

*Dedicated to Professor Costel Sârbu on the  
Occasion of His 65<sup>th</sup> Anniversary*

## **ANALYSIS OF PHYTOCONSTITUENT PROFILE OF FENUGREEK –*TRIGONELLA FOENUUM-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 Petropoulos (1973) had been suggesting categories like the Fluorescent, 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].

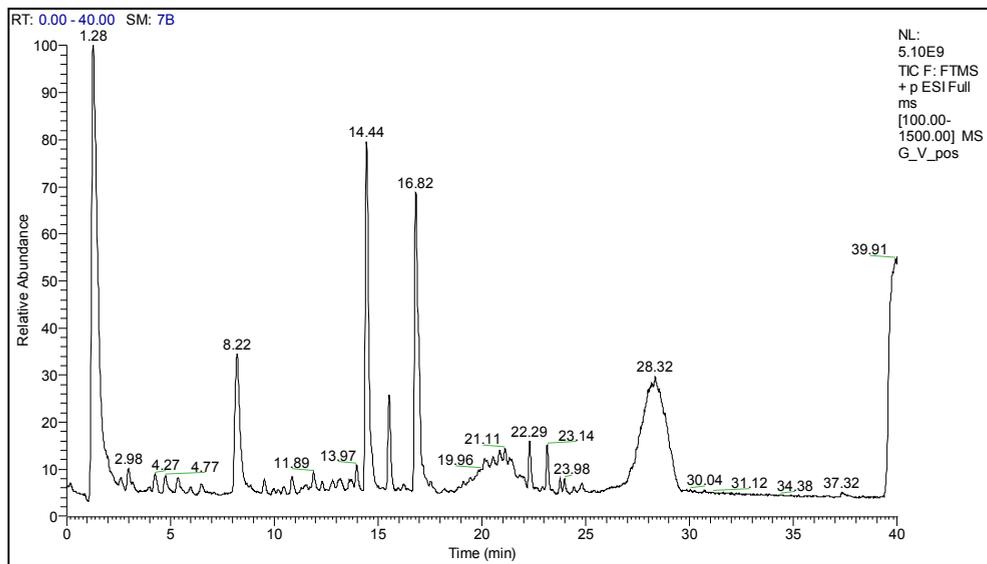
The fenugreek seeds contain (per 100g of edible portion): 369 calories, 7.8% moisture, 28.2 g protein, 5.9 g fat, 54.5 g total carbohydrate, 8g fibre, 3.6 g ash [4]. Fenugreek seeds containing diosgenin are considered one of the few natural sources of steroid saponin that is used for the synthesis of 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 from 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, stigmaterol,  $\beta$ -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, vicianin-1, vicianin-2, naringenin, kaempferol, 7,4'-dimethoxyflavanone were identified among flavonoids. Other phenolic compounds were detected in different parts of plants (root, shoot, and pod) like

scopoletin, 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].

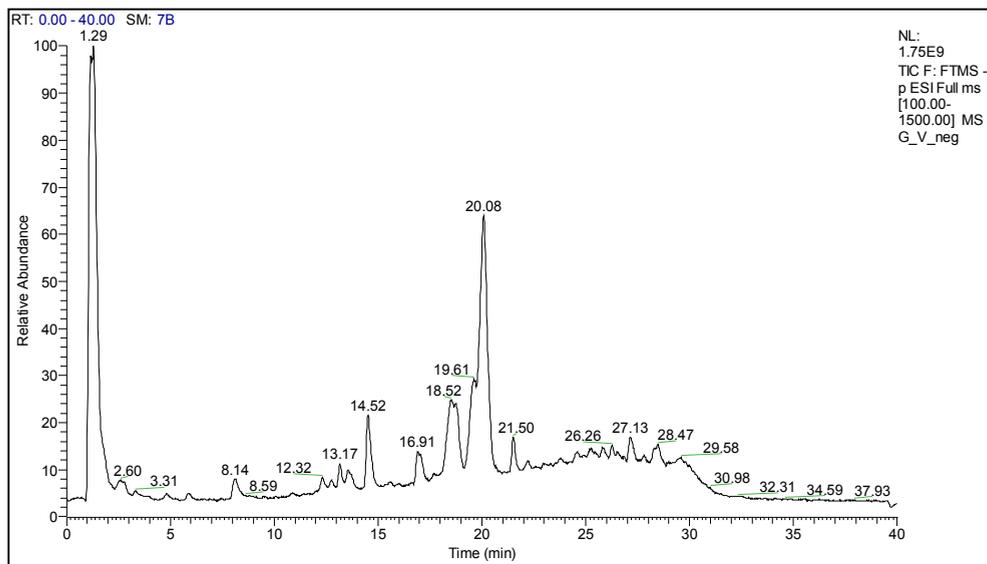
In the current paper we are reporting the UHPLC-ESI-MS chemomapping of aqueous and hydro-alcoholic fenugreek seed extracts that were found by us to induce dose dependent hormetic response and cytotoxicity in case of human breast cancerous cell lines [27]. We were able to detect 54 and 67 phytoconstituents 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.

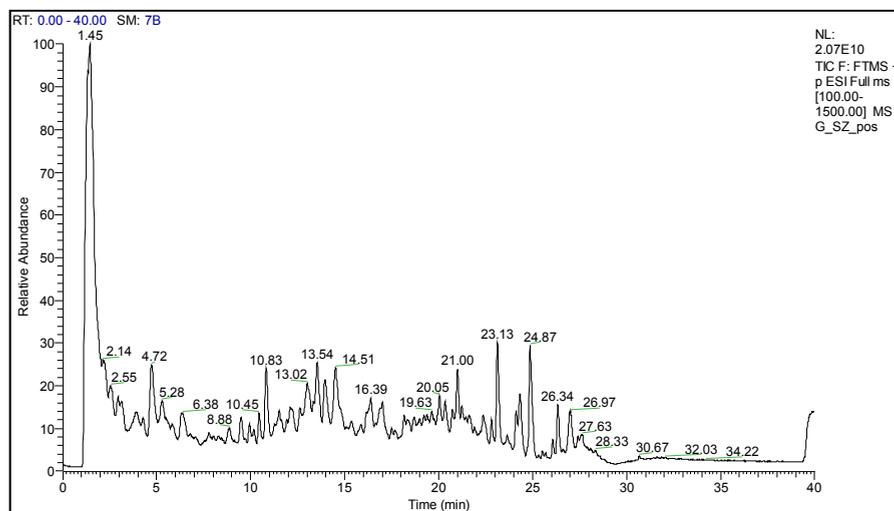
**Table 1.** Phytoconstituents identified in the aqueous fenugreek seed extract. Rt –retention time; [M+H]<sup>+</sup> - molecular ion masses; [M+H]<sup>-</sup> - the found fragment ion mass; Ref- references; (\*) [M]<sup>+</sup>; (\*\*) confirmed by standards.

| Peak | R <sub>t</sub> | [M+H] <sup>+</sup>     | [M+H] <sup>-</sup> | Formula   | Fragment<br>s found   | Assignment                                  | Ref. |
|------|----------------|------------------------|--------------------|---|-----------------------|---|------|
| 1    | 1.27           | 104.10754 <sup>*</sup> |                    | C <sub>5</sub> H <sub>14</sub> NO                               | 60.0814,<br>59.0735   | Choline                                     | 27   |
| 2    | 1.30           | 138.05550 <sup>*</sup> |                    | C <sub>7</sub> H <sub>8</sub> NO <sub>2</sub>                   | 110.0601,<br>96.0450  | Trigonelline                                | 27   |
| 3    | 1.31           | 175.11951              |                    | C <sub>6</sub> H <sub>14</sub> N <sub>4</sub> O <sub>2</sub>    | 158.0922,<br>130.0975 | Arginine <sup>**</sup>                      | 27   |
| 4    | 1.31           | 148.06099              |                    | C <sub>5</sub> H <sub>9</sub> NO <sub>4</sub>                   | 130.0863,<br>102.0553 | Glutamic acid                               | 27   |
| 5    | 1.31           | 118.08681              |                    | C <sub>5</sub> H <sub>11</sub> NO <sub>2</sub>                  | 59.0736,<br>58.0657   | Betaine                                     | 27   |
| 6    | 1.32           | 133.06132              |                    | C <sub>4</sub> H <sub>8</sub> N <sub>2</sub> O <sub>3</sub>     | 116.0342,<br>88.0397  | Asparagine <sup>**</sup>                    | 27   |
| 7    | 1.43           | 189.12392              |                    | C <sub>8</sub> H <sub>16</sub> N <sub>2</sub> O <sub>3</sub>    | 172.0961,<br>130.0863 | N-α-Acetyl-<br>lysine                       |      |
| 8    | 1.46           | 148.09737              |                    | C <sub>6</sub> H <sub>13</sub> NO <sub>3</sub>                  | 130.0862,<br>113.0598 | 4-Hydroxyiso-<br>leucine                    | 27   |
| 9    | 1.49           | 324.05968              |                    | C <sub>9</sub> H <sub>13</sub> N <sub>3</sub> O <sub>5</sub>    | 112.0507,<br>95.0243  | Cytidine <sup>**</sup>                      |      |
| 10   | 1.51           | 146.09296              |                    | C <sub>5</sub> H <sub>11</sub> N <sub>3</sub> O <sub>2</sub>    | 128.0821,<br>111.0555 | 4-Guanidino-<br>butyric acid                |      |
| 11   | 1.52           | 130.08681              |                    | C <sub>6</sub> H <sub>11</sub> NO <sub>2</sub>                  | 84.0812,<br>67.0548   | Pipecolic acid                              |      |
| 12   | 1.56           | 136.06233              |                    | C <sub>5</sub> H <sub>5</sub> N <sub>5</sub>                    | 119.0352,<br>94.0406  | Adenine                                     |      |
| 13   | 1.66           |                        | 283.06786          | C <sub>10</sub> H <sub>12</sub> N <sub>4</sub> O <sub>6</sub>   | 151.0248,<br>108.0188 | Xanthosine                                  |      |
| 14   | 1.73           |                        | 243.06171          | C <sub>9</sub> H <sub>12</sub> N <sub>2</sub> O <sub>6</sub>    | 200.0558,<br>153.0293 | Uridine                                     |      |
| 15   | 1.75           | 170.08172              |                    | C <sub>8</sub> H <sub>11</sub> NO <sub>3</sub>                  | 152.0704,<br>134.0600 | Pyridoxine <sup>**</sup>                    | 27   |
| 16   | 1.82           | 182.08172              |                    | C <sub>9</sub> H <sub>11</sub> NO <sub>3</sub>                  | 165.0545,<br>147.0439 | 2-<br>Hydroxyphenyl-<br>alanine             |      |
| 17   | 1.96           | 123.05584              |                    | C <sub>6</sub> H <sub>6</sub> N <sub>2</sub> O                  | 106.0287,<br>96.0447  | Nicotinamide <sup>**</sup>                  | 27   |
| 18   | 2.01           | 330.06035              |                    | C <sub>10</sub> H <sub>12</sub> N <sub>5</sub> O <sub>6</sub> P | 232.0828,<br>136.0617 | Adenosine 3',5'-<br>cyclic<br>monophosphate |      |
| 19   | 2.10           | 277.13997              |                    | C <sub>11</sub> H <sub>20</sub> N <sub>2</sub> O <sub>6</sub>   | 259.1286,<br>213.1231 | Saccharopine                                |      |
| 20   | 2.23           | 385.12942              |                    | C <sub>14</sub> H <sub>20</sub> N <sub>6</sub> O <sub>5</sub> S | 136.0618,<br>134.0271 | S-Adenosyl-<br>homocysteine                 |      |
| 21   | 2.60           | 152.05724              |                    | C <sub>5</sub> H <sub>5</sub> N <sub>5</sub> O                  | 135.0301,<br>128.0455 | Guanine                                     |      |

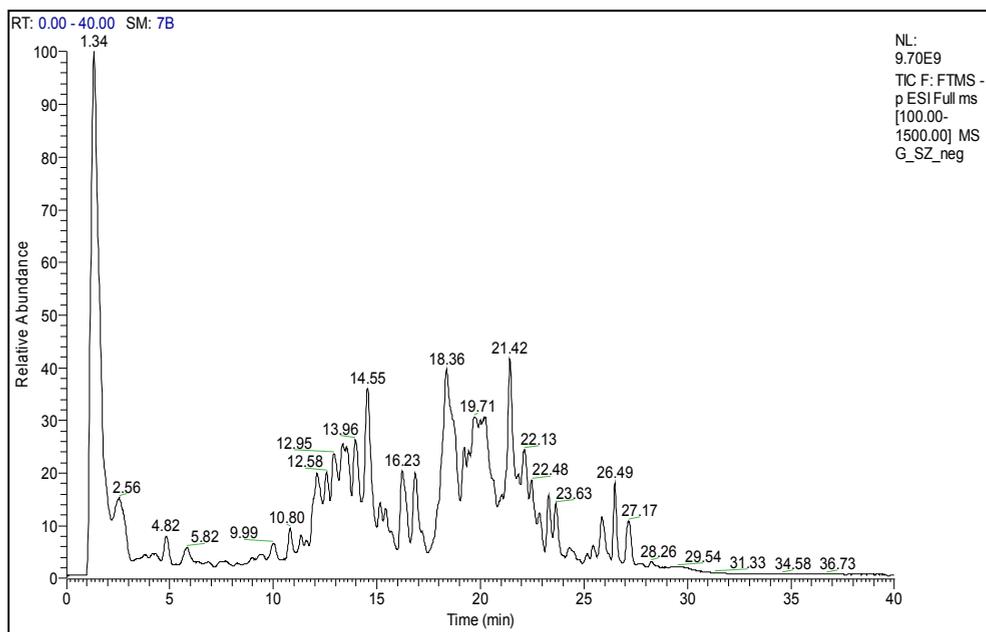
| Peak | R <sub>t</sub> | [M+H] <sup>+</sup> | [M-H] <sup>-</sup> | Formula   | Fragment<br>s found   | Assignment                                | Ref. |
|------|----------------|--------------------|--------------------|---|-----------------------|---|------|
| 22   | 2.63           |                    | 282.08385          | C <sub>10</sub> H <sub>13</sub> N <sub>5</sub> O <sub>5</sub> | 150.0407,<br>133.0142 | Guanosine                                 |      |
| 23   | 2.74           |                    | 163.03952          | C <sub>9</sub> H <sub>8</sub> O <sub>3</sub>                  | 119.0487,<br>93.0329  | p-Coumaric acid                           |      |
| 24   | 2.95           | 268.10458          |                    | C <sub>10</sub> H <sub>13</sub> N <sub>5</sub> O <sub>4</sub> | 136.0617,<br>119.0358 | Adenosine**                               |      |
| 25   | 3.10           | 252.10967          |                    | C <sub>10</sub> H <sub>13</sub> N <sub>5</sub> O <sub>3</sub> | 136.0618,<br>117.0548 | 2'-Deoxyadenosine                         |      |
| 26   | 3.21           | 166.08681          |                    | C <sub>9</sub> H <sub>11</sub> NO <sub>2</sub>                | 149.0598,<br>131.0493 | Phenylalanine**                           | 27   |
| 27   | 4.86           | 220.11850          |                    | C <sub>9</sub> H <sub>17</sub> NO <sub>5</sub>                | 202.1073,<br>184.0967 | Pantothenic acid**                        | 27   |
| 28   | 6.49           | 205.09771          |                    | C <sub>11</sub> H <sub>12</sub> N <sub>2</sub> O <sub>2</sub> | 188.0706,<br>170.0599 | Tryptophan**                              | 27   |
| 29   | 6.75           | 129.05517          |                    | C <sub>6</sub> H <sub>8</sub> O <sub>3</sub>                  | 111.0443,<br>101.0600 | Sotolone                                  | 27   |
| 30   | 8.31           | 190.05042          |                    | C <sub>10</sub> H <sub>7</sub> NO <sub>3</sub>                | 162.0547,<br>144.0435 | Kynurenic acid                            |      |
| 31   | 9.53           | 295.12940          |                    | C <sub>14</sub> H <sub>18</sub> N <sub>2</sub> O <sub>5</sub> | 278.1017,<br>232.0965 | γ-Glutamylphenylalanine                   |      |
| 32   | 11.56          | 186.11302          |                    | C <sub>9</sub> H <sub>15</sub> NO <sub>3</sub>                | 168.1017,<br>150.0909 | Ecgonine                                  |      |
| 33   | 12.32          |                    | 593.15065          | C <sub>27</sub> H <sub>30</sub> O <sub>15</sub>               | 503.1202,<br>473.1087 | Vicenin-2                                 | 28   |
| 34   | 12.75          |                    | 593.15065          | C <sub>27</sub> H <sub>30</sub> O <sub>15</sub>               | 503.1215,<br>473.1088 | Apigenin-di-C-hexoside (Vicenin-2-isomer) | 28   |
| 35   | 13.17          |                    | 563.14009          | C <sub>26</sub> H <sub>28</sub> O <sub>14</sub>               | 503.1187,<br>473.1091 | Vicenin-3                                 | 28   |
| 36   | 13.74          | 449.10839          |                    | C <sub>21</sub> H <sub>20</sub> O <sub>11</sub>               | 395.0760,<br>377.0658 | Isoorientin                               | 27   |
| 37   | 13.75          |                    | 563.14009          | C <sub>26</sub> H <sub>28</sub> O <sub>14</sub>               | 503.1194,<br>473.1096 | Vicenin-1                                 | 28   |
| 38   | 13.90          | 200.12867          |                    | C <sub>10</sub> H <sub>17</sub> NO <sub>3</sub>               | 182.1174,<br>100.0759 | Ecgonine methyl ester                     |      |
| 39   | 14.43          |                    | 577.15574          | C <sub>27</sub> H <sub>30</sub> O <sub>14</sub>               | 503.1193,<br>473.1097 | Apigenin-6-C-glucoside-8-C-rhamnoside     | 28   |
| 40   | 14.65          | 433.11348          |                    | C <sub>21</sub> H <sub>20</sub> O <sub>10</sub>               | 379.0805,<br>361.0709 | Isovitexin                                | 27   |
| 41   | 18.25          |                    | 1195.57478         | C <sub>56</sub> H <sub>92</sub> O <sub>27</sub>               | 705.3873,<br>609.3632 | Trigofoenoside G                          | 31   |
| 42   | 18.49          |                    | 905.47461          | C <sub>44</sub> H <sub>74</sub> O <sub>19</sub>               | 773.4326,<br>611.3798 | Trigoneoside Ia                           | 29   |
| 43   | 18.62          |                    | 1063.53252         | C <sub>51</sub> H <sub>84</sub> O <sub>23</sub>               | 609.3646,<br>447.3091 | Protoyuccagenin-S4                        | 31   |
| 44   | 18.83          |                    | 919.49026          | C <sub>45</sub> H <sub>76</sub> O <sub>19</sub>               | 773.4315,<br>611.3812 | Trigoneoside Xa                           | 30   |

| Peak | R <sub>t</sub> | [M+H] <sup>+</sup> | [M-H] <sup>-</sup> | Formula   | Fragment<br>s found   | Assignment   | Ref. |
|------|----------------|--------------------|--------------------|---|-----------------------|--|------|
| 45   | 18.84          |                    | 905.47461          | C <sub>44</sub> H <sub>74</sub> O <sub>19</sub> | 773.4336,<br>611.3795 | Trigoneoside Ib  | 29   |
| 46   | 19.61          |                    | 919.49026          | C <sub>45</sub> H <sub>76</sub> O <sub>19</sub> | 773.4322,<br>611.3808 | Trigoneoside Xb  | 30   |
| 47   | 19.73          |                    | 887.46405          | C <sub>44</sub> H <sub>72</sub> O <sub>18</sub> | 593.3680,<br>431.3171 | Trigoneoside VIII                                      | 30   |
| 48   | 19.78          |                    | 1225.58534         | C <sub>57</sub> H <sub>94</sub> O <sub>28</sub> | 1077.2218<br>901.4799 | Trigoneoside<br>XIIIa                                  | 30   |
| 49   | 19.88          |                    | 889.47970          | C <sub>44</sub> H <sub>74</sub> O <sub>18</sub> | 757.4387,<br>595.3850 | Trigoneoside IIa                                       | 29   |
| 50   | 19.98          |                    | 1063.53252         | C <sub>51</sub> H <sub>84</sub> O <sub>23</sub> | 755.4216,<br>593.3688 | Trigoneoside IVa                                       | 29   |
| 51   | 20.00          |                    | 1065.54817         | C <sub>51</sub> H <sub>86</sub> O <sub>23</sub> | 757.4368,<br>595.3844 | Trigofoenoside C                                       | 29   |
| 52   | 20.10          |                    | 1047.53760         | C <sub>51</sub> H <sub>84</sub> O <sub>22</sub> | 755.4216,<br>575.3581 | Asparasaponin I<br>(Protodioscin,<br>Trigonelloside C) | 31   |
| 53   | 20.30          |                    | 901.47970          | C <sub>45</sub> H <sub>74</sub> O <sub>18</sub> | 755.4237,<br>593.3704 | Trigoneoside<br>XIIa                                   | 30   |
| 54   | 20.37          |                    | 903.49535          | C <sub>45</sub> H <sub>76</sub> O <sub>18</sub> | 757.4390,<br>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]<sup>+</sup> - molecular ion masses; [M+H]<sup>-</sup> - the found fragment ion mass; Ref- references; (\*) [M]<sup>+</sup>; (\*\*) confirmed by standards.

| Peak | R <sub>t</sub> | [M+H] <sup>+</sup>     | [M-H] <sup>-</sup> | Formula  | Fragments found       | Assignment              | Ref. |
|------|----------------|------------------------|--------------------|--|-----------------------|-------------------------|------|
| 1    | 1.22           | 104.10754 <sup>*</sup> |                    | C <sub>5</sub> H <sub>14</sub> NO                            | 60.0814,<br>59.0736   | Choline                 | 27   |
| 2    | 1.32           | 138.05550 <sup>*</sup> |                    | C <sub>7</sub> H <sub>8</sub> NO <sub>2</sub>                | 110.0604,<br>96.0447  | Trigonelline            | 27   |
| 3    | 1.31           | 175.11951              |                    | C <sub>6</sub> H <sub>14</sub> N <sub>4</sub> O <sub>2</sub> | 158.0923,<br>130.0975 | Arginine <sup>**</sup>  | 27   |
| 4    | 1.31           | 148.06099              |                    | C <sub>5</sub> H <sub>9</sub> NO <sub>4</sub>                | 130.0863,<br>102.0553 | Glutamic acid           | 27   |
| 5    | 1.31           | 118.08681              |                    | C <sub>5</sub> H <sub>11</sub> NO <sub>2</sub>               | 59.0736,<br>58.0657   | Betaine                 | 27   |
| 6    | 1.40           | 189.12392              |                    | C <sub>8</sub> H <sub>16</sub> N <sub>2</sub> O <sub>3</sub> | 172.0962,<br>130.0862 | N-α-Acetyl-lysine       |      |
| 7    | 1.42           | 146.09296              |                    | C <sub>5</sub> H <sub>11</sub> N <sub>3</sub> O <sub>2</sub> | 128.0810,<br>111.0556 | 4-Guanidinobutyric acid |      |

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| Peak | R <sub>t</sub> | [M+H] <sup>+</sup> | [M-H] <sup>-</sup> | Formula   | Fragments found       | Assignment                       | Ref. |
|------|----------------|--------------------|--------------------|---|-----------------------|----------------------------------|------|
| 8    | 1.46           | 130.08681          |                    | C <sub>6</sub> H <sub>11</sub> NO <sub>2</sub>                  | 84.0812,<br>67.0547   | Pipecolic acid                   |      |
| 9    | 1.63           | 124.03986          |                    | C <sub>6</sub> H <sub>5</sub> NO <sub>2</sub>                   | 96.0448,<br>80.0500   | Nicotinic acid**                 | 27   |
| 10   | 1.72           |                    | 283.06786          | C <sub>10</sub> H <sub>12</sub> N <sub>4</sub> O <sub>6</sub>   | 151.0248,<br>108.0188 | Xanthosine                       |      |
| 11   | 1.73           |                    | 243.06171          | C <sub>9</sub> H <sub>12</sub> N <sub>2</sub> O <sub>6</sub>    | 200.0556,<br>153.0292 | Uridine                          |      |
| 12   | 1.72           | 170.08172          |                    | C <sub>8</sub> H <sub>11</sub> NO <sub>3</sub>                  | 152.0705,<br>134.0601 | Pyridoxine**                     | 27   |
| 13   | 1.79           | 182.08172          |                    | C <sub>9</sub> H <sub>11</sub> NO <sub>3</sub>                  | 165.0545,<br>147.0440 | 2-Hydroxyphenyl-<br>alanine      |      |
| 14   | 1.93           | 123.05584          |                    | C <sub>6</sub> H <sub>6</sub> N <sub>2</sub> O                  | 106.0288,<br>96.0448  | Nicotinamide**                   | 27   |
| 15   | 2.09           | 277.13997          |                    | C <sub>11</sub> H <sub>20</sub> N <sub>2</sub> O <sub>6</sub>   | 259.1282,<br>213.1233 | Saccharopine                     |      |
| 16   | 2.20           | 385.12942          |                    | C <sub>14</sub> H <sub>20</sub> N <sub>6</sub> O <sub>5</sub> S | 136.0617,<br>134.0270 | S-Adenosyl-<br>homocysteine      |      |
| 17   | 2.62           |                    | 282.08385          | C <sub>10</sub> H <sub>13</sub> N <sub>5</sub> O <sub>5</sub>   | 150.0408,<br>133.0142 | Guanosine                        |      |
| 18   | 2.66           |                    | 163.03952          | C <sub>9</sub> H <sub>8</sub> O <sub>3</sub>                    | 119.0487,<br>93.0331  | p-Coumaric acid                  |      |
| 19   | 2.95           | 268.10458          |                    | C <sub>10</sub> H <sub>13</sub> N <sub>5</sub> O <sub>4</sub>   | 136.0617,<br>119.0358 | Adenosine**                      |      |
| 20   | 3.10           | 252.10967          |                    | C <sub>10</sub> H <sub>13</sub> N <sub>5</sub> O <sub>3</sub>   | 136.0617,<br>117.0547 | 2'-<br>Deoxyadenosine            |      |
| 21   | 3.17           | 166.08681          |                    | C <sub>9</sub> H <sub>11</sub> NO <sub>2</sub>                  | 149.0600,<br>131.0492 | Phenylalanine**                  | 27   |
| 22   | 3.37           | 153.04126          |                    | C <sub>5</sub> H <sub>4</sub> N <sub>4</sub> O <sub>2</sub>     | 136.0142,<br>110.0351 | Xanthine                         |      |
| 23   | 4.80           | 220.11850          |                    | C <sub>9</sub> H <sub>17</sub> NO <sub>5</sub>                  | 202.1071,<br>184.0967 | Pantothenic acid**               | 27   |
| 24   | 6.33           | 205.09771          |                    | C <sub>11</sub> H <sub>12</sub> N <sub>2</sub> O <sub>2</sub>   | 188.0705,<br>170.0597 | Tryptophan**                     | 27   |
| 25   | 6.75           | 129.05517          |                    | C <sub>6</sub> H <sub>8</sub> O <sub>3</sub>                    | 111.0443,<br>101.0601 | Sotolone                         | 27   |
| 26   | 8.35           | 190.05042          |                    | C <sub>10</sub> H <sub>7</sub> NO <sub>3</sub>                  | 162.0547,<br>144.0444 | Kynurenic acid                   |      |
| 27   | 9.50           | 295.12940          |                    | C <sub>14</sub> H <sub>18</sub> N <sub>2</sub> O <sub>5</sub>   | 278.1019,<br>232.0964 | γ-Glutamylphenyl-<br>alanine     |      |
| 28   | 9.94           | 134.04534          |                    | C <sub>4</sub> H <sub>7</sub> NO <sub>4</sub>                   | 116.0344,<br>88.0397  | Aspartic acid                    |      |
| 29   | 9.94           | 298.09739          |                    | C <sub>11</sub> H <sub>15</sub> N <sub>5</sub> O <sub>3</sub> S | 163.0423,<br>145.0318 | 5'-S-Methyl-5'-<br>thioadenosine |      |
| 30   | 10.92          |                    | 455.09680          | C <sub>17</sub> H <sub>21</sub> N <sub>4</sub> O <sub>9</sub> P | 255.0886,<br>241.0725 | Flavin<br>mononucleotide         |      |

| Peak | R <sub>t</sub> | [M+H] <sup>+</sup> | [M-H] <sup>-</sup> | Formula   | Fragments found       | Assignment                                    | Ref. |
|------|----------------|--------------------|--------------------|---|-----------------------|---|------|
| 31   | 11.53          | 186.11302          |                    | C <sub>9</sub> H <sub>15</sub> NO <sub>3</sub>  | 168.1018,<br>150.0914 | Ecgonine                                      |      |
| 32   | 11.81          | 271.06065          |                    | C <sub>15</sub> H <sub>10</sub> O <sub>5</sub>  | 253.0483,<br>243.0648 | Genistein                                     | 56   |
| 33   | 12.10          |                    | 593.15065          | C <sub>27</sub> H <sub>30</sub> O <sub>15</sub> | 503.1193,<br>473.1087 | Vicenin-2                                     | 28   |
| 34   | 12.58          |                    | 593.15065          | C <sub>27</sub> H <sub>30</sub> O <sub>15</sub> | 503.1192,<br>473.1088 | Apigenin-di-C-hexoside                        | 28   |
| 35   | 12.81          | 193.05009          |                    | C <sub>10</sub> H <sub>8</sub> O <sub>4</sub>   | 178.0259,<br>165.0544 | Scopoletin                                    | 27   |
| 36   | 13.10          |                    | 563.14009          | C <sub>26</sub> H <sub>28</sub> O <sub>14</sub> | 503.1177,<br>473.1092 | Vicenin-3                                     | 28   |
| 37   | 13.17          | 229.08647          |                    | C <sub>14</sub> H <sub>12</sub> O <sub>3</sub>  | 211.0754,<br>183.0804 | Resveratrol                                   |      |
| 38   | 13.52          | 449.10839          |                    | C <sub>21</sub> H <sub>20</sub> O <sub>11</sub> | 395.0754,<br>377.0649 | Isoorientin                                   | 27   |
| 39   | 13.70          |                    | 563.14009          | C <sub>26</sub> H <sub>28</sub> O <sub>14</sub> | 503.1196,<br>473.1077 | Vicenin-1                                     | 28   |
| 40   | 13.82          | 200.12867          |                    | C <sub>10</sub> H <sub>17</sub> NO <sub>3</sub> | 182.1176,<br>100.0602 | Ecgonine methyl ester                         |      |
| 41   | 13.96          | 433.11348          |                    | C <sub>21</sub> H <sub>20</sub> O <sub>10</sub> | 415.1003,<br>397.0918 | Vitexin                                       | 27   |
| 42   | 14.33          |                    | 577.15574          | C <sub>27</sub> H <sub>30</sub> O <sub>14</sub> | 503.1193,<br>473.1084 | Apigenin-6-C-glucoside-8-C-rhamnoside         | 28   |
| 43   | 14.51          | 433.11348          |                    | C <sub>21</sub> H <sub>20</sub> O <sub>10</sub> | 379.0811,<br>361.0699 | Isovitexin                                    | 27   |
| 44   | 14.77          |                    | 461.10839          | C <sub>22</sub> H <sub>22</sub> O <sub>11</sub> | 371.0772,<br>353.0667 | Scoparin                                      |      |
| 45   | 15.69          | 493.13461          |                    | C <sub>23</sub> H <sub>25</sub> O <sub>12</sub> | 331.0806,<br>316.0572 | Tricin-7-O-glucoside                          | 57   |
| 46   | 16.18          | 595.14517          |                    | C <sub>30</sub> H <sub>26</sub> O <sub>13</sub> | 431.0971,<br>413.0861 | Luteolin-8-C-(2"-O-(E)-p-coumaroyl-glycoside) | 58   |
| 47   | 17.53          |                    | 271.06065          | C <sub>15</sub> H <sub>12</sub> O <sub>5</sub>  | 227.0709,<br>177.0181 | Naringenin                                    |      |
| 48   | 18.12          |                    | 1195.57478         | C <sub>56</sub> H <sub>92</sub> O <sub>27</sub> | 705.3867,<br>609.3640 | Trigofoenoside G                              | 31   |
| 49   | 18.36          |                    | 905.47461          | C <sub>44</sub> H <sub>74</sub> O <sub>19</sub> | 773.4312,<br>611.3799 | Trigoneoside Ia                               | 29   |
| 50   | 18.49          |                    | 1063.53252         | C <sub>51</sub> H <sub>84</sub> O <sub>23</sub> | 609.3642,<br>447.3113 | Protoyuccagenin-S4                            | 31   |
| 51   | 18.75          |                    | 919.49026          | C <sub>45</sub> H <sub>76</sub> O <sub>19</sub> | 773.4318,<br>611.3799 | Trigoneoside Xa                               | 30   |
| 52   | 18.91          |                    | 905.47461          | C <sub>44</sub> H <sub>74</sub> O <sub>19</sub> | 773.4330,<br>611.3799 | Trigoneoside Ib                               | 29   |

| Peak | R <sub>t</sub> | [M+H] <sup>+</sup> | [M-H] <sup>-</sup> | Formula   | Fragments found       | Assignment   | Ref. |
|------|----------------|--------------------|--------------------|---|-----------------------|--|------|
| 53   | 18.97          | 331.08178          |                    | C <sub>17</sub> H <sub>14</sub> O <sub>7</sub>  | 316.0573,<br>315.0494 | Tricin   | 27   |
| 54   | 19.05          |                    | 299.05556          | C <sub>16</sub> H <sub>12</sub> O <sub>6</sub>  | 284.0326,<br>256.0375 | Chrysoeriol  | 32   |
| 55   | 19.57          |                    | 887.46405          | C <sub>44</sub> H <sub>72</sub> O <sub>18</sub> | 593.3685,<br>431.3164 | Trigoneoside VIII                                      | 30   |
| 56   | 19.61          |                    | 919.49026          | C <sub>45</sub> H <sub>76</sub> O <sub>19</sub> | 773.4322,<br>611.3808 | Trigoneoside Xb  | 30   |
| 57   | 19.62          |                    | 1225.58534         | C <sub>57</sub> H <sub>94</sub> O <sub>28</sub> | 1077.98729<br>01.4840 | Trigoneoside XIIIa                                     | 30   |
| 58   | 19.87          |                    | 271.09704          | C <sub>16</sub> H <sub>14</sub> O <sub>4</sub>  | 243.1017,<br>161.0595 | Medicarpin   | 27   |
| 59   | 19.67          |                    | 889.47970          | C <sub>44</sub> H <sub>74</sub> O <sub>18</sub> | 757.4375,<br>595.3850 | Trigoneoside IIa                                       | 29   |
| 60   | 19.69          |                    | 935.48518          | C <sub>45</sub> H <sub>76</sub> O <sub>20</sub> | 757.4380,<br>595.3840 | Protoneogitogenin<br>-S5                               | 31   |
| 61   | 19.79          |                    | 1063.53252         | C <sub>51</sub> H <sub>84</sub> O <sub>23</sub> | 755.4224,<br>593.3696 | Trigoneoside IVa                                       | 29   |
| 62   | 19.74          |                    | 1065.54817         | C <sub>51</sub> H <sub>86</sub> O <sub>23</sub> | 757.4377,<br>595.3851 | Trigofoenoside C                                       | 29   |
| 63   | 19.97          |                    | 1047.53760         | C <sub>51</sub> H <sub>84</sub> O <sub>22</sub> | 755.4224,<br>575.3589 | Asparasaponin I<br>(Protodioscin,<br>Trigonelloside C) | 31   |
| 64   | 20.19          |                    | 901.47970          | C <sub>45</sub> H <sub>74</sub> O <sub>18</sub> | 755.4287,<br>593.3687 | Trigoneoside XIIa                                      | 30   |
| 65   | 20.29          |                    | 903.49535          | C <sub>45</sub> H <sub>76</sub> O <sub>18</sub> | 757.4388,<br>595.3852 | Trigoneoside IIIa                                      | 29   |
| 66   | 21.47          |                    | 941.51100          | C <sub>48</sub> H <sub>78</sub> O <sub>18</sub> | 733.4561,<br>615.3893 | Soyasaponin I  |      |
| 67   | 27.22          | 457.36818          |                    | C <sub>30</sub> H <sub>48</sub> O <sub>3</sub>  | 411.3607,<br>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 |
|--------------------|--|-------------------------|---------------------------|
| <b>Alkaloids</b>   | Ecgonine   | +                       | +                         |
|                    | Ecgonine methyl ester                                      | +                       | +                         |
|                    | Kynurenic acid   | +                       | +                         |
|                    | Trigonelline   | +                       | +                         |
| <b>Amino acids</b> | 2-Hydroxyphenylalanine                                     | +                       | +                         |
|                    | 4-Guanidinobutyric acid                                    | +                       | +                         |
|                    | Arginine   | +                       | +                         |
|                    | Asparagine <sup>a</sup>                                    | +                       |                           |
|                    | Betaine (Trimethylglycine)                                 | +                       | +                         |
|                    | Glutamic acid  | +                       | +                         |
|                    | Aspartic acid <sup>b</sup>                                 |                         | +                         |
|                    | Phenylalanine  | +                       | +                         |
|                    | Pipecolic acid   | +                       | +                         |
|                    | Tryptophan   | +                       | +                         |
|                    | N- $\alpha$ -Acetyl-lysine                                 | +                       | +                         |
|                    | 4-Hydroxyisoleucin <sup>a</sup>                            | +                       |                           |
|                    | $\gamma$ -Glutamylphenylalanine                            | +                       | +                         |
|                    | <b>Coumarins</b>   | Scopoletin <sup>b</sup> |                           |
| <b>Flavonoids</b>  | Naringenin <sup>b</sup>                                    |                         | +                         |
|                    | Chrysoeriol <sup>b</sup>                                   |                         | +                         |
|                    | Tricin <sup>b</sup>  |                         | +                         |
|                    | Luteolin-8-C-(2''-O-(E)-p-coumaroylglycoside) <sup>b</sup> |                         | +                         |
|                    | Tricin-7-O-glucoside <sup>b</sup>                          |                         | +                         |
|                    | Genistein <sup>b</sup>                                     |                         | +                         |
|                    | Vitexin (Apigenin-8-C-glucoside) <sup>b</sup>              |                         | +                         |
|                    | Isovitexin (Apigenin-6-C-glucoside)                        | +                       | +                         |
|                    | Medicarpin <sup>b</sup>                                    |                         | +                         |
|                    | Scoparin (Chrysoeriol-8-C-glucoside) <sup>b</sup>          |                         | +                         |
|                    | Vicenin-2 (6,8-Di-C-glucosylapigenin)                      | +                       | +                         |
|                    | Apigenin-di-C-hexoside (Vicenin-2-isomer)                  | +                       | +                         |
|                    | Vicenin-3 (6-C-Glucosyl-8-C-xylosylapigenin)               | +                       | +                         |
|                    | Isorientin (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 |
|--|---|----------------------------|---------------------------|
| <b>Other metabolites</b>                         | Sotolone(3-Hydroxy-4,5-dimethyl-2(5H)furanone)    | +                          | +                         |
|  | Choline   | +                          | +                         |
|  | Saccharopine                                      | +                          | +                         |
| <b>Polyphenols</b>                               | Resveratrol <sup>b</sup>                          |                            | +                         |
|  | p-Coumaric acid                                   | +                          | +                         |
| <b>Purines and pyrimidine</b>                    | 5'-S-Methyl-5'-thioadenosine <sup>b</sup>         |                            | +                         |
|  | 2'-Deoxyadenosine                                 | +                          | +                         |
|  | Adenine <sup>a</sup>                              | +                          |                           |
|  | Adenosine   | +                          | +                         |
|  | Adenosine 3',5'-cyclic monophosphate <sup>a</sup> | +                          |                           |
|  | Cytidine <sup>a</sup>                             | +                          |                           |
|  | Flavin mononucleotide (FMN) <sup>b</sup>          |                            | +                         |
|  | Guanine <sup>a</sup>                              | +                          |                           |
|  | Guanosine   | +                          | +                         |
|  | S-Adenosylhomocysteine                            | +                          | +                         |
|  | Uridine   | +                          | +                         |
|  | Xanthine <sup>b</sup>                             |                            | +                         |
|  | Xanthosine  | +                          | +                         |
|  | <b>Saponins</b>                                   | Soyasaponin I <sup>b</sup> |                           |
| 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 <sup>b</sup>                |   |                            | +                         |
| Protoyuccagenin-S4                               | +   | +                          |                           |
| <b>Terpenoid</b>                                 | Ursolic acid <sup>b</sup>                         |                            | +                         |
| <b>Vitamines</b>                                 | Nicotinamide                                      | +                          | +                         |
|  | Nicotinic acid (B3, niacin) <sup>b</sup>          |                            | +                         |
|  | Pantothenic acid (B5)                             | +                          | +                         |
|  | Pyridoxine (B6)                                   | +                          | +                         |

<sup>a</sup>Compounds to be found only in aqueous extract,

<sup>b</sup>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- $\kappa$ B 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-coumaroyl)glycoside, isoorientin, vicenin-1, vicenin-2, vicenin-3 (6-C-glucosyl-8-C-xylosyl)apigenin), apigenin-6-C-glucoside-8-C-rhamnoside, chrysoeriol and triclin had been reported already in fenugreek seeds [5, 28-33]. However, flavonoids like, triclin-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 rich 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 low 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, isovitexin), 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 fenugreek 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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