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# Iridoids of fenugreek (*Trigonella-foenum-graecum L.*) seed extract detected via LC-QTOF-MS analysis

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ABSTRACT

Fenugreek seed is a traditional medicinal plant with a wide biological activity. In this study, iridoids (Asperulosidic acid (**1**), 7-O-Methylmorroniside (**2**), Gentiopicroside (**3**), Rehmannioside A (**4**), Loganic acid-6′-O-*β*-Dglucoside (**5**), Sweroside (**6**), Penstemoside (**7**), 6′-O-*β*-D-Glucosyl Gentiopicroside (**8**), Oleuropein (**9**)) in optimized microwave-assisted extract of fenugreek seed were detected for the first time. The identification of iridoid compounds was carried out by liquid chromatography quadrupole time-of-flight mass spectrometry coupled with electrospray ionization (LC-QTOF-MS-ESI) in positive ion modes and Fourier Transform Infrared Spectroscopy (FTIR) analysis. More than 400 compounds were detected via LC-QTOF-MS, while among them only 9 were iridoids. The presence of iridoid compounds was also confirmed with FTIR analysis. In positive ion mode, iridoids with formic acid as mobile phase associated in formation of three adducts [+ H, + Na + K]. However, in the case of negative ion no iridoid compound was observed in the extract.

## 1. Introduction

Fenugreek (*Trigonella-foenum-graecum L.*) is among the plant with wide range of bioactivity used from the ancient year as a food source, natural medicine and for many other purposes. The seed if this plant is rich in phenols, flavonoids, alkaloids, saponins, and other bioactive compounds (Akbari et al., 2020; Luan et al., 2018; Rayyan et al., 2010). However, investigation on the presence of iridoid compounds in the seeds of this plant has not been carried out so far. Iridoids are secondary metabolites belong to the group of monoterpenoids with a cyclopentanopyran skeleton found in many medicinal plants used as antipyretics, sedatives, cough medicines, remedies for skin disorders and wound healing (Kucharska and Fecka, 2016; Tundis et al., 2008). These compounds are classified as glycosides and comprise a six ring where each containing an oxygen atom. There are almost 600 iridoids glycosides from 75 plant families. Generally, due to their bitter test they act as plant defender against pathogens and herbivores (Ludwiczuk et al., 2017; Yamane et al., 2010). According to the integrity of cyclopentane, the iridoids are classified into two groups named iridoid glycosides and secoiridoid glycosides (Wang et al., 2020). The most suitable analysis method for identification of these compounds in plant extracts is liquid chromatography-mass spectrometry (LC-MS), particularly when it is coupled with quadrupole time-of-flight and electrospray ionization (QTOF- ESI). This method allows to measure the compounds with high accuracy in specific retention time, chemical formula, ion fragmentation, observed mass (m/z) and detection of adducts in both positive and negative ion modes. There are also other methods for analyzing iridoid compounds with liquid chromatography-nuclear magnetic resonance (LC-NMR) and (LC-MS-NMR). However, these methods are reported to be expensive and complicated (Emwas, 2010; Kucharska and Fecka, 2016). Extraction method is also one of the key points in obtaining the bioactive compounds of foods. In contrast with the Soxhlet extraction, maceration, hydrodistillation and other conventional methods of extraction, microwave-assisted extraction has the advantages of fast extraction, easy application, high yield, low energy and time consumption (Lovrić et al., 2017). However, the drawback of this method is the requirement of big space for the equipment than maceration and Soxhlet extraction.

The aim of this study was to determine the iridoid compounds in optimized microwave-assisted extract of fenugreek seed via LC-QTOF-MS analysis. In addition, the chemical structure and biological activities of the identified compounds was also reported. FTIR analysis was also carried out to confirm the presence of iridoid compounds in the extract. To the best of our knowledge, this investigation has not been carried out/published before.

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## 2. Material and methods

### 2.1. Chemicals and materials

Acetonitrile, formic acid and methanol with LC-MS grade and ethanol (analytical grade for extraction) was purchased from Sigma Aldrich (M) Sdn. Bhd.Malaysia. All the chemicals used in this research had high purity. Edible fenugreek seed was obtained from a local market based in Kuantan, Pahang, Malaysia. Before extraction, the seeds were separated from the unknown species and dried under 50 °C in an oven, grinded (Retsch ZM-200, Germany) in powdered form, and then stored in 4 °C until further application.

### 2.2. Extraction of seed

The seed was extracted via microwave-assisted extraction (MAE) method under optimized parameters of 63.68% ethanol, 2.84 min extraction time, 572.50 W, 1:9 g/mL feed to solvent ratio and 70 °C extraction temperature. The optimization was carried out using response surface methodology (RSM) under Design-Expert (DOE) 7.0 Software. Ethos closed system microwave extractor (1000 W, 2450 F, Milestone, Italy) were used to extract the seed. Further details of the extraction process are available in our previous publication (Akbari et al., 2019).

### 2.3. Identification of iridoid compounds via LC-QTOF-MS

The analysis of LC-QTOF-MS was performed using a mass spectrometer (Vion IMS QTOF, Waters, USA) with an electrospray ionization (ESI) source. Identification of the compounds was carried out in both positive and negative ion modes. In positive ion acetonitrile, formic acid and water were used as the mobile phase at different concentrations at flow rate of 0.5mL/min and injection volume 20μL. Water + 0.1%formic acid was named as solvent A and acetonitrile was named as solvent B. The gradient program sequence was as follows: 1.25 min, 90%A, 10%B–4.17min, 45% A, 55%B–6.25min, 10% A, 90%B–8.34min, 90% A, 10%B. In negative ion mode, only water and acetonitrile were used as mobile phase without adding formic acid with the same gradient program. Mass spectra (MS) was between 100-1000 m/z, the C18 column (100mm, x 2.1mm, 1.8μm particle size), capillary voltage 1.50 kV, gas flow rate 800 L/h, the temperature of column, desolvation and sample were 40, 550 and 15 °C, respectively. Compound identification was performed using UNIFI software with scientific library. Further details of the analysis are available at (Akbari et al., 2021).

### 2.4. Fourier transform infrared spectroscopy

The FTIR analysis of fenugreek seed and Aloe vera leave extracts obtained in the optimal condition and performed using Nicolet iS5 FTIR spectrometer equipped with a DTGS detector and OMNIC software. FTIR spectrum of the extract was recorded between 4000–400 $cm^{-1}$ using KBr pellet methods at room temperature. The dry sample was flatted on plate surface containing a diamond crystal and the lid was closed to measure the bonding structure directly.

## 3. Results and discussion

The ethanolic extract of fenugreek seed in optimized condition of microwave assisted extraction was analyzed by the LC-QTOF-MS method. The compounds were identified by their chemical formula, mass spectra, mass errors, observe retention time (Rt) and adducts by comparing them with available data in previous studies. The result of LC-QTOF-MS analysis indicated 10 iridoid compounds namely: (Asperulosidic acid, 7-O-Methylmorroniside, Gentiopicroside, Sweroside, Rehmannioside A, Penstemoside, Oleuropein, 6′-O-$\beta$-D-Glucosyl Gentiopicroside, Loganic acid-6′-O-$\beta$-Dglucoside) as shown in Table 1. These iridoids were discovered for the first time in fenugreek seed extract. Each of these compounds are reported to have potential biological activities and health benefits. He et al. (2018) studied the anti-inflammatory effect of five iridoids including asperulosidic acid, asperuloside, desacetyl asperulosidic acid, E-6-O-p-coumaroyl scandoside methyl ester and scandoside methyl ester, they suggested that asperulosidic acid and asperuloside have the greatest anti-inflammatory effect when compared to 3 other iridoids. Tundis et al. (2008) stated that iridoid compounds have a wide range of bioactivity such as anti-inflammatory, hepatoprotective, antioxidant, anticancer and antimicrobic which make them a potent constituent for medical and pharmaceutical applications. The biological activity of the above iridoid compounds as anti-inflammatory, anticancer, antimicrobial, antiviral and antioxidant was also reported by other researchers (Alipieva et al., 2007; Cui et al., 2020; Omar, 2010; Jensen et al., 2007; Sun et al., 2019; Szumny et al., 2015). In a study conducted by West et al. (2014), it is claimed that dietary source of iridoids derived from food source can be used as antiaging.

Iridoids with formic acid associated in formation of positive ions with adducts [+H, +Na+ K]. However, in the case of negative ion no iridoid compound was observed in the extract. In the positive ion mode, compounds that can be bound with proton, potassium, sodium or other cations can be theoretically ionized. Most of the organic compounds having nitrogen (N) and oxygen (O) can be ionized in the positive ion modes (Zhao et al., 2019). In addition, another reason might be the absence of formic acid during identification process in negative ion mode. The retention time and mass spectra (100–1000 m/z) of identified iridoid compounds versus intensity are shown in Figs. 1–3. Compounds **1–3**, with Rt 0.66 min, 0.77 min and 1.14 min indicated molecular ions at m/z = 455.1159, m/z = 433.1525, and m/z = 379.1002 produced sodium adducts $[M + Na]^+$ and indicated 2, 2 and 1 fragments, respectively (Fig. 1 compounds **1–3** and Table 2). Compound **4** with Rt time 1.59 min displayed a molecular ion at m/z = 524.1753 with hydrogen adducts $[M + H]^+$ and indicated 6 fragments (Fig. 1 compound **4** and Table 2). In this vein, compounds **5–9** all indicated potassium adducts $[M + K]^+$ with molecular ion at m/z = 538.1917, m/z = 358.1258, m/z = 406.1495, m/z = 518.1632 and m/z = 540.1862 with retention times of 2.13 min, 2.41 min, 2.58 min, 2.62 min and 3.28 min, respectively. In addition, the total fragments found in these compounds were 6, 3, 5, 9, and 5, respectively (Fig. 1 compounds **5–9** and Table 2). The most common observed adduct ion in positive ion mode in ESI/MS analysis are sodium, potassium and in some cases hydrogen. This is due to the mobile phase containing 0.1% formic acid, solvent impurity or glassware used during the operation (Kruve and Kaupmees, 2017).

It is also seen in Figs. 1–3 that all the iridoid compounds were detected in less than 4 min of retention time. This might be due to the molecular structure of iridoid compounds with a smaller number of carbon and hydrogen atoms. The specific compound in the mass spectra figures is highlighted with light green color and the chemical structures are also presented. The LC-QTOF-MS analysis detected more than 400 compounds including, phenolics, saponins, alkaloids, flavonoids, steroids, terpenoids, 9 iridoids and glycosides.

### 3.1. Fourier transform infrared spectroscopy

The extract of fenugreek seed was also characterized using FTIR spectroscopy. Fig. 4 Shows the detected peaks observed at the wavelength of 4000–500 $cm^{-1}$. The peak at 3328.70 $cm^{-1}$ represents the presence of OH group attributed to the phenol groups in the extract. The peaks from 2922.10 to 2852.35 $cm^{-1}$ show the stretching vibration $CH_3$ and $CH_2$ corresponding to aldehyde, glycosides, aromatic rings and carboxyl groups (Keshari et al., 2018). The bounds around 1740.34–1457.48 $cm^{-1}$ are assigned to iridoids glycosides (Yang et al., 2014). The peak at 1377.30 $cm^{-1}$ can be assigned to the binding vibration of methyl groups. The bonds at 1220.32–913.93 $cm^{-1}$ are attributed to the stretching vibration of C-O and C-OH, showing the presence of glycosides, phenols and terpenes (Qi et al., 2017).

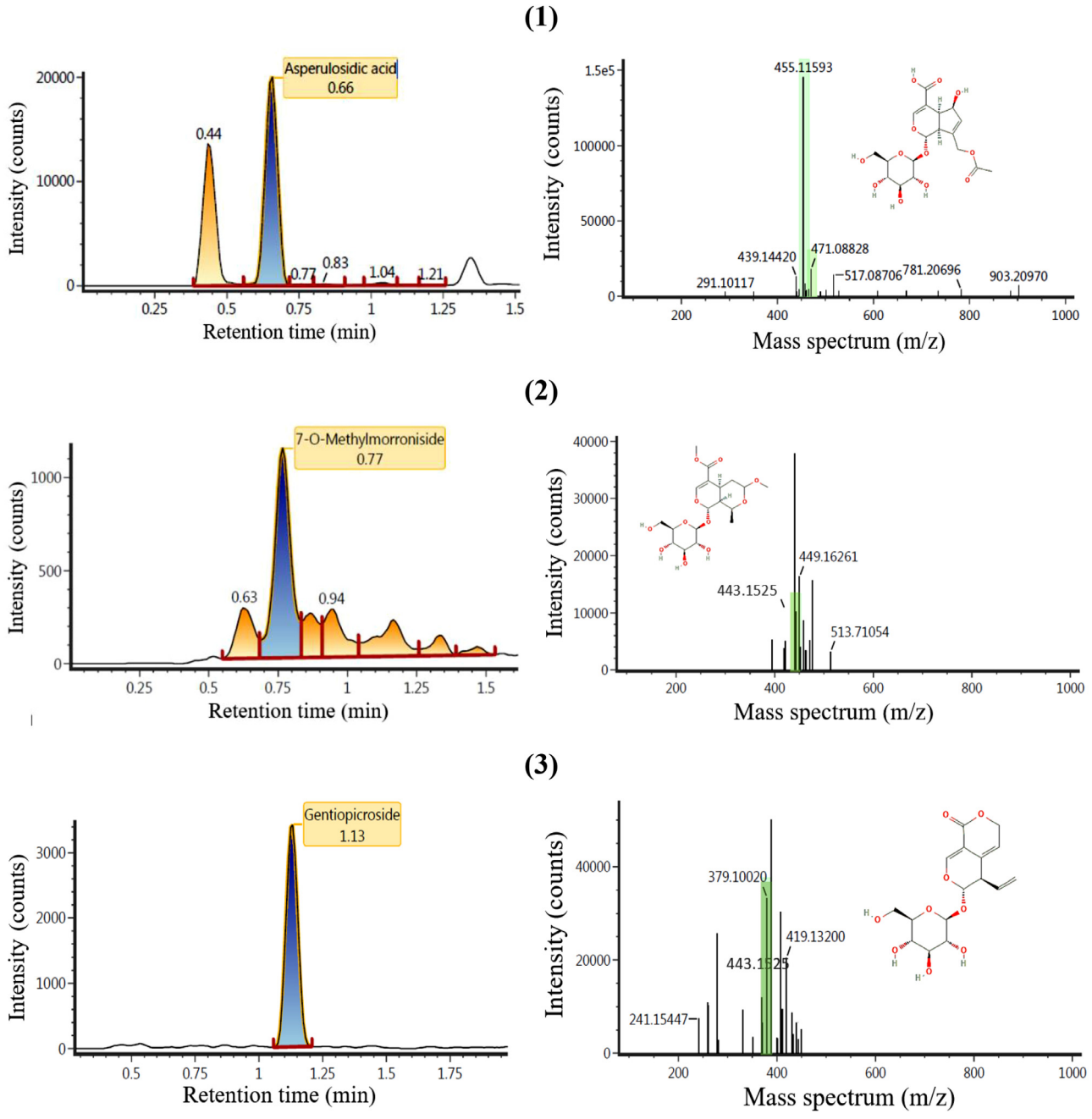



**Fig. 1.** Mass spectrum and retention time of compounds **1–3** versus intensity with sodium adducts (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.).

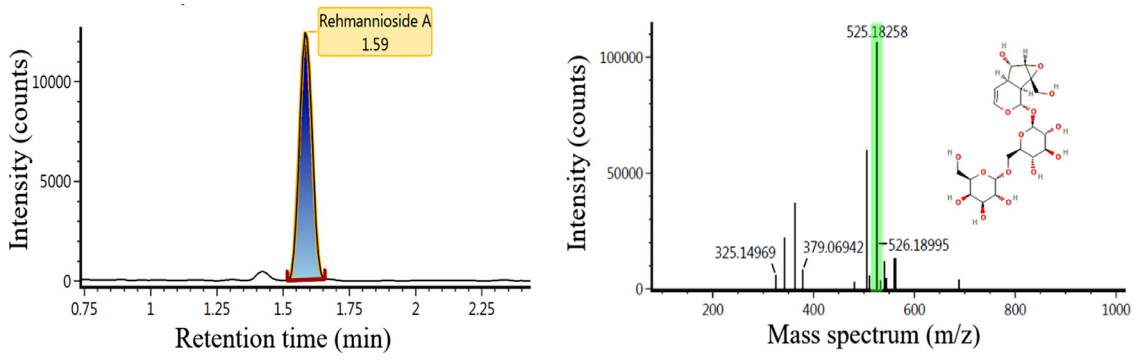



**Fig. 2.** Mass spectrum and retention time of compound **4** versus intensity with sodium adducts (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.).

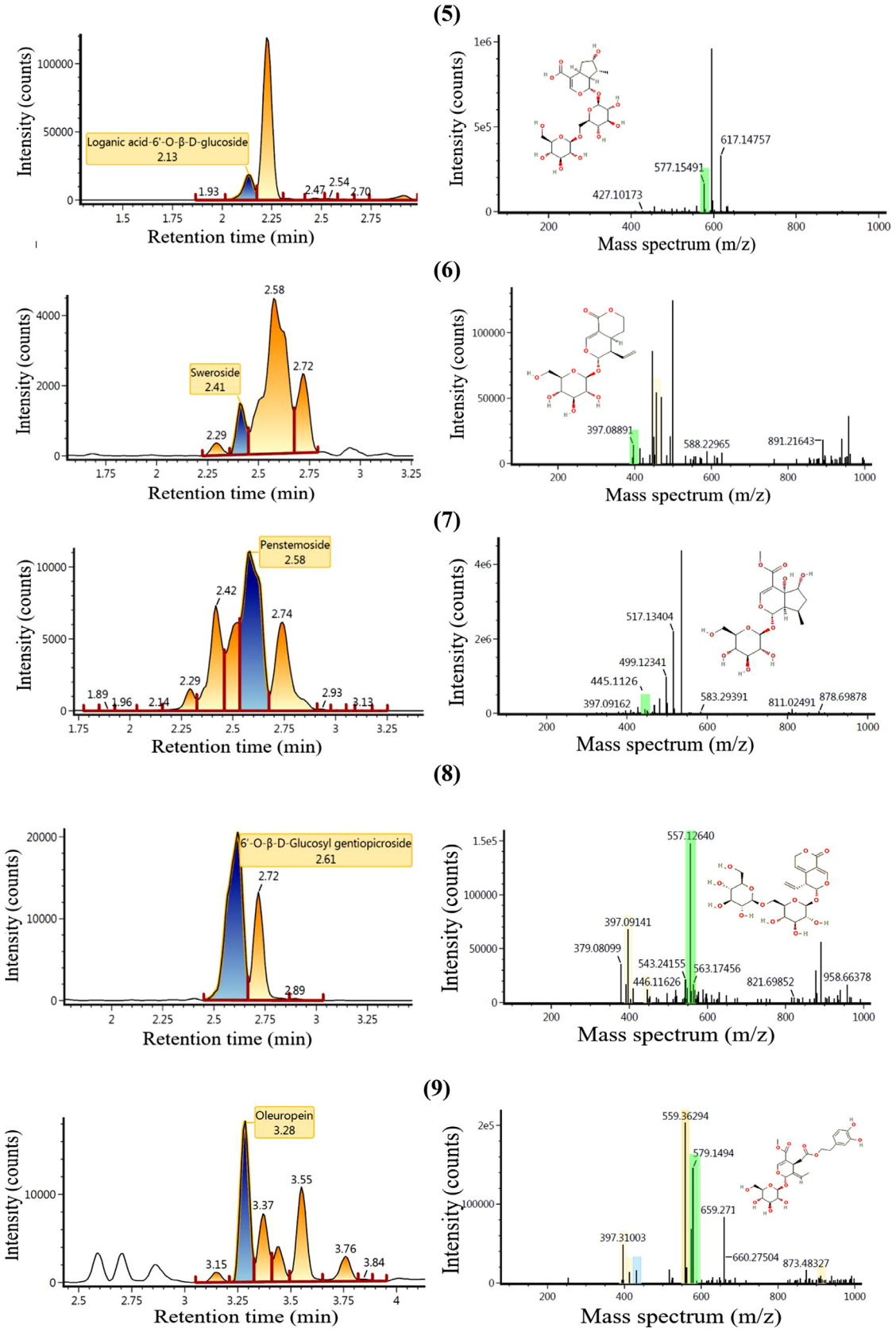
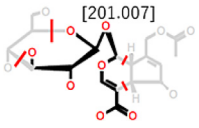
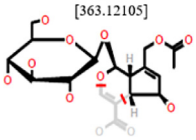
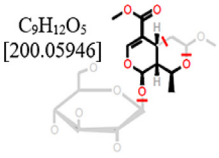
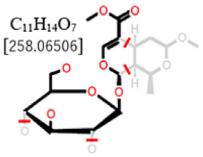
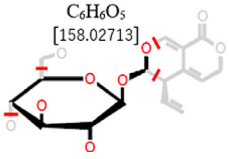
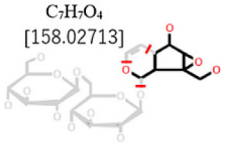
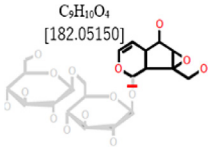
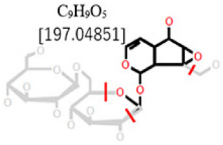
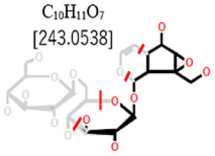
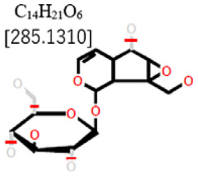
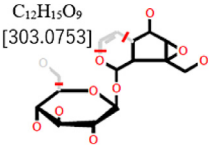
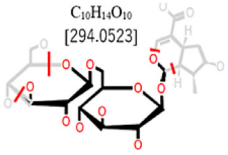
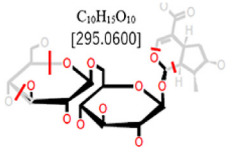
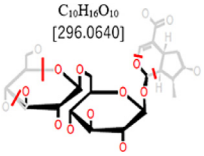
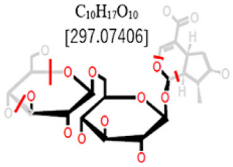
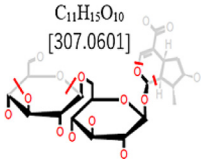
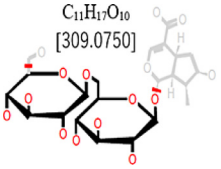



**Fig. 3.** Mass spectrum and retention time of compound **5–9** versus intensity with potassium adducts (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.).

**Table 1**
Identified iridoid compounds of fenugreek seed via LC-QTOF-MS analysis.

| No | Component name | Formula | Observed neutral mass (Da) | Observed m/z | Mass error (mDa) | Mass error (ppm) | Observed Rt (min) | Response | Adducts | Total Fragments Found |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Asperulosidic acid | $C_{18}H_{24}O_{12}$ | 432.1267 | 455.1159 | -0.1 | -0.1 | 0.66 | 20860 | + Na | 2 |
| 2 | 7-O-Methylmorroniside | $C_{18}H_{28}O_{11}$ | 420.1632 | 443.1525 | 0.1 | 0.2 | 0.77 | 1428 | + Na | 2 |
| 3 | Gentiopicroside | $C_{16}H_{20}O_9$ | 356.1110 | 379.1002 | 0.2 | 0.6 | 1.13 | 4352 | + Na | 1 |
| 4 | Rehmannioside A | $C_{21}H_{32}O_{15}$ | 524.1753 | 525.1826 | 1.2 | 2.2 | 1.59 | 16381 | + H | 6 |
| 5 | Loganic acid-6′-O-$\beta$-Dglucoside | $C_{22}H_{34}O_{15}$ | 538.1917 | 577.1549 | 2.0 | 3.4 | 2.13 | 26548 | + K | 6 |
| 6 | Sweroside | $C_{16}H_{22}O_9$ | 358.1258 | 397.0889 | -0.6 | -1.6 | 2.41 | 1904 | + K | 3 |
| 7 | Penstemoside | $C_{17}H_{26}O_{11}$ | 406.1495 | 445.1126 | 2.0 | 4.4 | 2.58 | 15172 | + K | 5 |
| 8 | 6′-O-$\beta$-D-Glucosyl gentiopicroside | $C_{22}H_{30}O_{14}$ | 518.1632 | 557.1264 | -0.3 | -0.6 | 2.61 | 23400 | + K | 9 |
| 9 | Oleuropein | $C_{25}H_{32}O_{13}$ | 540.1862 | 579.1494 | 2.0 | 3.4 | 3.28 | 23590 | + K | 5 |

**Table 2**
Fragmentation of iridoid compounds of fenugreek seed.

| | Compound name | Formula | Fragments | | |
|---|---|---|---|---|---|
| 1 | Asperulosidic acid | $C_{18}H_{24}O_{12}$ | $C_7H_5O_7$ [201.007]  | $C_{15}H_{23}O_{10}$ [363.12105]  | |
| 2 | 7-O-Methylmorroniside | $C_{18}H_{28}O_{11}$ | $C_9H_{12}O_5$ [200.05946]  | $C_{11}H_{14}O_7$ [258.06506]  | |
| 3 | Gentiopicroside | $C_{16}H_{20}O_9$ | $C_6H_6O_5$ [158.02713]  | | |
| 4 | Rehmannioside A | $C_{21}H_{32}O_{15}$ | $C_7H_7O_4$ [158.02713]  | $C_9H_{10}O_4$ [182.05150]  | $C_9H_9O_5$ [197.04851]  |
| | | | $C_{10}H_{11}O_7$ [243.0538]  | $C_{14}H_{21}O_6$ [285.1310]  | $C_{12}H_{15}O_9$ [303.0753]  |
| 5 | Loganic acid-6′-O-$\beta$-Dglucoside | $C_{22}H_{34}O_{15}$ | $C_{10}H_{14}O_{10}$ [294.0523]  | $C_{10}H_{15}O_{10}$ [295.0600]  | $C_{10}H_{16}O_{10}$ [296.0640]  |
| | | | $C_{10}H_{17}O_{10}$ [297.07406]  | $C_{11}H_{15}O_{10}$ [307.0601]  | $C_{11}H_{17}O_{10}$ [309.0750]  |

(*continued on next page*)

**Table 2** (*continued*)

| | Compound name | Formula | Fragments | | |
|---|---|---|---|---|---|
| 6 | Sweroside | $C_{16}H_{22}O_9$ | $C_9H_5O_4$ [177.018] | $C_{12}H_{16}O_5$ [240.099] | $C_{13}H_{14}O_9$ [314.069] |
| 7 | Penstemoside | $C_{17}H_{26}O_{11}$ | $C_9H_5O_4$ [177.0185] | $C_{10}H_{15}O_9$ [279.0667] | $C_{13}H_{17}O_9$ [317.0816] |
| | | | $C_{14}H_{19}O_9$ [331.0960] | $C_{15}H_{19}O_9$ [343.0969] | |
| 8 | 6′-O-$\beta$-D-Glucosyl gentiopicroside | $C_{22}H_{30}O_{14}$ | $C_7H_5O_2$ [121.0283] | $C_9H_5O_4$ [177.0192] | $C_{11}H_9O_4$ [205.0489] |
| | | | $C_{15}H_{11}O_6$ [287.0581] | $C_{12}H_{19}O_9$ [307.1058] | $C_{13}H_{21}O_{10}$ [337.1186] |
| | | | $C_{16}H_{21}O_{11}$ [389.1028] | $C_{15}H_{22}O_{12}$ [394.10180] | $C_{17}H_{17}O_{11}$ [397.0756] |
| 9 | Oleuropein | $C_{25}H_{32}O_{13}$ | $C_8H_7O$ [119.0491] | $C_9H_7O_2$ [147.0436] | $C_8H_{12}O_6$ [204.0663] |
| | | | $C_{16}H_{11}O_5$ [283.0609] | $C_{14}H_{21}O_6$ [285.1264] | |

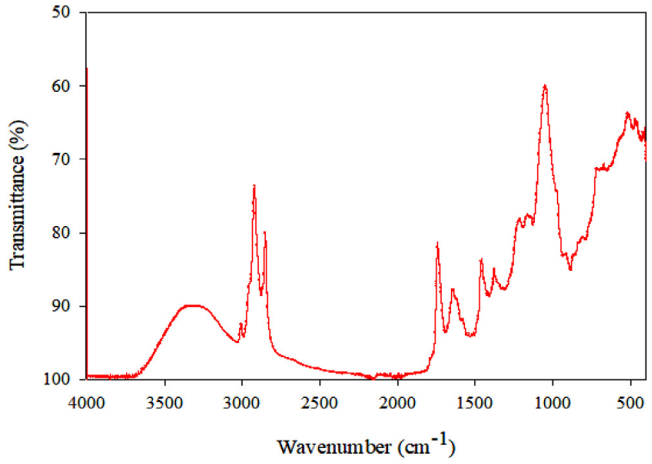



**Fig. 4.** FTIR spectra of the extract.

## 4. Conclusion

The application of LC-QTOF-MS analysis enabled the identification of 9 iridoid compounds in the optimized microwave-assisted extract of fenugreek seed. FTIR analysis was also confirmed the presence of iridoid compounds in the extract. The iridoids were detected in optimized extract of fenugreek seed for the first time. Each compound was identified with the specific compound name, molecular formula, molecular weight, retention time, fragmentation number and structure. The analyses were done in both positive and negative ion modes and the results came with 9 iridoids in positive mode while no iridoid compound was detected in negative ion. Results indicated that iridoids with formic acid associated in formation of positive ions with adducts [+ H, + Na+ K]. However, in the case of negative ion no iridoid compound was observed in the extract. Identification of iridoid compounds in fenugreek seed can be further investigated in future studies.

## Authors declaration

We wish to confirm that there are no known conflicts of interest associated with this publication and the financial support provided by the University Malaysia Pahang (UMP's) grant No. PGRS 1803105 has been acknowledged and mentioned in the manuscript. We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us. We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing we confirm that we have followed the regulations of our institutions concerning intellectual property. We further confirm the ethical approval of all relevant bodies and that such approvals are acknowledged within the manuscript. We understand that the Corresponding Author is the sole contact for the Editorial process (including Editorial Manager and direct communications with the office). He is responsible for communicating with the other authors about progress, submissions of revisions and final approval of proofs. We confirm that we have provided a current, correct email address which is accessible by the Corresponding Author and which has been configured to accept email from abrahman@ump.edu.my. Here, all authors confirm the submission of manuscript entitled '**Iridoids of fenugreek (Trigonella-foenum-graecum L.) seed extract detected via LC-QTOF-MS analysis**' To ***Future Foods.***

## Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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