ABSTRACT
We have previously reported the antioxidant and neuroprotective activities of the aqueous extract of four varieties of Ficus deltoidea (Fd) (Moraceae) namely var kunstleri (Fdvk), var angustifolia (Fdva), var deltoidea (Fdvd) and var intermedia (Fdvi). In this study, flavonoid constituents in aqueous leaf and fig extracts of the four varieties were analyzed and characterized using liquid chromatography mass spectrometer quadrupole-time of flight (LCMS-Q-TOF) via hierarchical cluster analysis (HCA) technique. The HCA dendrogram revealed that the abundant flavonoids among the eight samples are epicatechin, quercetin-3-rutinoside, quercetin 5,4’-di-O-beta-D-glucopyranoside, myricetin and naringenin. The study found that the distribution of the flavonoids differed between the four varieties and varied within the plant parts. To date, the flavonoid distribution of the different plant parts of the four varieties has not been documented. A positive correlation was observed between flavonoid constituents present and radical scavenging activities of the aqueous extracts.

Keywords
Ficus deltoidea (Fd); Flavonoid, Liquid Chromatography Mass Spectrometer Quadrupole-Time of Flight (LCMS-Q-TOF); Hierarchical Cluster Analysis (HCA)
1. INTRODUCTION

Ficus deltoidea (Mas Cotek) has long been used as traditional medicine in Malaysia and exists in seven varieties [39]. Malay traditional practitioners extensively consume Mas Cotek as tea. It is also used both externally and internally to treat an extensive list of conditions; including wounds, headache, fever [20], diabetes [31], toothache and as herbal drink for women to strengthen the uterus after childbirth [27] as well as other diseases related to oxidative stress. The reported biological activities of this plant include antioxidant, antihyperglycemic, anti-inflammatory, antilulcerogenic and antinociceptive ([36]; [15]; [26]; [27]; [2]). [2] reported that almost all of the parts of Fd plant including the root, bark, leaf and fig have their own medicinal properties. Previous studies on the aqueous extracts showed that it contained antioxidant flavonoids including flavan-3-ol monomers namely catechin, epicatechin, gallocatechin and epigallocatechin [22]. It was earlier reported to contain polyphenols, flavonoids and tannins but was absent of alkaloids, triterpenes and saponins ([38]; [9]).

[33] have reported flavonoids as the antioxidant scavengers of a wide range of ROS and inhibitors of lipid peroxidation. This class of natural products represents the most abundant antioxidants in the diet and they have gained tremendous interest as potential therapeutic agents against a wide variety of diseases, most of which involve oxidant damage [25]. Flavonoids may interfere in several of the steps that lead to the development of malignant tumors, including protecting DNA from oxidative damage, inhibiting carcinogen activation, and activating carcinogen-detoxifying systems [7,13].

Today, liquid chromatography mass spectrometer quadrupole-time of flight (LCMS-Q-TOF) has been shown to be a powerful tool in the search of new biomarkers for disease and drugs. Analysed data from LCMS-Q-TOF has been used to examine the untargeted metabolites in plant samples and to find the ones with statistically significant variations in abundance within a set of experimental versus internal and external database [28]. In analysing LCMS-Q-TOF data, hierarchical clustering analysis (HCA) is an accurate technique in which results are represented as a two-dimensional diagram known as a dendrogram. HCA demonstrate a clear clustering of plant specimens selecting the highest discriminating ions given by the complete data analysis and this lead to the specific identification of metabolites [12].

Continuing our interest in the biological activities of the plant, this study was aimed at identifying flavonoids in the aqueous leaf and fig extracts of four varieties of Fd namely var angustilolia (Fdva), var deltoidea (Fdvd), var intermedia (Fdvi), and var kunstleri (Fdvk) via LCMS-Q-TOF method. Distribution of the flavonoids was determined by HCA technique and correlation of flavonoid constituent and radical scavenging activities was made.

2. METHODOLOGY

Plant Materials

The fresh leaf and fig of four varieties of Fd namely Fdva, Fdvd, Fdvi, and Fdvk, were collected from Kuala Pilah, Negeri Sembilan, Malaysia and were identified by Mr. Kamarudin Saleh (botanist) from Forest Research Institute Malaysia (FRIM). The samples were cleaned, dried under room temperature and finely ground. 100 g of each ground sample was heated separately in 500 ml of distilled water by continuous stirring at 60°C for 1 hour. The aqueous extracts were then filtered, concentrated under reduced pressure and stored at -20°C before being subjected to further analysis.

Determination of Antioxidant Activities of the Aqueous Extracts

2.2.1 Total Flavonoids Content (TFC) Assay

Total flavonoids content were measured by a colorimetric assay developed by [37]. A 1 ml aliquot of appropriately diluted sample or standard solutions of catechin (20, 40, 60, 80, and 100 mg/ml) was added to a 10 ml volumetric flask containing 4 ml distilled water. At zero time, 0.3 ml 5% NaNO2 was added to the flask. After 5 minutes, 0.3 ml 10% AlCl3 was added. At 6 minutes, 2 ml 1 M NaOH was added to the mixture. Immediately, the reaction flask was diluted to volume with the addition of 2.4 ml of distilled water and thoroughly mixed. Absorbance of the pink coloured mixture was determined at 510 nm against water blank. Total flavonoid of extract was expressed in milligram catechin equivalents (mg CE/g extract). All analyses were run in three replicates and mean values were recorded.

2.2.2 1,1-diphenyl-2-picrylhydrazyl (DPPH) Radical Scavenging Assay

This assay was carried out as described by [28] with some modification [4]. Stock solutions of crude extracts were prepared as 1 mg/ml in methanol. The solutions were diluted to different concentrations (250, 125, 62.5 and 31.3 μg/ml in methanol) in a 96-well microtiter plate. Then, 5 μl of DPPH solution (prepared as 10 mg/ml in methanol) were added to each well. The plate was shaken gently and placed in the dark for 30 minutes at room temperature. The absorbance was then measured at 517 nm. Percentage of inhibition was calculated using the following formula:

% Inhibition = \( \frac{\text{Abs (control)} - \text{Abs (sample)}}{\text{Abs (control)}} \times 100\% \)
Identification of Compounds Using LCMS Q-TOF Analysis

2.3.1 Liquid Chromatography Conditions

Chromatography was performed on Liquid Chromatography of 1200 Rapid Resolution Series (Agilent Technologies, Santa Clara, CA, USA) consisting of a binary pump, degasser, 96-well plate autosampler with thermostat, thermostat column compartment and 6520 Q-TOF mass spectrometer equipped with a dual-ESI source. The column used was a Zorbax Eclipse Plus C18 with column ID of 1.8 μm particle size and 2.1 x 100 mm column dimensions [3]. The temperature was maintained at 40°C during the run.

2.3.2 Solvent System Conditions

The mobile phase (A) consisted of 0.1% formic acid (Supelco, Inc) in water and (B) 0.1% formic acid in acetonitrile. The flow rate was 0.25 mL/min and the injection volume was 2 μL. A linear gradient was developed over 36 minutes from 5% to 95% of mobile phase (B). Total run time was 48 minutes for each analysis. ESI source settings were as follows: V Cap 4000 V, skimmer 65 V and fragmentor 125 V.

2.3.3 Mass Spectral Conditions

Mass spectral acquisition range selected was from 50 to 1700 m/z. The nebulizer was set at 45 psig and the drying gas nitrogen was set at a flow rate of 12 L/min. Drying gas temperature was maintained at 350°C. Data was acquired at a rate of 2.5 spectra/second with a stored mass range of 50 to 1000 m/z. Internal reference ions were used to correct mass accuracy. Autocalibration parameters were chosen to average five scans and reference mass correction was enabled throughout the run.

3. RESULTS AND DISCUSSION

3.1 Determination of Total Flavonoids Content (TFC) and Radical Scavenging Activities of the Aqueous Extracts

The total flavonoids content in the aqueous extracts of each of the four variety was determined through a linear catechin standard curve (y = 0.002x + 0.014; R² = 0.999) where x is the catechin concentration in mg/L and y is the absorbance reading at 510 nm. The total flavonoids content of each extract of the four varieties of Fd were expressed in mg catechin/g extract or (mg CE/g extract). Table 1 shows the TFC of extracts of the plant varieties. The leaf extract of Fdvd showed the highest TFC (362.25 mg CE/g extract) followed by Fdvi (63.25 mg CE/g extract), Fdvk (52.65 mg CE/g extract), and Fdva (26.6 mg CE/g extract). The TFC values of the fig extract were exhibited in following order: Fdvd > Fdvi > Fdva > Fdvk. Among the varieties, Fdvi and Fdvd showed high TFC compared to Fdvk and Fdva. Furthermore, the fig extract of each variety showed higher TFC compared to the leaf extract. The TFC value for the leaf extract of Fdvd was more than five-fold higher than Fdvi. However, for the fig extract, the TFC value of Fdvd was only nearly two-fold the value of Fdvi.

[38] studied the content of flavonoids in the aqueous leaf extract of Fdva and Fdvd using colorimetric assay. The authors found that the TFC of the aqueous leaf extract of Fdvd was higher than the value for Fdva with 42.63 and 27.35 mg quercetin/g extract, respectively. Their results showed the same trend as found in our study (89.75 and 61.10 mg GAE/g extract for Fdvd and Fdva, respectively) with TFC value of Fdvd about 1.5 times higher than Fdva. The TFC values of the aqueous extracts of previous studies may be different with our study due to the employment of quercetin as a standard instead of catechin.

Table 1. Total Flavonoids Content of Aqueous Extracts of Four Fd Varieties

| Fd Variety | Part | TFC (mg CE/g extract) |
|------------|------|----------------------|
| Fdvk       | Leaf | 52.65<sup>a</sup>    |
|            | Fig  | 178.10<sup>a</sup>   |
| Fdva       | Leaf | 26.60<sup>b</sup>    |
|            | Fig  | 183.90<sup>b</sup>   |
| Fdvd       | Leaf | 362.30<sup>a</sup>   |
|            | Fig  | 742.60<sup>b</sup>   |
| Fdvi       | Leaf | 63.25<sup>b</sup>    |
|            | Fig  | 357.60<sup>a</sup>   |

Value was expressed in mean ± standard deviation (SD), (n =3); Pearson correlation: <sup>a</sup>p<0.05; <sup>b</sup>p<0.01
As shown in Table 2, among the extracts tested, the leaf and fig extracts of Fdvd and the fig extract of Fdvi were found to be strong free radical scavengers with IC\textsubscript{50} values of 15.6, 7.8 and 7.8 μg/ml, respectively. However, only moderate radical scavenging activity was found for the leaf extract of Fdvi with an IC\textsubscript{50} value of 31.3 μg/ml consistent with its TFC value, which is more than five times lower than its fig extract. The DPPH radical scavenging activity at most concentrations was found to correlate well with the TFC values. This is illustrated for the leaf and fig extract of Fdvd and Fdvi varieties at IC\textsubscript{50} value of 3.9, 7.8 and 15.6 μg/ml with values of the correlation coefficient ($R^2$) of 0.6284. To date, no report has been found on the radical scavenging activity of the aqueous extract on any of the varieties of Fd. However, a study on methanolic extracts of F.microcarpa found DPPH radical scavenging activity of 7.3 and 21.4 μg/ml of fruits and leaf, respectively [6]. These values are comparable to the values found in our study. 

**Table 2. Radical Scavenging Activity (%) of the Varieties of Fd**

| Fd variety | Plant part | Sample concentrations | IC\textsubscript{50} (µg/ml) |
|------------|------------|------------------------|-----------------------------|
|            |            | 3.9 µg/ml | 7.8 µg/ml | 15.6 µg/ml | 31.3 µg/ml | 62.5 µg/ml | 125 µg/ml | 250 µg/ml |
| Fdvk       | Leaf       | 25.67     | 28.37     | 31.07      | 54.32      | 56.32      | 64.43      | 71.98     | Na        |
|            | Fig        | 20.03     | 24.32     | 27.53      | 34.06      | 39.87      | 48.28      | 60.7      | Na        |
| Fdva       | Leaf       | 28.67     | 35.76     | 38.94      | 52.96      | 55.96      | 59.56      | 73.18     | Na        |
|            | Fig        | 25.86     | 37.97     | 46.74      | 49.43      | 50.08      | 52.42      | 56.54     | Na        |
| Fdvd       | Leaf       | 18.54     | 41.18     | 51.11      | 73.51      | 74.5       | 76.23      | 82.04     | 15.6      |
|            | Fig        | 34.92     | 47.19     | 48.85      | 61.93      | 67.16      | 71.88      | 81.43     | 7.8       |
| Fdvi       | Leaf       | 19.99     | 44.46     | 55.03      | 75.52      | 77.18      | 78.59      | 80.17     | 31.3      |
|            | Fig        | 48.31     | 49.64     | 53.85      | 64.97      | 68.57      | 74.56      | 78.13     | 7.8       |
| *F.microcarpa* | Leaf     |          |           |            |            |            |            |            | 21.4      |
|            | Fruit      |          |           |            |            |            |            |            | 7.3       |
| Quercetin  |            | 68.6      | 69.2      | 71.18      | 72.29      | 73         | 73.42      | 75.29     | Nd        |
| α-tocopherol |           | 74.92     | 80.72     | 82.3       | 83.32      | 83.9       | 83.96      | 84.69     | Nd        |

Value was expressed in mean ± standard deviation (SD), (n = 3). Na, Not active; Nd, Not detectable in experimental concentration range (3.9-250 µg/ml); strong radical scavenging activity, (IC\textsubscript{50} < 30 µg/ml); moderate radical scavenging activity, (30 µg/ml ≤ IC\textsubscript{50} ≤ 100 µg/ml); weak radical scavenging activity, (IC\textsubscript{50} > 100 µg/ml) [1].

*The IC\textsubscript{50} values found in methanolic extract of Ficus microcarpa [6].

### 3.2 Identification of Compounds Present in the Aqueous Leaf and Fig Extracts of Four Varieties of Fd

The leaf and fig aqueous extracts of the four varieties of Fd were analyzed by gradient reverse phase liquid chromatography (LC) with Q-TOF MS detection. The identification was confirmed by internal and external standards. In this study, five flavonoid compounds detected were epicatechin, quercetin-3-rutinoside, myricetin, naringenin and quercetin 5,4'-O-beta-D-glucopyranoside using LCMS-Q-TOF via HCA technique (Table 3).
Table 3. Mass Spectral Characteristics and Identity of Compounds Present in the Aqueous Extracts of Four Varieties of *Fd*

| Peak | \( t_R \) (min) | Compound | \([M+H]^+\) (m/z) |
|------|----------------|----------|------------------|
| 1    | 6.747          | *Epicatechin* \([C_{15}H_{14}O_6}\), db=95.57, overall=95.57, KEGG ID=C09727, CAS ID=490-46-0 | 291.0868 |
| 2    | 9.361          | Quercetin-3-rutinoside \([C_{27}H_{30}O_{16}]\), db=83.99, overall=83.99, KEGG ID=C05625, CAS ID=153-18-4 | 611.1611 |
| 3    | 9.377          | Quercetin 5,4'-di-O-beta-D-glucopyranoside \([C_{27}H_{30}O_{17}]\), tgt=61.06 | 627.1587 |
| 4    | 9.553          | Myricetin \([C_{15}H_{10}O_6}\), db=88.79, overall=88.79, KEGG ID=C10107, CAS ID=529-44-2 | 319.0435 |
| 5    | 16.062         | Naringenin \([C_{15}H_{12}O_5}\), db=79.39, overall=79.39, KEGG ID=C00509, CAS ID=480-41-1 | 273.0770 |

\( t_R \): retention time; \([M+H]^+\): positively charged molecular ion

A study by [22] on boiled aqueous extract of *Fd* (the variety is not specified) collected from a local market in Pahang, Malaysia had identified flavonoids such as catechin, epicatechin, gallocatechin and epigallocatechin using High Performance Liquid Chromatography (HPLC) amongst other flavonoid glycosides. The reported quantities were 98, 99, 44 and 87 μmol/L, for catechin, epicatechin, gallocatechin and epigallocatechin respectively. However, in our study, we were not able to detect catechin, gallocatechin or epigallocatechin in any of the four varieties. Internal standards were used to confirm their absence. This might be due to differences in the origin of the sample and the method of extraction. Furthermore, our method employed 60°C as the extracting temperature while [22] employed a temperature of 100°C to extract the secondary metabolites.

Peak 1 \((t_R, 6.747 \text{ min}; [M+H]^+ \text{ at m/z, 291.0868})\) was identified as epicatechin as shown in spectrum Fig. 1(a) and confirmed by comparison with standard. Epicatechin is a flavan-3-ol belonging to catechin family and can be found in natural products or medicinal herbs. It commonly exists in monomeric and oligomeric forms [21]. Employing the DPPH method, [22] had identified 18% antioxidant activity of epicatechin \((t_R, 19.4 \text{ min}; m/z, 289 [M-H]-1)\) in the aqueous infusion of leaf of *Fd*. It showed a strong fluorescent peak using HPLC-PDA-MS², which gave rise to MS³ ions at m/z 245, 205, and 179. Their results were confirmed by co-chromatography with an authentic standard and it indicates that epicatechin found in *Fd* may be utilized as a potential antioxidant. Similarly, [6] also used DPPH method and found that epicatechin from the ethyl acetate fraction of aerial root and bark of *Ficus microcarpa* exhibited excellent antioxidant activity.

As shown in Fig. 1(b), peak 2 \((t_R, 9.361 \text{ min})\) indicated the presence of rutin (quercetin-3-rutinoside) \([M+H]^+\) at m/z 611.1611. Rutin is a flavonol and comprises quercetin and the disapacharride rutinosine [35]. The first documented report on flavonoids profiling in different varieties of the hot and cold aqueous extracts of *Fd* leaf namely *Fdvk* and *Fdva* by [14] using reversed-phase HPLC reported a higher rutin content in *Fdvk* compared to *Fdva*. In contrast, the results obtained in our study indicated that the content of rutin in the aqueous extracts of *Fdvk* is similar to *Fdva* based on the dendrogram shown in Fig. 3. A study by [24] found that rutin have beneficial effect on spatial memory along with the concentration of brain neurotransmitters in aged rats. Rutin also showed anti-diabetic potential in streptozotocin-induced diabeticistar rats as reported by [18].

Peak 3 \((t_R, 9.377 \text{ min})\) had a \([M+H]^+\) at m/z 627.1587 and was identified as quercetin 5,4'-di-O-beta-D-glucopyranoside as shown in Fig. 1(c), [19] had earlier reported a relative molecular mass of 626 for the compound from its FD-MS spectrum. Its aglycone, quercetin, has been often been proven by *in vitro* tests to act as a powerful antioxidant. The compound being a major constituent of the flavonoid intake, has been suggested to be a key in fighting several chronic degenerative diseases. It also exhibited a marked neuroprotective effect in *in vitro* experiments and this is mainly explained by its antioxidant capability and ability to scavenge free radicals [11]. To date, there has been no comparative study reported on the quercetin or quercitin derivatives from the four varieties of *Fd*.

Peak 4 \((t_R, 9.553 \text{ min})\) showed an m/z 319.0435 corresponding to \([M+H]^+\) and was identified as myricetin as shown in Fig. 1(d). Myricetin \((3,3',4',5',7\text{-hexahydroxyflavone})\) is a natural flavon with hydroxyl substitutions at the 3,3',4',5',7 positions \([34]\). A study by [8] had reported the myricetin capacity in protecting PC12 cells from oxidative insult \((H_2O_2)\) and at the same time, it increases the cell survival. A study by [32] found myricetin as the major flavonoid in the ethanolic leaf extract of *Ficus carica* using reversed-phase HPLC and analyzed by UV/Vis array and electrospray ionization (ESI)-mass spectrometry (MS) detectors. Interestingly, [22] who studied *Fd* did not find myricetin in the aqueous extract. There has also been no other report on its detection from any variety of *Fd* species.

Peak 5 \((t_R, 16.013 \text{ min}; m/z, 273.0770)\) was identified as naringenin as shown by Fig. 1(e). Naringenin \((4',5,7\text{-trihydroxyflavanone})\) is also known as aglycone of naringin and classified as a flavanon [23]. Naringenin extracted from the methanol leaf extract of *F. benjamina* was also reported to possess cytotoxicity against T-lymphoblastic leukemia (CEM-SS) cell line [5]. To date, there has been no report on the isolation or detection of this compound from *Fd* species.
3.3 Hierarchical Cluster Analysis (HCA) of Aqueous Leaf and Fig Extracts of Four Varieties of Fd

HCA is the most popular clustering technique to evaluate the quality of the medicinal plants [17] and it is a simple method of grouping a set of available data based on their similarities [30]. The presentation of HCA results as a dendrogram makes it such a way the relationships can be more readily visualized. In this study, HCA using Euclidean distances and average linkage method was used for clustering its ability to separate the data into four clusters. In the dendrogram shown in Table 3, the horizontal axis represents the plant parts of the varieties while the vertical axis represents the flavonoids obtained. The clusters were classified and numbered as follows: (I) fig of Fdva and Fdvk, (II) fig of Fdvd and Fdvi, (III) leaf of Fdvd and (IV) leaf of Fdva, Fdvi and Fdvk. The identified flavonoids in this study were epicatechin, quercetin-3-rutinoside, quercetin 5,4’-di-O-beta-D-glucopyranoside, myricetin and naringenin (as listed in Table 1) were grouped based on their distribution values (0.14, 0.31 and 0.55) as shown in Fig. 2.

In the first (I) cluster, the flavonoid quercetin-3-rutinoside showed the highest intensity in the fig extracts of Fdva and Fdvk and low intensity of epicatechin, quercetin 5,4’-di-O-beta-D-glucopyranoside and naringenin. In contrast, the flavonoids epicatechin and quercetin 5,4’-di-O-beta-D-glucopyranoside exhibited the highest intensity in the fig of Fdvd and Fdvi while naringenin showed the lowest intensity in the second (II) cluster. The leaf of Fdvd was grouped alone in the third (III) cluster with the highest intensity of epicatechin and the other four flavonoids namely quercetin-3-rutinoside, quercetin 5,4’-di-O-beta-D-glucopyranoside, myricetin and naringenin were indicated by low intensity. In the fourth (IV) cluster, the flavonoid naringenin exhibited the highest intensity in the leaf of Fdvk while low intensity can be observed in the distribution of epicatechin, quercetin-3-rutinoside, quercetin 5,4’-di-O-beta-D-glucopyranoside and myricetin.

To the best of our knowledge, no comparative study regarding this analysis of plant parts of the aqueous extracts of the four varieties of Fd has been documented. In addition, the distribution and intensity of flavonoid constituents in the aqueous leaf and fig extracts of the four varieties were seen to be consistent with their radical scavenging activities as measured by the DPPH assay. Both Fdvd and Fdvi (leaf and fig extracts) exhibited strong antioxidant potential which may be related to the high amount of epicatechin, in this plant variety. A study by [16] had reported the scavenging activity of hydroxyl groups present on ring B of flavonoids, specifically the structure 3’-4’ catechol (catechol moiety). The presence of a hydroxyl group in position 3 added to the catechol structure of flavonoid (as shown in Fig. 3) was found to increase ten-fold scavenger activity towards free radicals.
4. CONCLUSION

Flavonoid profiling of the Fd extracts using LCMS Q-TOF followed by HCA clustering generated a dendrogram representing four clusters of flavonoids distribution in the eight samples, which were grouped based on their distribution values represented by colour intensity. The identified flavonoids were epicatechin, myricetin and naringenin along with two flavonoid glucosides identified as quercetin 5,4’-di-O-beta-D-glucopyranoside and quercetin-3-rutinoside. Except for epicatechin, the other four flavonoids have not been reported to occur in any Fd species. A high amount of epicatechin found in Fdvd (leaf and fig) and Fdvi (fig) may be responsible for the strong radical scavenging activities found in the
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