.47461	$C_{44}H_{74}O_{19}$	773.4330, 611.3799	Trigoneoside Ib	29

Peak	R_t	$[M+H]^+$	$[M-H]^-$	Formula	Fragments found	Assignment	Ref.
53	18.97	331.08178		$C_{17}H_{14}O_7$	316.0573, 315.0494	Tricin	27
54	19.05		299.05556	$C_{16}H_{12}O_6$	284.0326, 256.0375	Chrysoeriol	32
55	19.57		887.46405	$C_{44}H_{72}O_{18}$	593.3685, 431.3164	Trigoneoside VIII	30
56	19.61		919.49026	$C_{45}H_{76}O_{19}$	773.4322, 611.3808	Trigoneoside Xb	30
57	19.62		1225.58534	$C_{57}H_{94}O_{28}$	1077.9872901.4840	Trigoneoside XIIIa	30
58	19.87		271.09704	$C_{16}H_{14}O_4$	243.1017, 161.0595	Medicarpin	27
59	19.67		889.47970	$C_{44}H_{74}O_{18}$	757.4375, 595.3850	Trigoneoside IIa	29
60	19.69		935.48518	$C_{45}H_{76}O_{20}$	757.4380, 595.3840	Protoneogitogenin -S5	31
61	19.79		1063.53252	$C_{51}H_{84}O_{23}$	755.4224, 593.3696	Trigoneoside IVa	29
62	19.74		1065.54817	$C_{51}H_{86}O_{23}$	757.4377, 595.3851	Trigofoenoside C	29
63	19.97		1047.53760	$C_{51}H_{84}O_{22}$	755.4224, 575.3589	Asparasaponin I (Protodioscin, Trigonelloside C)	31
64	20.19		901.47970	$C_{45}H_{74}O_{18}$	755.4287, 593.3687	Trigoneoside XIIa	30
65	20.29		903.49535	$C_{45}H_{76}O_{18}$	757.4388, 595.3852	Trigoneoside IIIa	29
66	21.47		941.51100	$C_{48}H_{78}O_{18}$	733.4561, 615.3893	Soyasaponin I	
67	27.22	457.36818		$C_{30}H_{48}O_3$	411.3607, 393.3505	Ursolic acid	

The phytoconstituents were defined based on specific retention time, accurate mass, isotopic distribution and fragmentation pattern, and by screening MS databases like Metlin, mzCloud and Massbank. All together the detected compounds could be rendered into ten categories of phytoconstituents, while the aqueous and hydro-alcoholic fenugreek seed extracts were featuring both similarities and differences with respect to their content (Table 3).

Table 3. Phytoconstituents identified in the aqueous and hydro-alcoholic fenugreek seed extracts. Compounds to be found only in aqueous extract are shown in blue, while compounds found only in hydro-alcoholic extracts are high lightened in yellow.

Phytoconstituents		Aqueous fenugreek	Hydro-alcoholic fenugreek
	Ecgonine	+	+
	Ecgonine methyl ester	+	+
	Kynurenic acid	+	+
Alkaloids	Trigonelline	+	+
Amino acids	2-Hydroxyphenylalanine	+	+
	4-Guanidinobutyric acid	+	+
	Arginine	+	+
	Asparagine[a]	+	
	Betaine (Trimethylglycine)	+	+
	Glutamic acid	+	+
	Aspartic acid[b]		+
	Phenylalanine	+	+
	Pipecolic acid	+	+
	Tryptophan	+	+
	N-α-Acetyl-lysine	+	+
	4-Hydroxyisoleucin[a]	+	
	γ-Glutamylphenylalanine	+	+
Coumarins	Scopoletin[b]		+
Flavonoids	Naringenin[b]		+
	Chrysoeriol[b]		+
	Tricin[b]		+
	Luteolin-8-C-(2”-O-(E)-p-coumaroylglycoside)[b]		+
	Tricin-7-O-glucoside[b]		+
	Genistein[b]		+
	Vitexin (Apigenin-8-C-glucoside)[b]		+
	Isovitexin (Apigenin-6-C-glucoside)	+	+
	Medicarpin[b]		+
	Scoparin (Chrysoeriol-8-C-glucoside)[b]		+
	Vicenin-2 (6,8-Di-C-glucosylapigenin)	+	+
	Apigenin-di-C-hexoside (Vicenin-2-isomer)	+	+
	Vicenin-3 (6-C-Glucosyl-8-C-xylosylapigenin)	+	+
	Isoorientin (Homoorientin, Luteolin-6-C-glucoside)	+	+
	Vicenin-1 (6-C-Xylosyl-8-C-glucosylapigenin)	+	+
	Apigenin-6-C-glucoside-8-C-rhamnoside	+	+

Phytoconstituents		Aqueous fenugreek	Hydro-alcoholic fenugreek
Other metabolites	Sotolone(3-Hydroxy-4,5-dimethyl-2(5H)furanone)	+	+
	Choline	+	+
	Saccharopine	+	+
Polyphenols	Resveratrol[b]		+
	p-Coumaric acid	+	+
Purines and pyrimidine	5'-S-Methyl-5'-thioadenosine[b]		+
	2'-Deoxyadenosine	+	+
	Adenine[a]	+	
	Adenosine	+	+
	Adenosine 3',5'-cyclic monophosphate[a]	+	
	Cytidine[a]	+	
	Flavin mononucleotide (FMN)[b]		+
	Guanine[a]	+	
	Guanosine	+	+
	S-Adenosylhomocysteine	+	+
	Uridine	+	+
	Xanthine[b]		+
	Xanthosine	+	+
Saponins	Soyasaponin I[b]		+
	Trigoneoside Ia	+	+
	Trigoneoside Ib	+	+
	Trigoneoside IIa	+	+
	Trigoneoside IIIa	+	+
	Trigoneoside IVa	+	+
	Trigoneoside VIII	+	+
	Trigoneoside Xa	+	+
	Trigoneoside Xb	+	+
	Trigoneoside XIIa	+	+
	Trigoneoside XIIIa	+	+
	Asparasaponin I (Protodisocin, Trigonelloside C)	+	+
	Trigofoneoside C	+	+
	Trigofoneoside G	+	+
	Protoneogitogenin-S5[b]		+
	Protoyuccagenin-S4	+	+
Terpenoid	Ursolic acid[b]		+
Vitamines	Nicotinamide	+	+
	Nicotinic acid (B3, niacin)[b]		+
	Pantothenic acid (B5)	+	+
	Pyridoxine (B6)	+	+

[a]Compounds to be found only in aqueous extract,
[b]Compounds found only in hydro-alcoholic extracts

According to our current knowledge, we are the first to identify among the **alkaloid** type of compounds the ecgonine methyl ester and ecognine in fenugreek seed extracts. In mice, ecgonine methyl ester was shown to protect against cocaine lethality. This effect is consistent with its vasodilatory effects [34]. Moreover, we are reporting for the first time in fenugreek extracts the presence of kynurenic acid that is produced via the kynurenine pathway of tryptophan amino acid catabolism, the latest to be found in our both fenugreek extracts [35]. The neuroprotective role of kynurenic acid has been demonstrated [36]. Our experiments confirm the presence of trigonelline in our both fenugreek seed extracts. Trigonelline was shown to inhibit Nrf2 together with blocking of Nrf2-dependent expression of proteasomal genes [37].

We were able to detect the 4-hydroxyisoleucin, the most abundant **amino acid** in fenugreek seeds together with asparagine both being only present in the aqueous extract. Aspartic acid was present only in the hydro-alcoholic fenugreek seed extract, while all the other amino acids listed in Table 3 could be found in both extracts.

Among **coumarins** we are reporting for the first time the identification of scopoletin in hydro-alcoholic extract of fenugreek seeds, a compound that has already described in fenugreek root, shoot, pod, stem and leaves [18, 38]. Scopoleptin was suggested to have an important anti-inflammatory effect by inhibiting the phosphorylation of NF-κB and p38 MAPK in mice [39], and to inhibit human tumor vascularization in xenograft models [40].

Flavonoids like naringenin, vitexin (apigenin-8-C-glucoside), luteolin-8-C-(2”-O-(E)-p-coumaroylglycoside, isoorientin, vicenin-1, vicenin-2, vicenin-3 (6-C-glucosyl-8-C-xylosylapigenin), apigenin-6-C-glucoside-8-C-rhamnoside, chrysoeriol and tricin had been reported already in fenugreek seeds [5, 28-33]. However, flavonoids like, tricin-7-O-glucoside, genistein, isovitexin (apigenin-6-C-glucoside), medicarpin, scoparin and apigenin-6-C-glucoside-8-C-rhamnoside are revealed for the first time in fenugreek seed extracts.

The scoparin is a chrysoeriol glucoside and its biological effects are not known. In case of chrysoeriol was shown to partly inhibit adipogenesis by blocking the accumulation of triacylglycerol in the 3T3-L1 cells [41]. Moreover, it was demonstrated that chrysoeriol is a PI3K-AKT-mTOR pathway inhibitor with potent antitumor activity against human multiple myeloma cells in vitro [42].

The genistein is an estrogen agonist phytoestrogen, and when isolated from soy, it is reported to display neuroprotective effects against neuronal death in animal models [43]. Experimental data suggested that genistein may exhibit anticancer properties on HT29 colon cancer cells by modulating caspase-3 and p38 MAPK pathway at different transcriptional and protein levels [44].

The isovitexin (apigenin-6-C-glucoside), an isomer of vitexin, generally occurring together with vitexin, and together are exhibiting diverse biological

activities like anti-oxidant, anti-cancer, anti-inflammatory, anti-hyperalgesic, and neuroprotective effects [45].

The medicarpin was shown to have osteogenic activity promoting bone regeneration by activating Wnt and Notch signalling pathway [46]. Medicarpin it was suggested to have pro-apoptotic effects against drug-sensitive (P388) and multidrug resistant P388 leukemia cells [47].

The **polyphenol** content of fenugreek seeds was also analysed, and the presence of resveratrol had been demonstrated for the first time in our hydro-alcoholic extract. Resveratrol was shown to affect lipids and arachidonic acid metabolisms, and together with its antioxidant activity elicited a great research interest in fields such as cancer, neurodegenerative and cardiovascular diseases and metabolic disorders [48].

Trigocoumarin and caffeic acid seemed to be present at a low abundance in our aqueous fenugreek seed extract as suggested by the molecular mass corresponding peaks, and the structure confirming isotopic pattern, but no fragmentation profiles were generated hence they have not been included in the tables with phytoconstituents.

The quercetin, p-coumaric acid and chlorogenic acid were poorly detectable in both extracts, and again the fragmentation profile based evidences are missing, yet molecular masses and isotopic patterns are available. Nevertheless they have not been included into the tables with phytoconstituents.

Among **metabolites** we were able to identify sotolone (3-Hydroxy-4,5-dimethyl-2(5H)furanone), choline and saccharopine as new phytoconstituents in fenugreek seed extracts. We have to admit that sotolone was also detected in fenugreek hairy root cultures [49]. The sotolone is known to impart powerful Madeira-oxidized-curry-walnut notes to various hydro-alcoholic beverages. It has been much studied in oxidized Jura flor-sherry wines, dry white wines, aged Roussillon sweet wines, and old Port wines, in which it contributes to the characteristic "Madeira-oxidized" aroma of these beverages [50]. However, the sotolone biological effects are not known, though it was shown to interfere with the maple syrup urine disease, which is a rare autosomal-recessive metabolic disorder caused by a deficit of oxidative decarboxylation of branched-chain amino acids [51].

The choline is another phytoconstituent that we show to be present in both of our fenugreek seed extracts. It has been demonstrated that choline supplementation in insulin resistant (IR) mice would ameliorate muscle function by remodelling glucose and fatty acid (FA) metabolism [52]. This will be achieved by the reduction of glucose utilization for FA and triglyceride (TAG) synthesis, and increased muscle storage of glucose as glycogen. It was demonstrated that a choline reach diet would prevent non-alcoholic fatty liver.

We have identified for the first time the saccharopine in fenugreek seed extracts. Lysine is catabolized in developing plant tissues through the saccharopine pathway, and have been shown to be involved in the development of maize seed and stress responses [53]. In the case of mammalian myotubes, saccharopine was shown to stimulate Akt and mTOR signalling that has suppressed autophagic-proteolysis, and might reduce muscle wasting [54].

Purines and pyrimidine such as 5'-S-Methyl-5'-thioadenosine, 2'-deoxyadenosine, adenine, adenosine, adenosine 3',5'-cyclic monophosphate, cytidine, flavin mononucleotide (FMN), guanine, guanosine, S-adenosyl-homocysteine, uridine and xanthine have been identified for the first time in fenugreek seed extracts. The presence of 5'-S-methyl-5'-thioadenosine in apples was correlated with the conversion of methionine related to ethylene biosynthesis [55]. S-adenosylhomocysteine is the by-product of all S-adenosylmethionine-dependent transmethylation reactions, and its presence seems to be related to cardiovascular disease, kidney disease, diabetes, and obesity [56].

The presence of **saponins** was extensively studied in the case of fenugreek including the vegetative organs and seeds of the plant [6-11]. The trigoneoside profile of our fenugreek seeds was different from that described for those originated from India and Egypt, respectively. Trigoneosides such as Ia, Ib, IIa, IIIa and IVa were present, while trigoneosides like IIb, IIIb, Va, Vb, VI, VIIb, VIIIb and IX were absent from our extracts as compared to the Indian fenugreek seed. On the other hand, trigoneosides like Xa, Xb, XIIa and XIIIa were identified, though trigoneosides like XIb and XIIb were missing from our fenugreek seed extracts as compared to the seeds of Egyptian origin. We were able to detect soyasaponin I in our fenugreek seed hydro-alcoholic extract. Soyasaponin I was shown to inhibit the Renin- Angiotensin- Aldosterone System, so it could be considered a potent native anti-hypertensive compound that has to be further tested [57]. However, diosgenin, gitogenin, tigogenin and betulin were poorly detectable in our hydro-alcoholic seed extract, while only traces of graecunin B, lupeol and betulinic acid were found in both of our seed extracts. We have to admit that due to the relatively law abundance of the above mentioned saponins in our extracts, we were unable to generate fragmentation profiles, so that their presence, was defined by the molecular mass corresponding peaks, and the structure confirming isotopic pattern. Neotigenin and fenugrin B were absent from our fenugreek seed extracts. It seems therefore likely that our fenugreek seed features a specific saponin profile, and that is clearly distinct from that were previously described. This it means that the hypocholesterolemic activity attributed to the saponin content of earlier described fenugreek seeds has to be carefully re-examined in the case of the fenugreek seed used in our experiments [12].

We were able to identify in our fenugreek seed extracts some of the already reported B group **vitamins** like niacin, pantothenic acid and pyridoxine, while nicotinamide was detected for the first time, but biotin was absent [24,25]. The thiamine, riboflavine, cyanocobalamin and vitamin C were hardly detectable in our fenugreek seed extracts, so in the absence of fragmentation profiles, their presence could only be confirmed by the molecular mass corresponding peaks, and the structure specific isotopic pattern. It has been suggested that some of the B vitamins act as cancer risk reduction agent [58], and having anti-inflammatory effects associated with atherosclerosis and autoimmunity [59].

We were also able to identify for the first time among terpenoids the ursolic acid that was present only in the hydro-alcoholic fenugreek seed extract. It has been demonstrated that the ursolic acid exerted anti-oxidative and anti-inflammatory effects on mouse brain injury model by activating the Nrf2-ARE pathway [60], while its anti-cancer and anti-metastatic effects were also proven [61,62].

CONCLUSIONS

The comparative chemomapping of aqueous and hydro-alcoholic fenugreek seed extracts revealed already known and new phytoconstituents that further support the antidiabetic effects of fenugreek seeds. Originally, these antidiabetic effects were attributed mainly to galactomannan, 4-hydroxyisoleucin (4-OH-Ile), diosgenin and trigonelline [63]. It had been shown that these compounds featured direct antidiabetic properties in clinical studies by increasing insulin secretion (4-OH-Ile), decreasing insulin resistance and glucose resorption (galactomannan), and improvement in B-cells regeneration (trigonelline). Moreover, the presence of such phytoconstituents in our extracts is expected to improve blood lipid spectre (4-OH-Ile, diosgenin), and to show reno-protective (4-OH-Ile, trigonelline), neuroprotective (trigonelline) and antioxidant (diosgenin, trigonelline) properties. Other phytoconstituents identified in our seed extracts plead for a more substantial neuroprotective (kynurenine, genistein, vitexin, isovitexian), anti-inflammatory (trigonelline, scopoletin, ursolic acid, vitamins), hypocholesterolemic (saponins), muscle and/or hepatic insulin resistance reducing (choline) effects. However, when the phytoconstituent profile of saponins from Hungarian seeds was compared to the previously reported Indian and African seeds some differences were imminent. These differences were of qualitative nature but it seems logic to envision other dissimilarities at the quantitative level too. The ecological and cultivation conditions together with the genome based specificities are going to influence the qualitative and quantitative phytoconstituent profile of any fenugreek cultivated variety. This is the reason

why the careful assessment of chemical composition of fenugreek seeds from different sources is of great importance especially if they are intended for human consumption.

Given the large body of phytoconstituents found in fenugreek seed with effects that span across a wide health promoting spectrum, the future studies are expected to shed light on the quantitative parameters, and the cellular mechanisms attributed to such extracts. In this respect, remains to be elucidated whether such a multi phytoconstituent extract elicits an overcompensation to a disruption of homeostasis or a direct stimulatory response. It is expected that both overcompensation/disruption of homeostasis or stimulatory response will be below the toxic threshold, yet highly consistent with the hormetic concentration-response model [64]. This is exactly the case for our aqueous fenugreek seed extract that at very low concentrations increases the viability and division rate of human breast cancerous cells, while at high concentrations is exceedingly cytotoxic [27]. Moreover, our hydro-alcoholic fenugreek seed extract features only cytotoxicity and no evident dose-dependent hormetic response. Taken together our paper is one such an attempt that tries to correlate the phytoconstituent profile of fenugreek seed extracts with their corresponding biological effect seen in case of human breast cancerous cell lines. More system biology type of experiments are needed to unravel the complexity of beneficial effects of fenugreek.

EXPERIMENTAL SECTION

x. Materials and methods

x.1. Chemicals and reagents

Acetonitrile, water and formic acid were procured from Fisher Scientific (Geel, Belgium), while ammonium acetate and ammonium formate were from Sigma-Aldrich (Munich, Germany).

x.2. Plant material

The fenugreek seeds were obtained from TRIGONELLA MED. LTD., Mosonmagyaróvár, Hungary.

x.3. Sample preparation

The aqueous extract was obtained by boiling 5g fenugreek dried seeds in 100 ml water for 5 minutes then left to cool down at room temperature and centrifuged for 10 minutes at 4000 rpm. The obtained

supernatant was filtered through Whatman filter paper, and aliquots stored in 15 ml Falcon tubes at -20°C freezer up until their use.

To obtain the hydro-alcoholic (ethanol : water 1:1) extract, 5 g dried fenugreeks seeds were extracted two times with 500 ml ethanol–water (1:1) by stirring for 4h at 40 °C. The generated primary extract was centrifuged at 4000 rpm for 10 min at room temperature, and finally the ethanol was removed using a rotation vacuum evaporator. The ethanol free extract was filtered using a 45 µm Milipore filter unit and stored at 4°C until further studies.

x.4. UHPLC-ESI-MS analysis

A Dionex Ultimate 3000RS UHPLC system equipped with a Thermo Accucore C18 column, 100/2.1 with a particle size of 2.6 µm was coupled to a Thermo Q Exactive Orbitrap mass spectrometer equipped with an electrospray ionization source (ESI), and the measurement accuracy was within 5ppm. The mass spectrometer was operated at 320°C capillary temperature, 4.0 kV in positive mode and 3.8 kV in negative mode of spray voltage, and a resolution of 35,000 in the case of MS, while 17,500 was for MS/MS. The 100-1000 m/z was the scanned mass interval. For MS/MS scans the collision energy was 40NCE. The difference between measured and calculated molecular ion masses were always below 5 ppm.

In case of positive ionization mode UHPLC separation, a specific eluent A (500 ml of water containing 10 ml of acetonitrile, 0.5 ml of formic acid and 2.5 mM of ammonium formate) and eluent B (500 ml of acetonitrile containing 10 ml of water, 0.5 ml of formic acid and 2.5 mM of ammonium formate) combination was used.

For the negative ionization mode UHPLC separation, another combination of eluent A (500 ml of water containing 10 ml of acetonitrile and 2.5 mM of ammonium acetate) and eluent B (500 ml of acetonitrile containing 10 ml of water and 2.5 mM of ammonium acetate) was applied.

The flow rate was set for 200 µl/min, and the same gradient elution program was used both positive and negative ionization mode type of determinations (0-1 min, 95% A, 1-22 min, 20% A; 22-24 min, 20% A; 24-26 min, 95% A; 26-40 min, 95% A). 5 µl of aqueous or hydro-alcoholic fenugreek seed extracts were injected at every run.

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