



LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY FOR THE DETECTION AND VALIDATION OF QUERCETIN-3-*O*-RUTINOSIDE AND MYRICETIN FROM FRACTIONATED *Labisia pumila* var. *alata*

(Kromatografi Cecair Spektrometri Jisim Tandem untuk Pengesanan dan Pengesahsahihan Kuersetin-3-*O*-Rutinosida dan Mirisetin daripada Pemeringkatan *Labisia pumila* var. *alata*)

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Abstract

Quercetin-3-*O*-rutinoside and myricetin was reported to be important secondary metabolites in *Labisia pumila* (*L. pumila*). The reliability of the instrument, its sensitivity and methodology are the key in the analysis of a specific metabolite and liquid chromatography tandem mass spectrometry (LC-MS/MS) provides better accuracy and faster separation in detecting of such chemical compound. Therefore, this study aims to detect and validate the presence of quercetin-3-*O*-rutinoside and myricetin from fractionated extract of *L. pumila* (40% MeOH: water). The plant material was fractionated using octadecyl (C₁₈) silica solid phase and eluent with a ratio of 40% MeOH: water. The separation of compounds was carried out using a C₁₈ reversed phase column (Acquity, 150mm × 4.6mm × 1.7 μm). Negative ionization was utilized to fragment the precursor ion of *m/z* 609 for quercetin-3-*O*-rutinoside and *m/z* 317 for myricetin. Three fragments ions; *m/z* 447, 463, 301 was recognized as quercetin-3-*O*-rutinoside product ions within 3.3 minutes. Meanwhile, *m/z* 271, 137 and 151 represented characteristics ions for myricetin were recognized within 3.9 minutes. The concentration of quercetin-3-*O*-rutinoside and myricetin was 0.007 mg/g and 0.009 mg/g respectively. The linearity was found between 0.991-0.998. Detection based on (limit of detection) LOD and (limit of quantification) LOQ were found in the ranged of 0.03-0.04 μg/mL and 0.11-0.13 μg/mL, respectively. Both compounds showed good recovery of above 87%. Intra-day and inter-day (RSD) study was in the acceptable range (below 10%). Based on the result of this study, the LC-MS/MS was shown to be the best selective, fast and sensitive method to determine quercetin-3-*O*-rutinoside and myricetin in the plant fraction. These findings could be used as a guideline for the detection of the compounds and suitable for quality controls of *L. pumila* this and another medicinal herb containing quercetin-3-*O*-rutinoside and myricetin.

Keywords: *Labisia pumila*, quercetin-3-*O*-rutinoside, myricetin, validation

Abstrak

Kuersetin-3-*O*-rutinosida dan mirisetin telah dilaporkan sebagai metabolit sekunder yang penting dalam *Labisia pumila* (*L. pumila*). Kebolehppercayaan instrumen, kepekaan dan kaedah pembangunan menjadi aspek penting dalam analisis yang disasarkan kepada metabolit tertentu dan kromatografi cecair spektrometri jisim tandem (KC-SJ/SJ) menyediakan pengesanan lebih cepat dan tepat bagi pengesanan sebatian kimia. Oleh itu, kajian ini adalah bertujuan untuk mengesan dan menentusahkan kehadiran kuersetin-3-*O*-rutinosida dan mirisetin daripada pemeringkatan *L. pumila* (40% metanol:air). Bahan tumbuhan ini

diperingkatkan menggunakan fasa pepejal oktadesil silika (C_{18}) dengan kadar 40% metanol: air. Pengasingan komponen dicapai menggunakan fasa turus berbalik C_{18} (Acquity, 150 mm \times 4.6 mm \times 1.7 μ m). Pengionan negatif telah digunakan untuk mengasingkan pencetus ion m/z 609 untuk kuersetin-3-*O*-rutinosida dan m/z 317 untuk mirisetin. Tiga pecahan ion; m/z 447, 463, 301 dikenal pasti sebagai produk molekul ion dari kuersetin-3-*O*-rutinosida dalam selang masa 3.3 minit. Manakala m/z 271, 137 and 151 mewakili ciri ion untuk mirisetin telah dikenalpasti dalam selang masa 3.9 minit. Kepekatan kuersetin-3-*O*-rutinosida dan mirisetin masing-masing ialah 0.007 mg/g dan 0.009 mg/g. Kelinearan adalah di antara 0.991-0.998. Had pengesanan (LOD) dan had pengkuantitian (LOQ) adalah masing-masing dalam julat 0.03-0.04 μ g/mL dan 0.11-0.13 μ g/mL. Kedua-dua sebatian menunjukkan penghasilan yang baik, lebih daripada 87%. Kajian dalam-hari dan antara-hari (RSD) adalah dalam julat yang boleh diterima (bawah 10%). Berdasarkan keputusan melalui kajian ini, KC-SJ/SJ ditunjukkan sebagai kaedah terpilih, cepat dan sensitif bagi menentukan kuersetin-3-*O*-rutinosida dan mirisetin di dalam pemeringkat tumbuhan ini. Penemuan ini boleh digunakan sebagai panduan untuk pengesanan sebatian dan sesuai sebagai kawalan kualiti untuk *L. pumila* dan herba perubatan yang lain yang mengandungi kuersetin-3-*O*-rutinosida and mirisetin.

Kata kunci: *Labisia pumila*, kuersetin-3-*O*-rutinosida, mirisetin, pengesahsahihan

Introduction

Labisia pumila (*L. pumila*) is known as the best Malaysian herbal recognized in various medical benefits especially for women. Locally known as *Kacip Fatimah* (Myrsinaceae), it is a traditional herbal plant widely used by peoples from the Asian region, especially in Malaysia and Indonesia. This plant is exclusive for women's health due to the presence of phytoestrogen. It can relief menopausal syndrome as well as health booster during post-delivery and it is also suitable to be used during pregnancy [1-6]. Furthermore, it was reported earlier that the aqueous plant extract could assist in modulating the post-menopause adiposity in ovariectomized rats and the result was comparable to the estrogenic receptor replacement therapy (ERT) [7]. There was also no acute toxicity reported for the treatment of post-menopausal symptom based on the clinical studies conducted [8]. Additionally, the plant's root aqueous extract was also found to be effective in osteoporosis treatment [9] as it showed significant modulation effect in pharmacokinetic and pharmacodynamics properties [10]. Interestingly, the value of anti-aging and antioxidant property of the *L. pumila* has surged its application as cosmeceutical agent [11-14]. The plant aqueous extract also showed a prospective application as anticancer, anti-inflammatory and anti-fungal agent [13, 15]. The three types of *L. pumila* variety were recognized worldwide as *alata*, *pumila* and *lanceolata* [16] and the former two varieties were selected as most important valued sources due to its significant medicinal properties [3].

Recently, many types of phytochemicals have been identified from the leaves, roots and whole plant parts of *L. pumila* [13-15, 17-21]. Based on the analytical studies using various detection techniques, it was shown that the plant extract contained significant amount of polyphenols and the sub-group of polyphenols; flavonoids was the major constituents. In our initial work, qualitative analysis of *L. pumila* var. *alata* from the methanolic extract using LC-MS/MS in profiling 20 polyphenols constituents has been achieved [22]. Quercetin-3-*O*-rutinoside and myricetin have shown by its significant peaks in the chromatogram. In other related publication, the presence of these compounds was also detected in leaves and roots of the same plant variety [13, 14, 17, 18]. These compounds were the most beneficial flavonoids and commonly used in food and beverages and it was used and registered in over 130 medicinal formulations for its therapeutic effect [23]. In addition, the pharmacological properties of quercetin-3-*O*-rutinoside as anti-cancer, antioxidant, antidiabetic and anti-inflammatory agent were also studied [23]. From cosmeceutical view, quercetin-3-*O*-rutinoside has the sun light blocking properties as well as the ability to prevent skin diseases such as redness and skin lesions [24]. Myricetin were also used as a chemotaxonomic marker in *Chrysobalanaceae* family [25]. Relevant pharmacological activity from this compound has been reported earlier [25-26].

From the literature review, analytical procedure that has been developed to identify and quantify the quercetin-3-*O*-rutinoside and myricetin was using HPLC [13, 17]. However, high sensitivity and resolution with accurate mass can only be achieved using mass spectrum. Still, analysing complex matrices have become a challenge to create consistent signal in every sample injection. The use of LC-MS/MS with triple quadrupoles ion trap for quantitation offer faster and accurate results. Hence, it is essential to developed new analytical procedures that provide good compound resolution and sensitivity to identify quercetin-3-*O*-rutinoside and myricetin from plant. This analytical technique could be used together with chemical and biological assays of natural products.

The aim of this study was to develop a LC-MS/MS method to identify quercetin-3-*O*-rutinoside and myricetin. The quantitative results were further validated in this study for quality control purposes. Prior to analysis, to reduce sample matrix in the complex mixture, preparative fractionation by using packed C₁₈ column bed in the solid phase extraction (SPE) technique were used after the extraction process [22-27]. The analytical method developed could be essential for the discovery of natural product especially to identify quercetin-3-*O*-rutinoside and myricetin from *L. pumila* and other herbal plants.

Materials and Methods

Chemicals

Reagent grade methanol (MeOH) was purchased from Fisher Scientific (Pittsburgh, PA). HPLC grade methanol and acetonitrile were obtained from Merck (Darmstadt, Germany). Ultra-pure water was from a Barnstead Nano-pure Diamond water purification system (Thermo, Waltham, MA). The deionized water was produced from Barnstead E-pure water system (Thermo, Waltham, MA). The chemical standards of quercetin-3-*O*-rutinoside and myricetin were procured from Sigma-Aldrich (St Louis, MO) and the SPE cartridge, Strata™-X, (8B-S029 FBJ, 200 mg) was purchased from the local supplier (Phenomenex, Torrance, CA).

Plant material: Sources and preparation

L. pumila var. *alata* which was labeled as FRI 59810 was freshly taken from the herbal farm at Forest Research Institute Malaysia (FRIM), Selangor. After harvesting, the leaves were dried using conventional drying oven at 40 °C for 24 hours. The dried leaves were ground (mesh size approximate 200 micron) by using a warring blender and kept in seal plastic before storage at -20 °C, prior to further analysis.

Fractionation of *L. pumila* by solid phase extraction

The extraction and fractionation method were done based on method established by Chua et al. [22]. The fractionation of *L. pumila* was done by using C₁₈ sorbent type material. The SPE cartridge, Strata™-X, (8B-S029 FBJ, 200 mg) was procured from local supplier (Phenomenex, Torrance, CA). In this procedure, 40% MeOH-water mixture was used as the eluting solvent. The crude extract (50 mg) was suspended in 5 mL MeOH. The mixture was vortexed to allow homogenization. The homogenized mixture (2 mL) was further diluted with 3 mL of deionized water. The fractionation process was started by conditioning the cartridges with 15 mL of 100% MeOH and followed by 15 mL of deionized water, at a control flow rate of 10 mL/min, under vacuum. This procedure is important to remove the air bubbles trapped inside the cartridge. The sample (2 mL) was then added to the SPE cartridge at slow flow rate of 1 mL/min. An amount 5 mL of 5:95 MeOH: water in ratio 5:95 was loaded into the cartridge to remove the impurities. The fraction was then collected upon elution with 40% MeOH-water (15 mL). The fraction was dried using rotary evaporator and finally weighed and stored in the refrigerator before further analysis.

LC-MS/MS analysis

The method is referred from established method by Chua et al. [22] with minor changes for improvement. The plant fraction (10 mg) was diluted with 10 mL 40% MeOH-water. The sample was vortexed to homogenise and filtered using 0.20 µm syringe filters (Nylon membrane, Titan, Thermo Scientific, Germany). The filtered solution (1 mL) was inserted into HPLC vial to be injected into LC-MS/MS system. Chromatographic separation was done by using an Acquity, system (Waters Corporation, Milford, MA) and an Acquity reversed phase C₁₈ column (150 mm × 4.6 mm × 1.7 µm particle size) from Waters (Milford, MA). The detection was done *via* mass spectrometry 4000 QTRAP (Applied Biosystems-AB Sciex, Life Technologies Corporation, Carlsbad, CA). The analysis of the fraction was performed using the Analyst software (version 1.4.2). Gradient system was used to elute the sample before entering the mass spectrometry system. The mobile phase A consists of 0.1% formic acid in water and 0.1% formic acid in acetonitrile for mobile phase B. The mobile phase was developed using gradient system at 0-5 minutes, 10% B; 5-15 minutes, 10-90% B; 15-20 minutes, 90%B; 20-25 minutes, 90-10% B; 25-30 minutes, 10% B for the final wash and column equilibration before the next injection. The injection volume was 5 µL. The mobile phase was filtered using 0.2 µm nylon membrane filter. The flow rate was set at 0.25 mL/min and system was run at 15 minutes.

The mass spectrometry (MS/MS) was done by using electrospray ionization (ESI) in negative and positive ion mode. The mass spectra were acquired with the ranges molecular weight (m/z) of 100-1500 with 20 mass ion accumulations. The capillary and voltage of the ESI source were maintained at 400 °C and 4.5 kV, respectively (both negative and negative mode). The ESI parameters used were as follows: nitrogen was used as ion source gas for nebulization at 40 psi; solvent drying at 40 psi; nitrogen gas was set at 10 psi; collision gas was set at high; declustering potential of 30-50 V, and collision exit energy, 10-20 V. The scan rate was 100 amu/s. A low energy collision dissociation (CID) was used in quantitative by multiple reactions monitoring (MRM) mode. The MRM mode was used to generate the standard curve from a standard compound of aglycone. To develop the mass spectrum from targeted compounds, the compounds were directly delivered into ESI source. This procedure was called as the direct infusion where the sample was directly injected to the mass detector. Manual injection was done by using syringe pump and the injection flow rate was 0.5 mL/min at 1 µg/mL.

Validation of the quercetin-3-*O*-rutinoside and Myricetin

According to European Analytical Chemistry (EURACHEM) guideline for method validation, the fundamental of validation includes accuracy, precision, recovery, selectivity, calibration curve and stability [28]. This fundamental work can be applied to determine the quantity of metabolites from the complex matrices. The EURACHEM guidance provides general references on the best practice of chemical analysis and its criteria can be selected depending on the specific test and analytical method applied. The validation of the quercetin-3-*O*-rutinoside and myricetin was selected based on four criteria which are the calibration curve, sensitivity, accuracy, and precision, as suggested [28, 29].

Linearity

Linearity was evaluated through the determination of six standards working solutions containing 0.1-100 µg/mL of the standard compounds in triplicate preparations. The peak area and concentration were subjected to least-squares linear regression analysis to calculate the calibration equation and correlation coefficients.

Accuracy

The accuracy was calculated through the recovery studies by adding a known amount of the solution of each standard compound into triplicate aliquots of extract. The sample and the spiking solutions were followed by the fractionation process before subjected to LCMS/MS analysis. The recovery of the standard compounds was calculated in the percentage unit. Recovery of the standard compounds was calculated in percentage as follows:

$$\text{Recovery (\%)} = \frac{C1 - C2}{C3} \quad (1)$$

where C1 is define as concentration of spiked sample, C2 is concentration of sample and C3 is concentration of the standard solution.

Sensitivity

The sensitivity of the method was evaluated by utilizing the limit of detection (LOD) and limit of quantitation (LOQ) analysis. The LOD was described as the minimum detection (signal) of instrument response a significant difference between background, blank and analyte signals [28]. LOQ was described as the quantitative result which can be reported at a high degree of confidence. The LOD and LOQ of the LCMS/MS method for the standards were established at a standard deviation of the average value of 3 and 10, respectively, which were experimentally verified by six injections of each of the substances at the LOQ concentrations.

Precision

To study the precision, the analysis was carried out in intra-day and inter-day with the aim to produce reproducibility. The aliquots were subjected to LCMS/MS injection. For intra-day studies, aliquots were injected at four different times on the same day. The peak area was analyzed and compared between injection samples at 4 different times. The percentage of the relative standard deviation (% RSD) was calculated. The inter-day studies were evaluated by injecting the aliquots on the first day, second day, after 1 week and after 1 month. The good reproducibility for the investigated compounds was observed, which is evident by the RSD value which was less than 10%.

Results and Discussion

Optimization of LC-MS/MS

The 40% MeOH-water fraction of *L. pumila* was found having glycosides which include quercetin-3-*O*-rutinoside [22]. Further detection of quercetin-3-*O*-rutinoside and myricetin to improved quantitative analysis is necessary. In this study, several parameters were considered for compound optimization which basically signalled by the chromatographic separation as well as the mass spectra that was used for the compound characterisation. The parameter of the mobile phase was investigated using both isocratic and gradient separation. The isocratic separation was done *via* a combination of mobile phase A and mobile phase B consisting of mixtures of water: acetonitrile and water-methanol. The compound resolution using water: methanol (90:10) mixture was found to be lower when compared with the water-acetonitrile (90:10) mixture using isocratic separation system. The gradient system employed could improve the compounds resolution and eliminate the effect of peak tailing. The addition of low concentration, of 0.1% formic acid was able to improve the chromatographic feature and the mass spectra quality. Therefore, 0.1% formic acid was added into water as mobile phase A and into acetonitrile as mobile phase B in the gradient system with the system set at a fast flow rate; 0.25 mL/min. Fast separation was obtained between 10 minutes for both quercetin-3-*O*-rutinoside and myricetin. The ratio of mobile phase A and B was 90:10 for the first elution and was changed to 10:90 after 3 minutes and stay to equilibrate in the system for the next 6 minutes before return to the initial ratio in 8.10 to 10 minutes in a total runtime.

The MS/MS detection method for product ion characterisation was designated to acquire the mass signal. In this study, the mass was set up to render the mass of interest. The identification of product ion for compound verification was done by enhancing the product ion (EPI) scan mode. Different energy was used to produce ion from the electro-spray ionisation (ESI) source. The parameter included studying entering declustering potential (DP, Volt, V) and collision exit energy (CE, Volt, V) before it was identified. Meanwhile, the quantification of quercetin-3-*O*-rutinoside and myricetin were done using the multiple reaction monitoring (MRM) scan mode. This MRM scan mode is the best operational mode to localize the accuracy with specific ion fragmentation from the precursor ion (by mean parent ion) to its specific fragmentation ion (by mean daughter ion). The DP energy was set up from 30-50 V which is from high to lower energy. Meanwhile, CE was acquired from 10-20 V. Negative fragmentation produced better sensitivity detection signal for quercetin-3-*O*-rutinoside. The optimized DP energy for EPI scan mode was achieved at 40 V, while CE at 10 V. The optimized DP and CE for MRM scan mode were accomplished at 50V and 20V, correspondingly for both mass of interests.

Figure 1 showed the molecular ion m/z 609 at two different DP energy which produce characteristics ions of m/z 463, 447, 301, 271, 285, 179 and 151. The presence of four ions, m/z 271, 285, 179 and 151 were attributed to the major characteristics of quercetin [30]. The mass spectra showed an intense peak with clear characteristics ions at DP 40 V rather than DP 55 V with reference to the Figure 1. However, by increasing the DP energy, the product of quercetin was fragmented to intense quercetin peaks, however, the other three peaks of m/z 463, 447 and 609 had disappeared from the mass spectrum. From the EPI mass spectral, the loss of 146 Da (rhamnoside residual) from m/z 609 formed the m/z 463, and occurrence of m/z 447 was due to the cleavage of glucoside unit (162 Da) from the main quercetin structure. It was reported that m/z 609 was quercetin-3-*O*-rutinoside [31]. The loss of galactose, glucose or mannose from m/z 609 showed a significant peak at m/z 463 which was similar with previous article reported by Abad and his team [31]. The peak corresponding to m/z 463 was identified as quercetin-3-*O*-galactoside [30, 32]. The presence of m/z 447 was designated as quercitrin or commonly known as a quercetin-3-rhamnoside where one type of sugar was attached to its basic aromatic rings. The m/z 447 corresponded to the loss of one-unit sugar (146 Da) to form the quercetin m/z 301 together with the product ion m/z 179 and m/z 151 [30, 33]. The rhamnose unit was easily deprotonated with the loss of 146 Da, resulting in fragments at m/z 301, 179 and 151 (Figure 1). Pikulski and Brodbelt [33] reported that the deprotonation of quercitrin apparently by homolytic bond cleavage. This compound was commonly found in Brazilian cherry and South America berries fruits [32, 34].

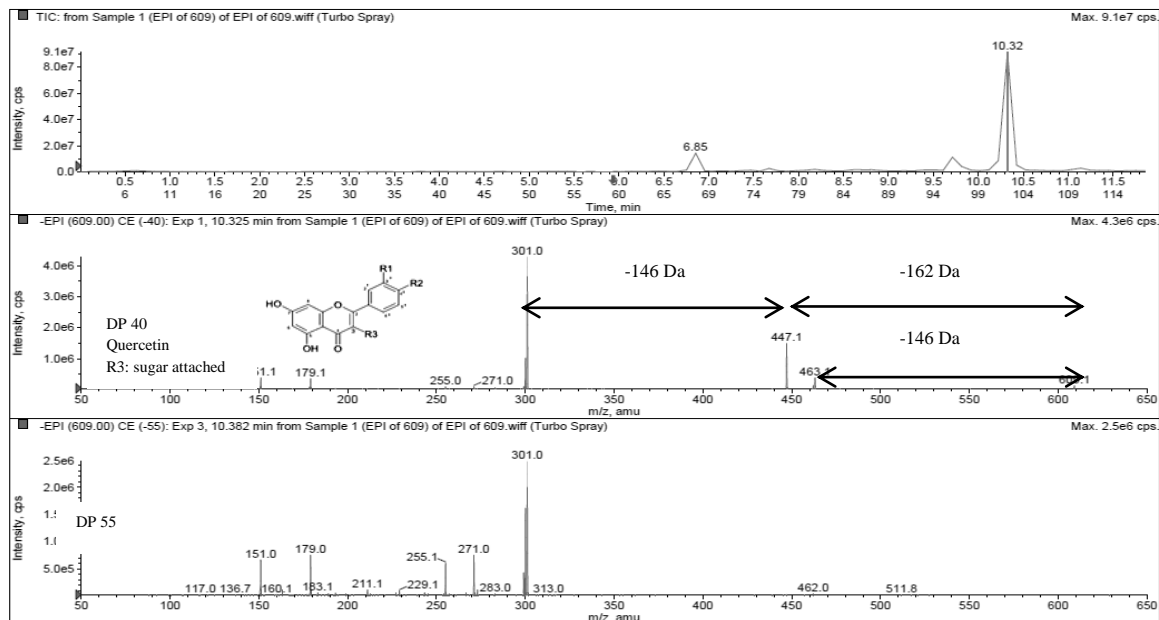


Figure 1. EPI mass spectra of quercetin-3-*O*-rutinoside from *L. pumila* fraction (40% MeOH: water) at different DP energy (DP 40 V and DP 55 V)

Identification of quercetin-3-*O*-rutinoside and myricetin from the fraction

Identification of quercetin-3-*O*-rutinoside and myricetin were carried out in negative and positive ionisation mode by comparing the MS/MS spectra in EPI scan. The $[M-H]^-$ was selected because it produces extensive MS/MS fragmentation compare to $[M+H]^+$. Quercetin-3-*O*-rutinoside (m/z 610) has loss 1-unit hydrogen in $[M-H]^-$ producing m/z 609 as shown in Figure 1. Myricetin (m/z 318) has also loss 1 unit of hydrogen to formed molecular ion, m/z 317 (Figure 2). The structure of quercetin-3-*O*-rutinoside and myricetin were shown in Figure 3.

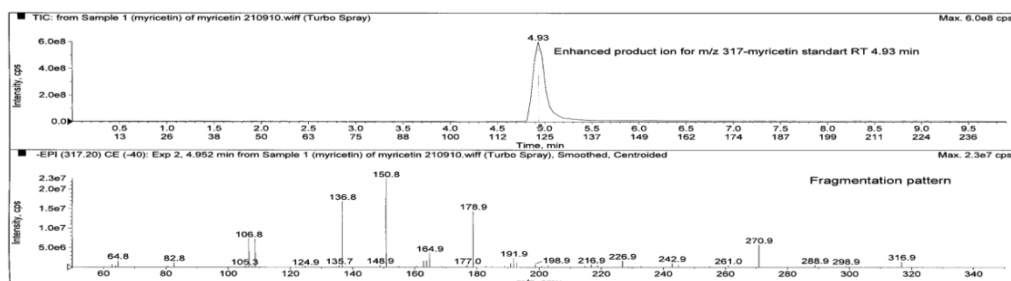


Figure 2. The EPI mass spectra of myricetin *L. pumila* fraction (40% MeOH: water) at different DP energy (DP 40 V and DP 55 V)

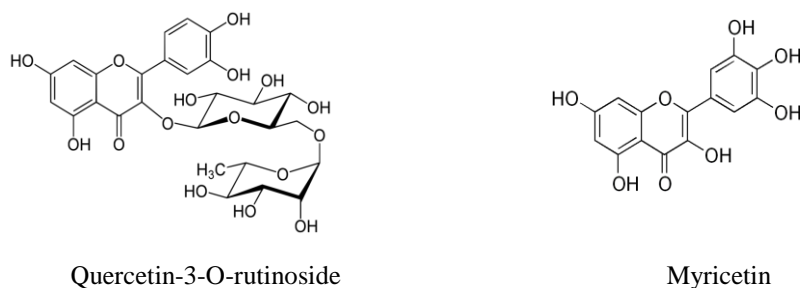


Figure 3. Structure of quercetin-3-O-rutinoside and myricetin

Myricetin with molecular ion m/z 318 was ionised leading to loss 1 unit of hydrogen to generate negative ion $[M-H]^-$ m/z 317 which was then fragmented to four characteristics ion of m/z 271, 179, 151, 137 (Figure 1) that is similar to the aglycone of quercetin. This occurrence was due to the breakdown of the aglycone myricetin structure, with the loss of 92 Da, 28 Da ($2CH_2$) and 14 Da (CH_2) with reference to the formation of peaks of m/z 179, 151 and 137, respectively. The presence of two peaks of m/z 179 and 151 were attributed to the major characteristics ion of myricetin [35, 36]. These four product ions were then confirmed by MRM scan, where consistent fragment ions matched to the standard myricetin. Myricetin is a flavonol-type which consists of three hydroxyl groups located at the B ring at position 3, 4 and 5. The sugar unit was attached to its basic aromatic ring to form a myricetin glycosides. This compound was also presence in fruits such as cherry, grapes and cranberry [25, 32, 37]. Our experiments showed that the best DP energy to generate the product ion was 40 V and the CE was at 40 V.

From the study, 40% MeOH fraction contained quercetin-3-*O*-rutinoside and myricetin and the presence of product ion is essential for the characterisation of these compounds. The generated DP energy at 40V was the most optimum energy to generate the product ion for both compounds in the enhance product ion scan mode (EPI).

Quantification and validation

The quantification step was done by using MRM scan to generate peaks from the precursor ion to product ion. Fragment of m/z 301 is the dominant product ion over quercetin-3-*O*-rutinoside. Meanwhile, m/z 137 was selected for myricetin in MRM scan mode. Using fast and accurate MRM scan mode, chromatographic data for both compounds including retention time, peak area, and peak height were monitored. As a requirement to verify and quantify the presence of these compounds, a standard working solution of quercetin-3-*O*-rutinoside and myricetin (99% purity) was used to build a standard calibration curve.

The peak of quercetin-3-*O*-rutinoside was detected at 3.3 minutes with a total running time of 10 minutes (Figure 4). The concentration of quercetin-3-*O*-rutinoside was determined to be at 0.007 mg/g in the plant fraction. Meanwhile, myricetin was detected at 3.9 minutes with the total running time set at 10 minutes (Figure 5). Significantly, myricetin was found to be higher in this plant fraction as compared to quercetin-3-*O*-rutinoside which was at 0.009 mg/g in the plant fraction.

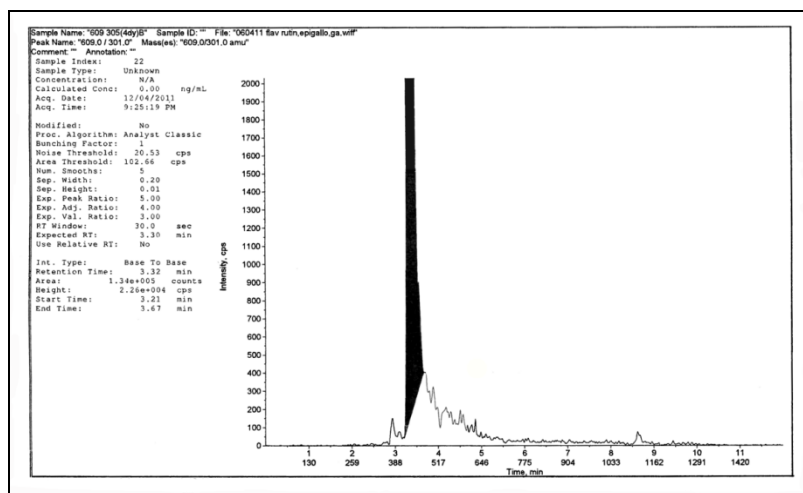


Figure 4. The MRM peak of quercetin-3-*O*-rutinoside from the *L. pumila* in 40% MeOH: water fraction

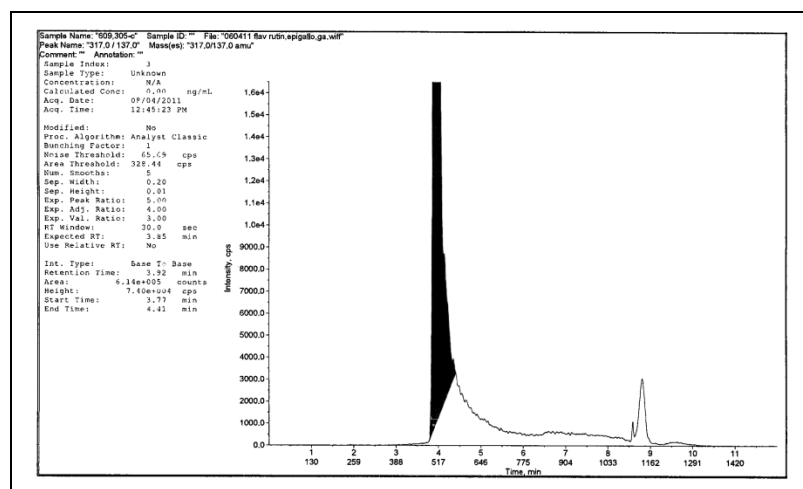


Figure 5. The MRM peak of myricetin from the *L. pumila* in 40% MeOH: water fraction

The LC-MS/MS validated method was determined based on its linearity, LOD, LOQ, precision and accuracy according to the guideline [28] suitable for quality control (Table 1). The response of quercetin-3-*O*-rutinoside in the range of 0.1–100 µg/mL produced linearity result with good regression equation and correlation coefficient ($r^2 = 0.998$ with the following equation; $y = 99956x - 89337$). The detection limit, LOD and LOQ for the quercetin-3-*O*-rutinoside were 0.04 µg/mL and 0.13 µg/mL, respectively. The developed LC-MS/MS method had successfully achieved high sensitivity detection of quercetin-3-*O*-rutinoside based on the LOD and LOQ which were comparable to the signal noise to ratio (S/N). This method was found fit for quantitation purpose. The recovery for quercetin-3-*O*-rutinoside was measured at 87% and most probably the 13% loss was probably due to the steps involved during the sample preparation. However, it can be considered as a good compound recovery since the percentage was above 80%. Good precision was expressed by the RSD (%) value, which is required to be less than 10%. The intra-day and inter-day for quercetin-3-*O*-rutinoside were found to be 5.01% and 9.18%, respectively. These two values showed that quercetin-3-*O*-rutinoside was stable for at least a month, but it is recommended to prepare the extract fresh for analysis to avoid any deterioration.

The detection method for myricetin was described based on the optimal detection condition (not shown). The calibration curve in order to obtain the linearity of myricetin was found to be straight linear concentration ranged from 0.1 to 100 µg/mL and had a linear regression of $r^2 = 0.991$ with the following equation; $y = 35125x - 13273$. The LOD and LOQ for myricetin were found to be at 0.03 µg/mL and 0.11 µg/mL, respectively. Analysis of the spiked myricetin standard for recovery study had showed an average recovery of 88.2%. The analysis of the compound in the solution on the same day had shown good reproducibility at 3.14%. The study on the different days (inter-day) also showed good precision of 3.18%. Therefore, the robustness of the method indicated that myricetin is possibly consistent for at least 1 month of storage based on these results. Table 1 summarises the results from the study.

Table 1. Linearity, LOD, LOD, recovery, intra-day and inter-day data of quercetin-3-*O*-rutinoside and myricetin in *L. pumila* fraction (40% MeOH: water) by LC-MS/M

Flavonoids	Linear Equations	LOD (µg/mL)	LOQ (µg/mL)	Recovery (%)	Intra- day (RSD) %	Inter- day (RSD) %	Concentration (mg/g)
Quercetin-3- <i>O</i> -rutinoside	$Y = 99956x - 89337$	0.04	0.13	87.00	5.01	9.18	0.007
Myricetin	$Y = 35125x - 13273$	0.03	0.11	88.20	3.14	3.18	0.009

Conclusion

The present work describes the application of LC-MS/MS procedure to analysed quercetin-3-*O*-rutinoside and myricetin in *L. pumila* fraction which was obtained from 40% MeOH: water. The validated method indicated that lower concentration of quercetin-3-*O*-rutinoside and myricetin can be identified and quantified in the plant fraction by using high throughput analytical technique via electrospray ionisation (ESI). Valuable information can be provided including mass identity together with characteristics fragmentation ion. This method is applicable for the analysis of plant containing quercetin-3-*O*-rutinoside and myricetin as well as for quality control and routine analysis purposes.

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