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Discriminating Factors for *Ficus deltoidea* Jack Varieties by HPTLC coupled with Chemometrics

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Keywords:
Vitexin,
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Ficus deltoidea Jack

ABSTRACT

Ficus deltoidea Jack (FD) is a highly potential herb. However, there is serious confusion on selecting the right plant as FD occurs in several varieties. In this study, a high-performance thin layer chromatography (HPTLC) method was developed to determine the discriminating factors of FD varieties. Eight varieties of methanol extract, five varieties of water extract and marker compounds, vitexin and isovitexin were analyzed. Chemometric analysis; principal component (PCA), hierarchical cluster (HCA) and discriminant (DA) on HPTLC R_f value were performed. For methanol extracts, total variance of PCA score was 66.78% and clustered apart from each other. Total variance of water extract was 82.25%, which grouped into three clusters. HCA analysis produced three clusters for both types of extracts. Discriminant analysis revealed that methanol and water extracts were 100% discriminated. This finding suggests that the discriminant factors between methanol extracts were unidentified compounds at R_f value of 0.51, 0.72 and isovitexin. Meanwhile, isovitexin and vitexin were identified as discriminant factors for water extract.

Keywords: Vitexin, Isovitexin, HPTLC, Chemometrics, *Ficus deltoidea* Jack

ABSTRAK

Ficus deltoidea Jack (FD) adalah herba yang berpotensi tinggi. Namun, terdapat kekeliruan dalam memilih tumbuhan yang betul kerana FD wujud dalam beberapa varieti. Dalam kajian ini, tatacara kromatografi lapisan nipis berprestasi tinggi (HPTLC) dibangunkan bagi menentukan faktor perbezaan antara varieti. Ekstrak metanol dari lapan varieti, ekstrak air dari 5 varieti dan sebatian penanda, vitexin dan isovitexin telah dianalisa. Analisis kemometrik; *principal component* (PCA), *hierarchical cluster* (HCA) and *discriminant* (DA) dijalankan keatas nilai R_f HPTLC. Bagi ekstrak metanol, jumlah varian untuk skor PCA adalah 66.78% dan terkumpul dalam kluster berasingan. Jumlah varian bagi ekstrak air adalah 82.25%, terkumpul dalam tiga kluster. Analisis HCA menghasilkan tiga kluster bagi kedua-dua ekstrak. DA menunjukan ekstrak metanol dan ekstrak air adalah 100% berbeza. Dapatan kajian ini mencadangkan bahawa sebatian tidak dikenalpasti pada R_f 0.51, 0.72 and isovitexin merupakan faktor perbezaan bagi ekstrak metanol. Isovitexin dan vitexin pula dikenalpasti sebagai faktor yang membezakan ekstrak air.

Kata kunci: vitexin, isovitexin, HPTLC, kemometrik, *Ficus deltoidea* Jack

INTRODUCTION

Herbal have widely been used since ancient times for therapeutic purposes. WHO estimates about 80% of the world population still consumes herbs as well as other traditional medicines for their primary health care needs. The main issue of herbal is adulteration where practitioner unintentionally or intentionally used the wrong plant material. Thus, authentication of herbal material is very important. *Ficus deltoidea* Jack (FD), known as mas cotek by locals has been used by traditional practitioners as herbal remedies to treat several illnesses like headaches, hypertension and hyperglycaemia (Abd Samah et al., 2012; Adam et al 2007; Adam et al 2010; Adam et al 2012). Several studies have shown that this plant has antioxidant and neuroprotection activities (Choo et al., 2012; Dzolin et al., 2010; Farsi et al., 2011), antinociceptive activity (Gazzaz et al 2012), antidiabetic activity (Hakiman & Maziah, 2009; Jing et al 2011; Martinez & Martinez, 2005) and antimicrobial activity (Misbah et al., 2013). In addition, at least 25 flavonoids were identified, and the main constituents have been identified to be flavan-3-ol monomer, proanthocyanidins, and C-linked flavone glycosides (Mohd et al., 2014). Vitexin and isovitexin were used as marker compounds for identification of FD (Mohd et al., 2014; Musa, 2005). FD occurred in several varieties in Malaysia including, *Ficus deltoidea* var. *deltoidea,* var. *trengganuensis,* var. *kunstleri,* var. *motleyana,* var. *intermedia,* var. *borneensis,* var. *bilobata and* var. *angustifolia*. Mohd et al. (Mustafa et al., 2011) have utilized chemometric analysis on FTIR data to classify FD and found that all eight varieties tested were significantly discriminated. However, the actual compounds that contribute to the discrimination remain unclear.

The aim of this study was to determine the effect of marker compounds namely vitexin and isovitexin in the discrimination of FD varieties via principal component analysis (PCA), hierarchical cluster analysis (HCA), and discriminant analysis (DA). The identification of FD varieties using marker compounds approach was performed by HPTLC and its chromatographic retention data was acquired for discriminant analysis. The results from the chromatographic fingerprinting studies based on marker compounds will be useful for differentiating between varieties.

MATERIAL AND METHODS

Chemical and reagents

The pure compounds vitexin and isovitexin were purchased from Fluka-Sigma. The solvents methanol, ethyl acetate, and formic acid are products of Merck (Darmstadt, Germany). HPTLC plates pre-coated with silica gel 60 F_{254} (layer thickness of 0.2 mm) were purchased from Merck.

Sample Preparation

The leaves of eight FD varieties, FD var. *deltoidea* (FDD/FDDW), var. *kunstleri* (FDK), var. *angustifolia* (FDA), var. *motleyana* (FDM/FDMW), var. *bilobata* (FDB) and var. *trengganuensis* (FDTG/FDTGW) were obtained from Universiti Sultan Zainal Abidin (UniSZA) living collection, Gong Badak Campus, Kuala Terengganu. FD var. *intermedia* (FDI/FDIW) was collected from mount Brinchang, Cameron highland and var. *borneensis* (FDBN/FDBNW) was collected in Santubong, Sarawak, East Malaysia. Each sample was identified by Prof. Nashriyah Mat, UniSZA, Terengganu, Malaysia and deposited at UniSZA's herbarium. All leaves samples collected were dried in a conventional oven at 45°C and then ground into powder. Fifty grams of leaves powder were extracted with methanol and for water extract, 50g leaves powder was extracted at 100°C with distilled water for 1 hour. Both filtrates of methanol and water extraction were concentrated under pressure at 45°C and maintained at -20° prior analysis. However, there were only five varieties available for water extract.

Preparation of sample extract for HPTLC analysis

All crude extracts of FD varieties were prepared at a final concentration of 20 mg/mL in methanol for methanol extract and in a mixture of methanol: water (1:1) for water extract. All samples prepared were sonicated for 30 minutes and centrifuged. The supernatant was spotted on HPTLC plates.

HPTLC analysis

The protocol of HPTLC qualitative was performed according to Mohd et al. (2014). The samples were spotted in the form of narrow bands (8.0 mm) with a length of 8.0 mm from the bottom edge, 40 mm from the margin, and 13.3 mm apart at a constant rate of 100 nL/seconds using a nitrogen aspirator. The HPTLC plates were developed in mobile phase consisting of ethyl acetate: formic acid (0.1%): methanol (5:5:2 v/v/v) based on the separation of marker compounds. The migration distance was 8 cm with a migration time of 25 minutes. The developed plates were visualized under long wavelength of 365 nm using TLC visualizer. The densitometric analysis of the separated components was carried out using a Camag thin layer chromatography (TLC) scanner 3 (Camag, Switzerland) in the absorbance mode at 340 nm. The bands were scanned using deuterium and tungsten lamps, and the scanning speed was maintained at 20 mm/seconds with a macro slit dimension of 8.00 mm × 0.2 mm. The integration of chromatograms was performed using Camag TLC scanner system and winCATS software (Camag, Switzerland).

Data analysis for chromatographic fingerprinting

The HPTLC chromatographic data based on the separation of standard markers (vitexin and isovitexin) of FD varieties were exported and analysed using winCATS 1.2.5 software. The data of retention factor (R_f) value and peak areas of all samples were extracted manually into Microsoft Excel 2007.

The excel files were imported into a multivariate statistical software program, The Unscrambler X 10.1 (CAMO, Trondheim, Norway) and were normalized with area normalization. Baseline correction is not necessary for this data as this analysis was carried out on HPTLC silica gel plate, which the baseline noise was not critical in the observation. The normalized datasets were then subjected to chemometrics analysis. The PCA, HCA and DA classifications were performed using XLSTAT 2014.

RESULTS AND DISCUSSION

HPTLC fingerprints and densitogram of FD varieties

HPTLC of FD extracts were developed based on marker compounds separation (Figure 1). Chromatograms and spectra at a wavelength of 365 nm were shown in Figure 2. HPTLC chromatograms were aligned with 2D densitogram showing the separation of chemical compositions of FD varieties and different type of extracts. Vitexin and isovitexin were well separated at R_f value of 0.37 and 0.29 for methanol extract and R_f value of 0.39 and 0.31 for water extract, respectively. HPTLC chromatograms in Figure 2 show methanol extract has more intense peaks with a total of 28 peaks. As comparison, 23 peaks were separated from water extract. However, there were only specific peaks of compounds that were selected for chemometric analysis, which was shown in circles (Figure 2) including two marker compounds, vitexin and isovitexin. Those particular peaks were chosen due to their presence in all samples of FD varieties or at least in seven of methanol extract or four varieties in water extracts.

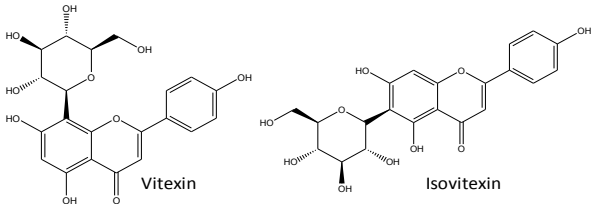

Figure 1 Structural formula of marker compounds

Principal component analysis

PCA is a technique that allowed the identification of a group of variables, which reduce the dimensionality of the data sets (Omar et al., 2011). PCA also provides information on the most important parameters that explain the entire data sets by excluding the less significant parameters and rendering data reduction with a minimum loss of original information (Omar et al., 2011). Due to the PCs generated by PCA are sometimes not readily interpreted, it is advisable to rotate the PCs by varimax rotation in order to produce the new groups of variables called varimax factors (VFs) (Retnam et al., 2013). In this study, the established marker compounds for FD; vitexin and isovitexin were identified in FD varieties in methanol and water extracts using HPTLC method adapted in Mohd et al. (2014). Multivariate HPTLC retention factor data obtained from the scanning of chemical compound bands on HPTLC plates based on separation of marker compounds, vitexin and isovitexin were then applied and analysed using PCA. Though these marker compounds occurred in all eight FD varieties, the chemometric analysis gives a better information and understanding on effect of compounds in the differentiation of FD varieties. The selected peaks of compounds used as variables in PCA for methanol extract were; isovitexin, vitexin, unidentified compounds at R_f value of 0.1, 0.51, 0.72 and 0.94. Figure 3a showed that the total variance of PCA score plot was accounted for 66.78% (PC1: 43.22%; PC2: 23.56%) for methanol extract of eight FD varieties. This PCA result indicates that there was variability between the samples though they were analysed using selected variables, which occurred in all samples.

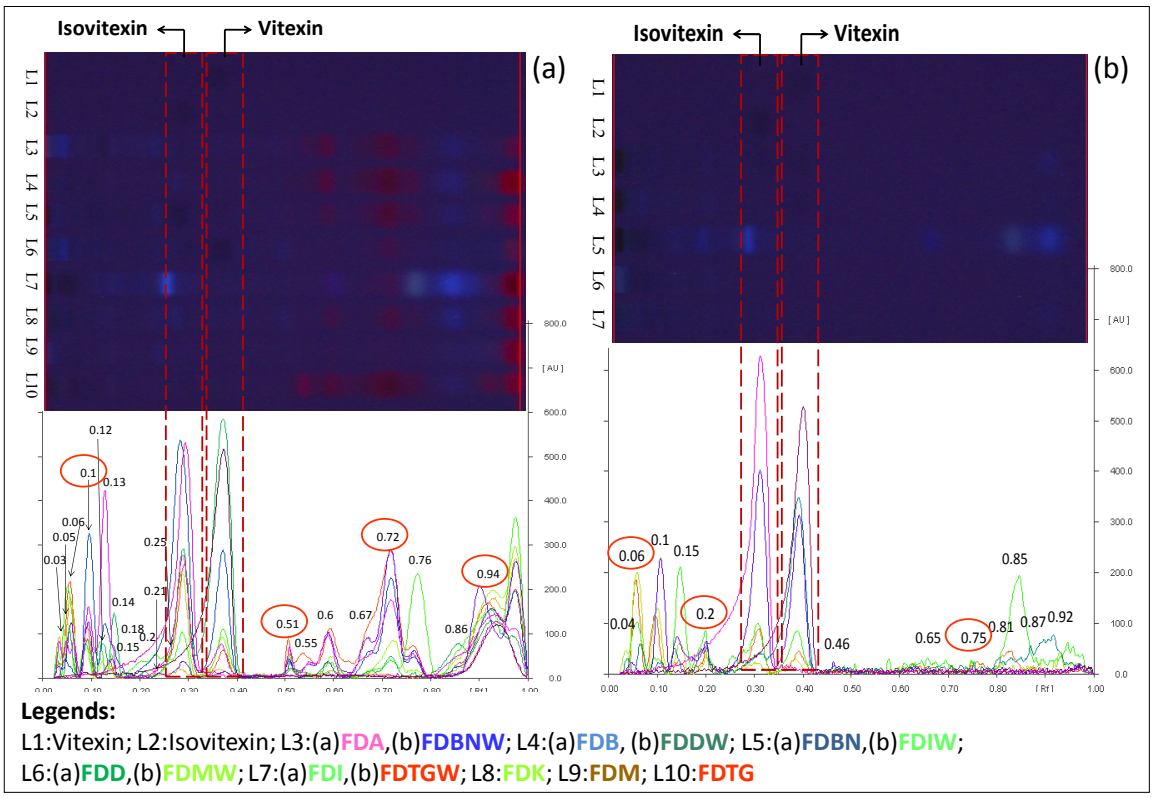

Figure 2 HPTLC chemical profile and 2D densitogram of (a) methanol extract of eight FD varieties and (b) water extract of five FD

As shown in Figure 3a, the peaks compound variables grouped all samples into four groups; Group I (FDA, FDK, FDI and FDM), Group II (FDB and FDTG), Group III (FDBN) and Group IV (FDD) for methanol extract. In Biplot scores (Figure 3c), isovitexin and vitexin were observed grouped along with FDBN and FDD, respectively. Unidentified compound at R_f value of 0.1 also grouped near to FDBN because it has the highest peak area in FDBN. Meanwhile, Group II clustered along with an unidentified compound at R_f value of 0.72 due to it has the highest peak areas in FDB and FDTG but absent in FDM, making Group II plotted far apart from FDM. Likewise, the unidentified compounds at R_f values of 0.51 and 0.94 were clustered near to FDM, FDK and FDI because of both compounds have the highest peak areas in FDK and FDM, respectively. The unidentified compound at R_f value of 0.94 also have comparable value of its peak area in FDK, FDI and FDA, which congregating into Group I. Figure 3b reveals that the selected variables that have strong loading in VF1 and VF2 were; unidentified compound at R_f value of 0.51 (VF1), 0.72 (VF2) and 0.94 (VF1), and isovitexin (VF1). Meanwhile, vitexin was classified as moderate loading (VF1 and VF2). Therefore, the most influential variables were unidentified compound at R_f values of 0.51 and 0.94, which contributed to the clustering of Group I. This followed by 0.72 for Group II and isovitexin and vitexin for Group III and IV, respectively. Although unidentified compound at R_f value of 0.1 was the highest peak area value in FDBN, it remains insignificant for the clustering of eight varieties FD of methanol extract as it plotted at the centre of the scores loading plot (Figure 3b).

In addition, for water extract, the total variance of PCA score plot was account for 82.25% (PC1: 41.89%; PC2: 40.37%) as shown in Figure 4a. The separation of compounds in water extract was observed to have slightly different with methanol extract. Moreover, the peaks of compounds been selected for water extract were unidentified compounds at R_f value of 0.06, 0.2, 0.75, isovitexin and vitexin as shown in Figure 2b. Samples in water extract were clustered into three groups; Group I (FDBNW), Group II (FDDW) and Group III (FDIW, FDMW and FDTGW).

The loading plot and Biplot (Figure 4b and 4c, respectively) clearly shown that particular variables have grouped those three for respective group; isovitexin for Group I (FDBNW), vitexin for Group II (FDDW), unidentified compounds at R_f value of 0.06, 0.2 and 0.75 for Group III (FDIW, FDMW and FDTGW).

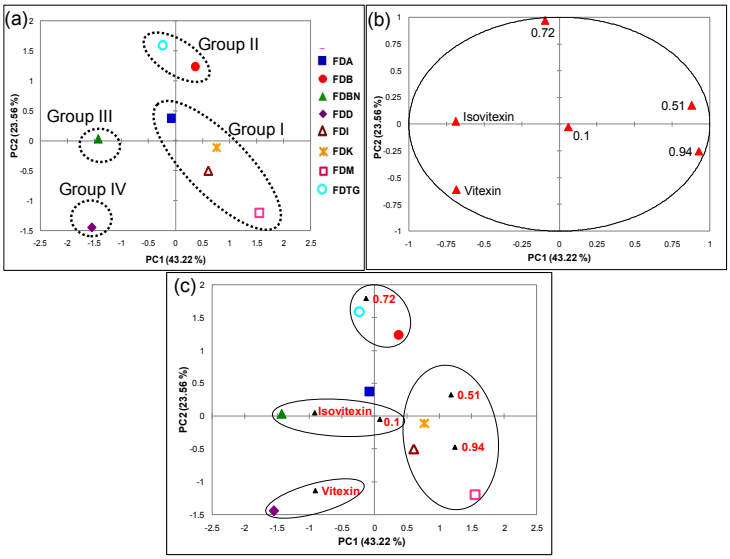

Figure 3 Scores plot of (a) PCA, (b) loading and (c) Biplot of methanol extract of eight FD varieties

These results were due to the abundance of the peak area of isovitexin and vitexin in FDBNW and FDDW, respectively. While the peak area of unidentified compounds at R_f value of 0.2 was highest in FDIW and FDTGW.

However, both unidentified compounds at R_f value of 0.06, and 0.75 were found absent in FDBNW, and abundance in FDMW for 0.06 and FDTGW for 0.75. For this reason why, Group III (Figure 4c) with unidentified compounds at R_f value of 0.2 was clustered near to FDIW and FDTGW while 0.06 and 0.75 were clustered near to FDMW and FDTGW, respectively. The variables that have strong loading in VF1 were unidentified compounds at R_f value of 0.06 and isovitexin while for VF2 were unidentified compounds at R_f value of 0.2 and vitexin (data not shown). This study found that the influence variable that accumulates together with the respective samples were due to their occurrences/ absence in particular variety and/or having the highest peak areas from other varieties. The discrimination of FD varieties in methanol and water extracts by selected peaks of compounds revealed that FD var. *deltoidea* and var. *borneensis* possess similar unique clustering pattern as compared with other varieties. It was found that vitexin and isovitexin do discriminate both var. *deltoidea* and var. *borneensis* from other varieties.

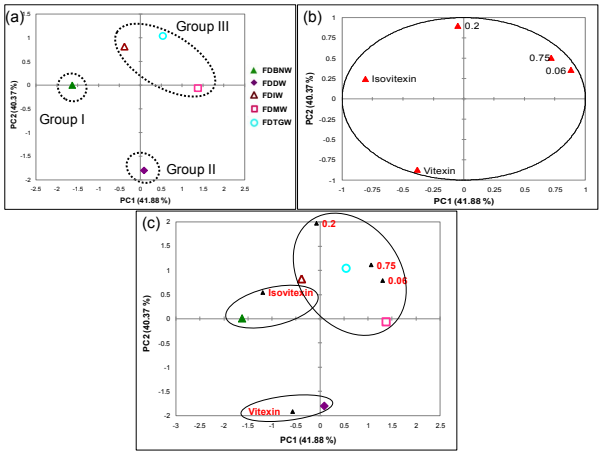

Figure 4 Scores plot of (a) PCA, (b) loading and (c) Biplot of water extract of five FD varieties

Hierarchical cluster analysis

Cluster analysis (CA) is a natural grouping of the unlabeled data sets without making earlier assumptions regarding on the possible structure formed of the datasets (Sulaiman et al., 2008). CA grouped samples based on quantitative characters [20]. However, hierarchical clustering analysis (HCA) method was the common approach method used (Retnam et al., 2013) and the most famous clustering technique in the quality evaluation of medicinal plants (Vega et al., 1998). The clustering method used in this study was Ward's method and the distance measure was Euclidean distance measure. Figure 5 showed that HCA for both FD extracts shown to have similar clustering and was in agreement with PCA scores plot (Figure 3 and 4). HCA results confirmed the PCA results, which showed that FDBNW was separately clustered from FDDW. Vitexin and isovitexin then were confirmed to be responsible as influence variables that separate both of them and grouped other varieties into another sub cluster, which in fact contain the lower concentration of standard markers.

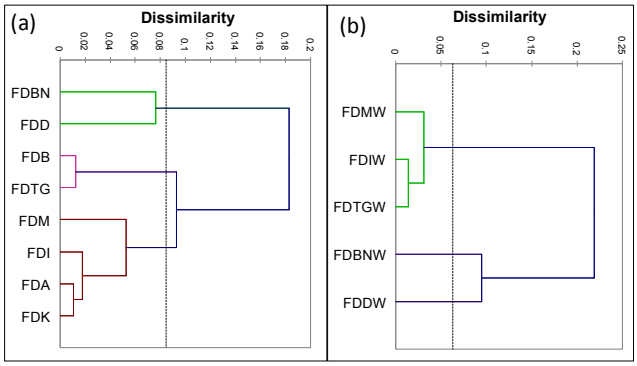

Figure 5 HCA dendrograms of (a) methanol extract of eight FD varieties and (b) water extract of five FD varieties

Discriminant analysis

The natural grouping in HCA does come out with good clustering observation of the samples. However, it still does not provide any details regarding their cluster characteristic that response to the samples clustering. Therefore, DA has been used for confirmation in HCA clustering and to define the variables that contribute to the discrimination the clusters in HCA. The clustering in HCA was further analyzed in DA as three main clusters for methanol extract but two clusters for water extract. For water extract, FDBNW and FDDW were classified into one group due to DA cannot be computed by the single sample in one group. Besides, FDBNW and FDDW were still branching from one same main clade. The classification matrices of samples datasets obtained from standard, forward stepwise and backward stepwise modes of DA shown in Table 1. For methanol extract, the standard mode yielded 100% correctly using 5 out of 6 variables (except unidentified compound at R_f value of 0.94) (Table 1). Both forward and backward stepwise modes also yielded 100% correctly using 3 variables (isovitexin, the unidentified compound at R_f value of 0.51 and 0.72). Meanwhile, for water extract, these three modes yielded 100% correctly using 3 variables (isovitexin, vitexin and unidentified compound at R_f value of 0.2) for standard mode, 1 variable (vitexin) for forward stepwise and two variables (isovitexin and vitexin) for backward stepwise modes. Thus, DA results suggest that isovitexin, the unidentified compound at R_f value of 0.51 and 0.72 are the significant parameters for methanol extract and vitexin for water extract in order to discriminate FD varieties. Figure 6 showed the clustering in HCA was 100% discriminated which methanol extract (F1: 95.49%; F2: 4.51%) and water extract (F1:100%) in DA plots. These results were corresponding to the PCA results. For methanol extract (Figure 6a), isovitexin was responsible to the clustering of Cluster 1, while unidentified compounds at R_f values of 0.51 and 0.72 were responsible for the clustering of Cluster 2 and Cluster 3, respectively. In addition, even Cluster 2 in water extract as shown in Figure 6b consist of FDBNW and FDDW, they still were clustered apart. This indicates that vitexin was responsible to the clustering in Figure 6b, which discriminate them according to the amount of vitexin in FD varieties of water extract.

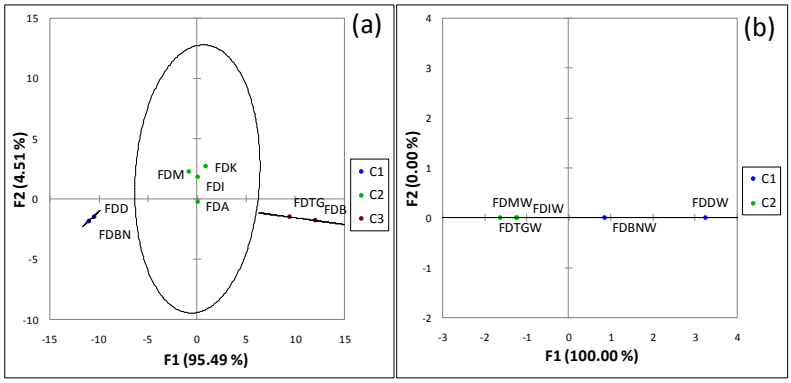

Figure 6 DA plots of (a) methanol extract of eight FD varieties and (b) water extract of five FD varieties

Table 1 The classification function of methanol and water extracts of FD varieties

Variables	Standard mode			Forward stepwise mode			Backward stepwise mode		
	Methanol extract of eight varieties of *Ficus deltoidea* Jack								
	C1	C2	C3	C1	C2	C3	C1	C2	C3
0.1	1259.781	750.293	878.792	0.000	0.000	0.000	0.000	0.000	0.000
Isovitexin	169.533	-258.766	-635.010	91.807	-265.955	-623.012	91.807	-265.955	-623.012
Vitexin	435.426	219.247	235.866	0.000	0.000	0.000	0.000	0.000	0.000
0.51	90.059	4152.105	7144.948	-323.790	3653.602	6429.467	-323.790	3653.602	6429.467
0.72	67.839	515.628	1104.995	-106.647	388.642	944.215	-106.647	388.642	944.215
0.94	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Water extract of five varieties of *Ficus deltoidea* Jack								
	C1	C2		C1	C2		C1	C2	
0.06	0.000	0.000		0.000	0.000		0.000	0.000	
0.2	-941.158	197.008		0.000	0.000		0.000	0.000	
Isovitexin	334.281	4.502		0.000	0.000		151.669	42.727	
Vitexin	448.846	23.215		42.197	8.625		245.259	65.831	
0.75	0.000	0.000		0.000	0.000		0.000	0.000	

CONCLUSION

The use of High Performance Thin Layer Chromatography (HPTLC) in this study provides simple, flexible, fast and inexpensive techniques for separation of the marker compounds used, namely vitexin and isovitexin for authenticity, identity and quality of the FD varieties. HPTLC also produced a reliable retention factor data for chromatographic fingerprints for further uses in the chemometric analysis. The chemometrics has been applied to differentiate plant cultivars, varieties or parts since the past decades. In this study, chemometric analysis (PCA, HCA and DA) was successfully identifying the discriminating factor for FD varieties. In combination with chemometric, HPTLC analysis of FD variety offers metabolic fingerprinting information that can be utilized for further investigation.

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