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Research paper



Pattern Recognition for Varieties of Malaysian Herb, *Ficus deltoidea* Jack through Chemometric Applications from GC-MS Fingerprinting

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Abstract

Pattern based approach has been used for quality control for identification and authentication of herbal medicines including plant that having varietal issue, *Ficus deltoidea* Jack (FD). Chemical fingerprinting of FD varieties were profiled by Gas Chromatography-Mass Spectrometry (GC-MS). The aim was to profile and classify the untargeted volatile compounds which commonly occur in FD varieties for pattern recognition purpose. Chromatographic data from GC-MS fingerprint of FD varieties were analyzed using chemometric applications; principal component analysis (PCA), hierarchical cluster analysis (HCA) and discriminant analysis (DA). Twenty-two major volatile compounds found were commonly occurred and stable in FD varieties. PCA give out total variance of 33.13%, while HCA generated three clusters. However, DA confirmed FD varieties only grouped into two groups, which suggested that the unidentified compounds at tR: 8.85, 14.80, 16.25, 16.55 and 29.06 were the most significant parameters ($p < 0.05$) to discriminate both grouping. This finding indicates that 22 volatile compounds that commonly occur in FD varieties do not contribute to the discrimination and var. *bilobata* revealed to have specific volatile compounds that distinguish it from other varieties. The use of GC-MS fingerprinting and chemometric techniques provides useful and promising information for authentication and quality control of herbal medicines.

Keywords: *Ficus deltoidea Jack; Quality control; Chemometrics; GC-MS Fingerprinting; Volatile compounds; Malaysian Herbs.*

1. Introduction

Herbal products or commonly been called as natural products derived from plants materials have been widely used since ancient times as medicines in order to accomplish the health care needs. WHO also give as estimation about 80% of world population still consumes herbs as well as other traditional medicines for their primary health care needs. As consumption of herbal medicines and products are increasing nowadays towards its primary health care needs, the uncontrollable quality in herbal productions can give an effect on efficacy and safety of starting material, preparations and finished products. Herbal materials contains complex mixtures of chemical constituents which possess variability due to many factors such as climate, harvest seasons, plant origins, drying, storage, extraction procedure, as well as adulteration which might lead to variant efficacy and safety [1-3]. Therefore, the quality control parameters should be developed for specific tested samples (herbal materials, extracts, preparations or finished products). As for herbal medicines, identification, determination and authentication techniques are commonly performed to fulfil the requirement of quality control. In [4] had been classified the authentication and quality control of herbal medicines into two approaches; compound-based and pattern-based approaches, and have been refined by [5] as compound-oriented and pattern-oriented approaches. Compound-based approach basically is the use of single marker approach for identification and quantification of chemical markers which represent the quality of herbal samples. While, in [5] refined the compound-oriented approach involves the marker approach and multi-compound approach which use the specific single or multiple compounds for identification of herbal samples. Multi-compound (more than one compound) was used as if the single compound failed to represent the herbal efficacy. Meanwhile, pattern-based approach (also known as fingerprint approach) is referring to the overall features of chromatogram or spectrum of herbal medicine in specific profiles and characteristics obtained by certain analytical techniques, which can give description including herbal raw materials, slices, semi-finished products and finished products in holistic [6]. Fingerprint pattern from chromatogram or spectrum is the reflection of the chemical components present in herbal materials, extracts or preparations. This fingerprint approach is more reliable as it been used as total pattern in order to identify the sameness and/ or differences between herbal samples analyzed. According to [7], the development of fingerprint method for quality control however involved two key issues: i) how to gain more effective and stable

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information and ii) how to evaluate the similarity and difference with chemometric method. Hereby, the chemical fingerprints coupled with chemometric deconvolution and resolution are the powerful methods in ensure the efficiency of the quality control for herbal materials [5]. In this study, we choose to apply this method on one Malaysian herb, *Ficus deltoidea* Jack (FD). FD has been known as potent herbal medicine yet to occur in varietal levels which give the confusedness on the variation and therapeutic effect within varieties.

Ficus deltoidea Jack, known locally as 'Mas Cotek' or mistletoe fig is the herbs with variety of therapeutic potential. This plant has been used by traditional practitioners as herbal remedies to treat several illnesses like headache, hypertension and hyperglycemia [8-9]. Several studies have shown that this plant has antioxidant and neuroprotection activities [10-11]. There was also report on antinociceptive activity from aqueous extract of this plant [12]. In addition, there are reports on its chemical constituents that characterized at least 25 flavonoids, and the main constituents have been identified to be flavan-3-ol monomer, proanthocyanidins, and C-linked flavones glycosides [13, 10]. To date, *Ficus deltoidea* Jack has been reported to occur at least in eight varieties in Malaysia namely, *Ficus deltoidea* var. *deltoidea* (FDD), var. *trengganuensis* (FDTG), var. kunstleri (FDK), var. *motleyana* (FDM), var. *intermedia* (FDI), var. *borneensis* (FDBN), var. *bilobata* (FDB) and var. *angustifolia* (FDA). To our knowledge, differentiation and identification of FD varieties have been mostly studied using morphological characterization. Recently, in [14] reported the work on identification of seven varieties of FD based on the maturity characterization of the leaf, together with their morphology and anatomy analysis. According to them, FD known as a complex species of subgen and regularly recognized by their several unique characteristics; which were figs, midrib dichotomous, golden dots on the surface of the lamina, leafy twigs and milky latex. The analysis revealed the morphological and anatomical characters of FD varieties possess variations. However, although the morphological identification and microscopic identification have been used in determining the authenticity of herbal medicines, the complexity of herbal medicines still cannot be elaborated [6]. For FD in particular, the morphological characterization is still insufficient in ensuring the quality of FD varieties as herbal products and not applicable for powder material after herbal processing. However, chemical profiling or fingerprinting can served several purposes in quality control including; finding out the adulteration, to ensure its consistency and efficacy, to understand the bioactivities and possible side effects and for finish products. Thus, it is justified that the chromatographic fingerprint coupled with chemometric analysis is the appropriate approach in studying the variation of FD varieties.

The aims of this study were to profile and classify the untargeted volatile compounds which commonly occur in methanol extract of eight FD varieties for pattern recognition purpose. To best our knowledge, this is the first attempting on discrimination study from GC-MS fingerprinting coupled with chemometric analysis for eight varieties of *Ficus deltoidea* Jack.

2. Materials and Methods

2.1. Chemical and Reagents

The solvent methanol (HPLC grade) is products of Merck (Darmstadt, Germany).

2.2 Sample Preparation

The leaves of eight FD varieties; FD var. kunstleri (FDK), var. *angustifolia* (FDA), var. *bilobata* (FDB) and var. *trengganuensis* (FDTG) were obtained from Universiti Sultan Zainal Abidin (UniSZA) living collection, Kuala Nerus Campus, Kuala Terengganu. FD var. *intermedia* (FDI) was collected from Brinchang Mount, Cameron highland. Meanwhile, FD var. *deltoidea* (FDD) was collected from Sarawak Forestry Centre (SFC), Semenggok Sarawak, and FD var. *motleyana* (FDM) was collected from Gunung Pueh, Sematan, Sarawak. FD var. *borneensis* (FDBN) was collected in Santubong, Sarawak, East Malaysia. Each sample was identified by expert and deposited at UniSZA's herbarium. All leaves samples collected were dried in conventional oven at 45°C and then were milled into powder. Fifty grams of leaves powder were extracted with methanol. The filtrate from methanol extraction was concentrated under pressure at 45°C and maintained at -20° prior analysis.

2.3 GC-MS Analysis

Agilent© 6890 Gas Chromatograph System equipped with Mass Selective Detector Transfer Line Heater was used in the analysis to identify the major and unknown compounds in the FD varieties leaf extracts. Helium was used as the carrier gas. GC-MS analysis was carried out using Agilent 19091S-433 HP-5MS capillary column (30m x 0.25mm inner diameter, 0.25 µm film thickness). The oven temperature was set for initial temperature at 70°C (2 min hold) to 280°C (20 min hold). A split mode of front inlet was used with split ratio 1:1 and the flow rate of helium gas (40 cm sec^{-1}) was 1.2 mL min^{-1}. Mass Selective Detector (MSD) Transfer Line Heater was 285°C. Injector temperature was 280°C and 1µL of sample extracts was injected.

2.3.1. Preparation of Sample Extract for GC-MS Analysis

Methanol extract of eight varieties of FD (10 mg) were dissolved in 1 mL of methanol (HPLC grade) in order to get final concentration of 10 mg mL-1 and was sonicated for 30 minutes. All samples were then centrifuged and the supernatant were transferred into new vials. The supernatant of all samples were used to be injected in GC-MS system.

2.3.2. Pre-Processing Data Analysis for GC-MS Fingerprinting

All samples were standardized prior the analysis into the same concentration for chromatographic data (10 mg mL^{-1} for GC-MS analysis). This step was taken in order to ensure the concentration factor would not affect the clustering in chemometrics analysis. GC-MS chromatographic data of all samples analyzed by GC-MS analysis were obtained from ChemStation GC-MS software. The assigned peaks were detected by MS detector and the total ion chromatogram of each peak were compared and matched with the library search report based on National Institute of Standards and Technology (NIST) library database (Library ID: NIST02.L). Thereafter, the peaks which matched with NIST database in the quality 80% and above were considered as the particular compounds. Those peaks in the quality of below 80% were labelled as unidentified compounds. The apex was minus the baseline at 20 minutes for baseline correction operation in ChemStation GC-MS software. The data of retention time and percentage area of all integrated peaks found in the samples were exported from the software and was extracted manually into Microsoft Excel 2007. The excel files were then imported into The Unscrambler X 10.1 (CAMO, Trondheim, Norway) and the datasets were normalized into area normalization. The normalized datasets were then subjected to chemometrics analysis.

2.4 Chemometric Approach

2.4.1. Principal Component Analysis (PCA)

PCA is a technique allowed the identification of a group between variables which reduce the dimensionality of the data sets [15]. PCA also provides information on the most important parameters that explain the entire data sets by excluding the less significant parameters [16-17] and rendering data reduction with a minimum

loss of original information [18]. 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) [19]. As the output of the analysis, PCA consisted of score plots and loading plots which must be interpreted together. The correlation between VFs and the original variables is given by factor loadings, while the individual transformed observations are called factor scores [18]. This varimax rotation function enables increasing of the weight of the higher factor loading values and reduction of the weight of the lower values; thus leads to better understanding of the data structure [20]. With the aim in obtaining the new groups of variables (varimax factors), varimax rotations was applied on the PCs with eigenvalues more than 1 are considered significant [21]. Varimax rotation ensures that each variable is maximally correlated with only one component and has a near zero association with other components [22]. The correlation between score plots and loading plots obtained were interpreted as which VF coefficient having correlation > 0.75 are considered 'strong' correlations, in the range of 0.74-0.50 are considered 'moderate' and those in the range of 0.49-0.30 are considered 'weak' significant factor loadings [23]. The higher the factor loading of that variable, the more the variable contributes to the variation accounted for the particular PC [24]. Thus, VF coefficient that gives strong significant factor loading which more than 0.75 both positive and negative will be discussed.

In this study, the normalized chromatographic data of FD varieties extracts from GC-MS fingerprinting was subjected to PCA analysis to look for the relationship between the variables and samples as well as the significant variances which could be visualized on the new coordinate (PCs) after PCA analysis. These relationships also could be discussed on the separation of the classes in terms of chemical similarity for score plots and the loading plots is an indication as to which compounds were significant with respect to the classification obtained in the score plots [25]. The first two PCs was used to see the total variances of the analysis as mentioned by [26], which the PCs are calculated by ordering the variables in such way that the first variable (PC) explains the largest proportion of variability within the original data and the second PCs explains the largest proportion of the variability that has not been explained by the first PC. For presentation of data sets in this study, each eight data set has 165 peaks variables which contain of rows (Y-data) as test samples datasets and columns (X-data) present the retention time of volatile compounds as variables. The selection of variables and observation (FD varieties) was done as followed the defined ranges mentioned earlier, which dependent variables in row sets was selected as observation (samples) and independent variables in columnset was selected as variables option, respectively. Once done selection of data sets for observations and variables, the analysis was analyzed resulting in scores plot, loading and biplot. PCs/VFs with eigenvalues more than 1 were selected to be analyzed one more time with varimax rotation function in order to obtain better understanding in samples clustering. The strong loadings obtained from the factor loading after varimax rotation was used to interpret together with the samples clustering in scores plot. The interpretation of PCA for GC-MS data was concerned on the compounds that have strong loading in PC1 and PC2 which responsible to differentiation within the sample varieties due to the samples clustering. The main PCs that been used in this study were PC1 and PC2. Biplot was used in the interpretation of PCA results which show the correlation between the samples and variables. PCA for chromatographic data was analyzed in order to observe the relationship between samples clustering with the responsible compound(s) that discriminate FD varieties.

2.4.2. Hierarchical Cluster Analysis (HCA)

Cluster Analysis (CA) is a natural grouping of the unlabelled datasets without making earlier assumptions regarding on the possible structure formed of the datasets [27]. CA grouping the samples based on quantitative characters [28] and applied in several methods in clustering observation such as hierarchical, partitioning, model-based and fuzzy methods [29]. However, hierarchical clustering analysis (HCA) method was the common approach method used [19, 30] and the most famous clustering technique in the quality evaluation of medicinal plants [28]. The clustering method used in this study was Ward's method and the distance measure was Euclidean distance measure. In this study, HCA was applied on the similar data sets applied in PCA, by using Ward's method and Euclidean distance measures. The natural grouping of FD varieties by HCA was performed in order to examine the dissimilarities between the samples varieties based on the variables inserted. All the data sets were selected for the row set (observations) and columnset (variables) as applied in PCA as mentioned in previous text. The natural clustering of GC-MS data were based on the separation of particular compounds. The automatic truncation was checked prior analysis in order to let the hierarchical tree being cutting off for significant cluster, which formed in dotted line perpendicular to the hierarchical tree. The higher relative distances between the samples in the dendrogram tree indicate the dissimilarities between the samples increased. However, as HCA give out the natural grouping within the samples, the specific variables that responsible to the sample clustering remained unknown. Therefore, the clustering of samples in HCA was proceeded to be analyzed in Discriminant Analysis (DA) in order to define the variables that corresponding to the dissimilarities between sample varieties.

2.4.3. Discriminant Analysis (DA)

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 responsible to the samples clustering. Therefore, DA has been used for confirmation in HCA clustering and to define the variables that most discriminate the clusters in HCA. For each group, this technique builds a discriminant factor (DF) taking the form [16, 31]:

$$f(G_i) = k_i + \sum_{j=1}^{n} w_{ij} \times p_{ij}$$

where i represents the number of groups (G), ki is the constant inherent to each group, n is the number of independent variables constitute the DF, and wj is the weight assigned by DFA to the selected parameter (pj). Discriminant analysis was carried out using three modes; standard modes, stepwise-forward and stepwise-backward modes. Stepwise forward was performed on the datasets use to include step-by-step variables beginning from the most significant variables until no variables changes obtained. Meanwhile, stepwise backward was carried out by remove step-by-step beginning the less significant variables until no variables changes obtained. In this study, DA was carried out on the same data sets used in PCA and HCA. However, one new columnset contain the number of cluster which used for discrimination was created based on the samples clustered in HCA. Those samples which clustered into particular clusters were analyzed in DA using three modes. Firstly, the data sets were imported into the software by selecting cluster columnset, variables column sets and samples row sets. The analysis was run using standard mode for the first analysis and the results were observed for discrimination of samples based on the cluster columnset. The same procedure was repeated and analyzed by DA for step-wise forward mode in order to see should there any most significant variables that responsible to the discrimination of the samples varieties. The analysis was further analyzed by using step-wise backward mode in order to observe the less significant variables that may not be take into account for samples discrimination. Those three modes were then being compared for their percentage of yields which 100% correctly and those variables from the particular modes been chosen and suggested as significant parameters that responsible to the

discrimination of samples. The percentage of cross validation results which also found 100% correctly were used to confirm the best DA mode to be chosen. The chemometrics analysis (PCA, HCA and DA classifications) were performed using XLSTAT Pro 2014 (Addinsoft, Paris, France), an add-in software program for Microsoft Excel 2007. These tools were used to discriminate the FD varieties.

3. Results and Discussion

3.1. GC-MS Fingerprinting of FD Varieties

Gas Chromatography (GC) is a well-known analytical technique for identification or characterization of volatiles compounds. In herbal medicine, most of pharmacologically active constituents are consists of volatile chemical components. GC-MS analysis was carried out to analyze and identify major peaks representing volatile compounds in all eight varieties of FD. All analyzed extracts of eight FD varieties showed good separation in chemical compositions. There were 165 peaks found in the overlay GC-MS chromatogram of eight FD varieties. In this study, 22 volatiles compounds were found commonly and stably occurring in methanol extract of all eight FD varieties, which aligned and overlapped in the same retention time and peaks (Fig. 1). Identification of peaks was based on Probability Based Matching (PBM) library search system using a NIST MS Spectral database as library references and was identified eight out of twenty-two major volatile compounds occur in FD varieties and another twelve peaks were unidentified compounds (Table 1).

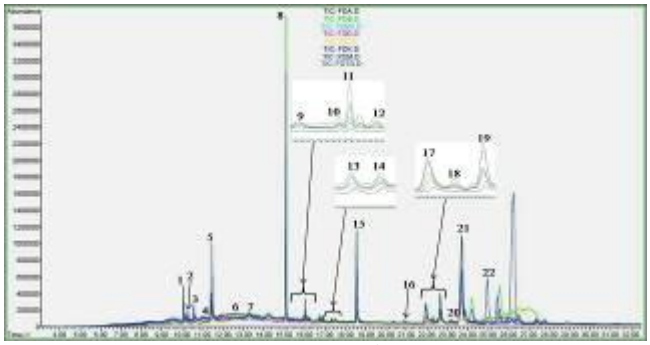


Fig. 1: Overlay GC-MS chromatogram of 22 major volatile compounds commonly occur in methanol extract of all eight varieties of *Ficus deltoidea* (FD). FDA: FD var. *angustifolia*; FDB: FD var. *bilobata*; FDBN: FD var. *borneensis*; FDD: FD var. *deltoidea*; FDI: FD var. *intermedia*; FDK: FD var. *kunstleri*; FDM: FD var. *motleyana*; FDTG: FD var. *trengganuensis*.

The comparison of GC-MS profiles and their unique chromatogram features of each eight FD varieties can be observed in Fig. 2. Although the presences of major peak compounds are similar within the varieties, there might be differing in peak intensities. Moreover, there might certain peaks that occur and/ or absent that vary them greatly. For example, it was found that unidentified compound at tr: 26.18± 0.035, occur as a strong peak occur in FDM, moderate in FDI, FDBN and FDD, weak in FDB and FDTG, but absent in FDA. This peak might be a unique characteristic that discriminate FD varieties.

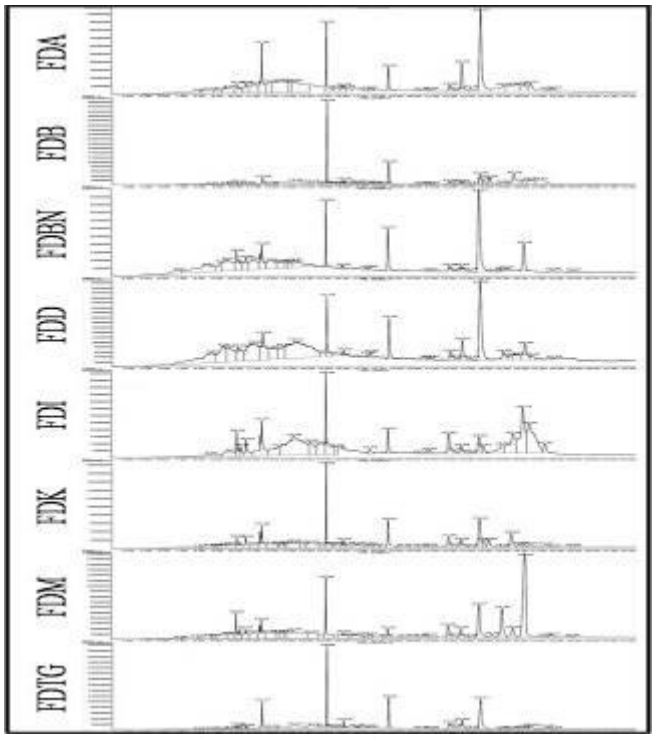


Fig. 2: Comparison of GC-MS profiles of volatile compounds present in methanol extract of eight varieties of *Ficus deltoidea* (FD). FDA: FD var. *angustifolia*; FDB: FD var. *bilobata*; FDBN: FD var. *borneensis*; FDD: FD var. *deltoidea*; FDI: FD var. *intermedia*; FDK: FD var. *kunstleri*; FDM: FD var. *motleyana*; FDTG: FD var. *trengganuensis*.

Therefore, further analysis using chemometric should have been employed in order to identify the main discriminant peaks. From Table 1, compound (17) (tr: 21.98 ± 0.026) was identified from NIST library search report matching as β-sitosterol and/or γ-sitosterol. This was revealed by percentage of similarity correspond to that peak, in which; FDB (97%) for β-sitosterol; FDBN (99%), FDD (94%) and FDK (99%) for γ-sitosterol. Meanwhile, FDA, FDI, FDM and FDTG respectively were assigned as β-sitosterol/ γ-sitosterol for 99% of similarity.

This uncertainty might due to the same molecular weight for both β-sitosterol and γ-sitosterol, 414.7067 g/mol. β-sitosterol is a definite structure which has C5-double bond and α-ethyl at C-24 [32]. Meanwhile, γ-sitosterol has been reported to be an epimer of β-sitosterol, which differ only at C24-ethyl substituent which present in the side of γ-sitosterol or described as C5 sterol and β–ethyl at C-24 [32-33]. Several decades ago, the confusion on separation method of β-sitosterol and γ-sitosterol has already been investigated. In [34] have been reported that the so-called 'γ-sitosterol' was found is a mixture of β-sitosterol and campesterol in ratio (1:1) using gas chromatography analysis. This result obtained by comparing the melting points and spectra rotations of IR spectra of mixture of 50% of β-sitosterol and campesterol, respectively with that of the so called γ-sitosterol', which showed completely same properties. This finding was reasonable as sitosterol, campesterol and stigmasterol have been found as the most abundant plant sterols [35].

In this study, GC-MS can be considered is a good tool to profile untargeted chemical fingerprint of FD varieties from methanol extract. The separation by GC-MS showed a reproducible chromatogram of volatile compounds in FD varieties. This finding revealed that, those 22 stable compounds might be significant pharmacologically active constituents of FD varieties. However, the overlapping of hundreds of peaks in eight FD varieties might be discriminated FD varieties according to their chemical components. Thus, GC-MS chromatographic data were subjected to chemometric analysis for discrimination study of FD varieties in term of volatile compounds.

Table 1: Lists of 22 major volatile compounds commonly occur in methanol extract of all eight varieties of *Ficus deltoidea* Jack.

Peak #	TICs (min) ±SD	Name of Compound	Area %							
			FDA	FDB	FDBN	FDD	FDI	FDK	FDM	FDTG
1	10.10 ± 0.027	unidentified compound	1.14	1.13	3.2	2.31	1.3	1.31	1.56	0.78
2	10.32 ± 0.014	unidentified compound	0.54	0.48	1.63	1.11	0.73	0.73	0.71	0.37
3	10.60 ± 0.023	**hexadecanoic acid, methyl ester**	**1.44**	**1.04**	**1.97**	**2.08**	**1.67**	**2.44**	**2.1**	**0.77**
4	11.06 ± 0.042	unidentified compound	1.91	0.86	4.55	4.75	0.57	2.47	2.75	0.7
5	11.49 ± 0.020	unidentified compound	5.55	2.98	4.8	3.1	4.47	5.17	2.49	5.62
6	12.62 ± 0.030	unidentified compound	5.25	1.41	1.89	1.9	1.54	1.4	1.15	0.87
7	13.28 ± 0.026	unidentified compound	2.49	2.73	8.26	5.8	4.17	3.58	2.17	6.19
8	15.10 ± 0.005	**squalene**	**3.99**	**11.46**	**4.11**	**2.57**	**5.01**	**5.87**	**3.55**	**9.79**
9	15.62 ± 0.007	unidentified compound	0.99	0.67	0.85	0.8	0.75	1.28	0.78	0.81
10	15.96 ± 0.005	unidentified compound	0.47	0.35	0.52	0.35	0.33	0.53	0.41	1.41
11	16.04 ± 0.008	unidentified compound	0.41	0.76	0.46	0.43	0.72	0.73	0.47	1.25
12	16.25 ± 0.012	unidentified compound	0.47	0.86	0.5	0.42	0.57	1.18	0.46	1.33
13	17.35 ± 0.013	**β-tocopherol**	**0.33**	**0.47**	**0.54**	**0.27**	**0.06**	**0.76**	**0.43**	**0.73**
14	17.53 ± 0.011	**γ-tocopherol**	**0.42**	**0.66**	**0.94**	**0.47**	**0.37**	**0.89**	**0.76**	**0.56**
15	18.57 ± 0.009	**α-tocopherol**	**2.08**	**6.02**	**3.57**	**2.65**	**1.95**	**4.65**	**1.94**	**7.9**
16	20.91 ± 0.024	unidentified compound	0.3	0.17	0.12	0.3	0.39	1.04	0.55	0.98
17	21.98 ± 0.026	**β-sitosterol / β-stigmasterol**	**1.15**	**1.58**	**0.85**	**1.00**	**3.09**	**2.89**	**2.04**	**3.06**
18	22.28 ± 0.013	unidentified compound	0.36	0.65	0.25	0.38	0.41	0.85	0.79	1.13
19	22.67 ± 0.014	**β-amyrin**	**4.07**	**1.52**	**0.69**	**2.06**	**1.19**	**2.57**	**2.01**	**4.02**
20	23.17 ± 0.014	unidentified compound	0.65	0.36	0.16	0.22	0.38	0.52	0.39	1.33
21	23.69 ± 0.014	**α-amyrin**	**13.47**	**4.43**	**11.62**	**9.53**	**2.49**	**6.52**	**5.67**	**13.52**
22	24.97 ± 0.017	unidentified compound	3.26	2.21	0.29	1.22	2.62	0.69	5.25	1.08

3.2. Identification source of FD varieties

Fig. 3(a) shows that eight FD varieties were clustered into three groups; Group I (FDM), Group II (FDA, FDB, FDBN, FDD, FDI and FDK), Group III (FDTG). The total variance of PCA score was 33.13% (PC1: 17.22%; PC2: 15.41%), indicates the small variance which may due to six varieties that grouping in Group II possess high similarities in term of the occurrence of other unknown volatile compounds. Table 2 revealed the strong loading of peaks compounds variables for VF1 were circled and labeled as B in PCA-biplot (Fig. 3c) assigning to Group III (FDTG). Meanwhile, the influence variables that have strong loading for VF2 circled as C which attributed to Group I (FDM). Whereas, the groups of variables circled as A in PCA-biplot show to have strong loading for VF3 to VF7 appointed to Group II which have overlapping six FD varieties (FDA, FDB, FDBN, FDD, FDI and FDK). Alpha tocopherol is one of many other unidentified compounds, which have clustered FDTG alone. The most important information to describe the most variance (VF1) of the grouping comprising the couple numbers of variables including β-tocopherol, γ-tocopherol, hexadecanoic acid methyl ester, β-amyrin and α-tocopherol. While the variables that used to differentiate the samples (VF2), consist of a list of unidentified peak compounds.

Table 2: Factor loading of all volatile compounds variables after rotated by varimax rotation of GC-MS data. The highlighted values in VFs columns indicate the strong loading (≥ 0.75) of variables. Eigenvalues, variability and cumulative for methanol extract of eight FD varieties are shown.

Retention Time (min)	VF1	VF2	VF3	VF4	VF5	VF6	VF7
5.21	-0.114	0.877	0.111	-0.116	0.414	-0.005	-0.144
5.42	-0.106	-0.091	0.083	-0.103	-0.040	0.031	0.980
6.45	0.967	-0.111	0.130	-0.121	-0.087	0.082	-0.081
6.83	-0.201	-0.214	0.209	-0.211	-0.295	-0.833	-0.213
6.9	-0.137	0.882	0.135	-0.136	-0.168	-0.337	-0.155
7.05	-0.134	-0.175	0.134	0.669	0.677	0.006	-0.167
7.18	0.361	0.232	-0.323	0.248	0.352	-0.663	0.296
7.32	-0.064	-0.113	-0.975	-0.118	-0.079	0.055	-0.098
7.53	-0.081	-0.122	0.077	0.978	-0.075	0.057	-0.086
7.76	0.340	-0.167	0.133	0.896	-0.112	0.093	-0.120
7.87	-0.319	0.064	0.320	-0.241	0.046	-0.695	0.499
8.04	-0.361	-0.232	0.323	-0.248	-0.352	0.663	-0.296
8.12	-0.168	-0.257	0.352	-0.274	-0.372	0.685	-0.315
8.26	-0.123	-0.126	0.124	-0.134	0.955	0.000	-0.156
8.35	-0.219	0.202	0.225	-0.227	-0.309	-0.811	-0.237
8.42	-0.252	-0.266	0.259	-0.268	0.385	-0.706	-0.284
8.52	-0.379	-0.140	0.377	0.299	-0.073	-0.665	-0.398
8.56	0.967	-0.111	0.130	-0.121	-0.087	0.082	-0.081
8.62	-0.124	-0.121	-0.179	-0.135	-0.062	0.046	0.956
8.76	-0.058	0.991	0.053	-0.054	-0.052	-0.006	-0.072
8.85	-0.017	-0.108	0.058	0.989	-0.053	0.011	-0.067
8.91	-0.064	-0.113	-0.975	-0.118	-0.079	0.055	-0.098
8.99	-0.136	-0.150	-0.067	-0.158	0.948	0.011	-0.177
9.07	-0.054	-0.104	0.053	0.988	-0.049	0.008	-0.064
9.17	-0.158	-0.159	0.017	-0.169	0.939	0.052	-0.192
9.22	-0.191	-0.200	0.190	-0.210	-0.196	-0.413	0.796
9.4	-0.116	0.742	-0.495	0.391	-0.117	0.031	-0.153
9.52	-0.309	-0.200	0.302	-0.310	-0.199	-0.768	0.218
9.6	-0.260	0.236	0.249	0.144	0.827	0.165	-0.287
9.64	0.287	-0.141	0.098	0.934	-0.079	0.036	-0.092
9.7	-0.340	-0.269	-0.424	-0.286	-0.343	0.580	-0.310
9.81	0.225	0.958	0.091	-0.089	-0.078	0.018	-0.096
9.9	-0.106	-0.091	0.083	-0.103	-0.040	0.031	0.980
10.1	-0.440	-0.067	0.260	-0.184	-0.307	-0.722	0.290
10.22	-0.096	0.416	0.420	0.387	0.289	0.472	-0.430
10.32	-0.462	-0.137	0.344	-0.115	-0.347	-0.681	0.229
10.53	-0.251	-0.265	0.259	-0.266	0.214	-0.778	-0.279
10.60 (hexadecanoic acid, methyl ester)	-0.604	0.273	0.394	0.523	-0.137	-0.212	0.261
10.83	0.967	-0.111	0.130	-0.121	-0.087	0.082	-0.081
10.92	-0.208	-0.201	0.191	-0.211	-0.200	-0.430	0.781
11.06	-0.329	0.109	0.279	0.055	-0.055	-0.677	0.582
11.22	-0.361	-0.232	0.323	-0.248	-0.352	0.663	-0.296

11.44	-0.106	-0.091	0.083	-0.103	-0.040	0.031	0.980
11.49	0.345	-0.585	0.458	0.207	0.338	-0.080	-0.406
11.61	-0.058	0.991	0.053	-0.054	-0.052	-0.006	-0.072
11.82	-0.064	-0.113	-0.975	-0.118	-0.079	0.055	-0.098
11.88	-0.058	0.991	0.053	-0.054	-0.052	-0.006	-0.072
11.9	-0.360	-0.215	0.192	0.185	0.267	-0.816	-0.124
12.02	0.967	-0.111	0.130	-0.121	-0.087	0.082	-0.081
12.08	-0.144	0.487	0.142	-0.151	0.816	-0.004	-0.182
12.21	-0.062	0.026	0.370	-0.019	0.245	-0.460	0.766
12.37	0.187	0.233	0.218	0.264	0.855	0.031	-0.246
12.44	-0.453	-0.350	0.017	-0.365	-0.483	0.375	-0.406
12.53	-0.216	0.017	0.197	-0.218	-0.199	-0.392	0.821
12.62	-0.293	-0.216	0.146	-0.160	0.900	-0.084	-0.054
12.67	0.967	-0.111	0.130	-0.121	-0.087	0.082	-0.081
12.74	0.967	-0.111	0.130	-0.121	-0.087	0.082	-0.081
12.81	0.135	0.259	0.173	0.514	-0.119	0.055	0.777
12.93	-0.127	-0.144	0.106	0.534	-0.066	0.032	0.813
13.03	-0.448	-0.020	0.411	-0.091	0.254	0.621	-0.415
13.11	-0.183	-0.234	-0.714	-0.237	-0.256	-0.481	-0.220
13.28	0.235	-0.464	0.384	-0.226	-0.454	-0.539	0.187
13.46	-0.364	0.674	0.072	-0.326	0.435	0.334	-0.045
13.55	-0.119	-0.111	-0.086	-0.125	-0.054	0.041	0.973
13.61	-0.178	-0.186	-0.868	-0.196	-0.190	0.263	-0.191
13.7	-0.017	-0.373	0.449	0.039	0.004	0.694	-0.419
13.84	-0.106	-0.091	0.083	-0.103	-0.040	0.031	0.980
14.04	-0.140	0.177	0.140	-0.150	0.936	-0.002	-0.178
14.19	-0.054	-0.104	0.053	0.988	-0.049	0.008	-0.064
14.24	0.668	0.053	0.050	0.022	0.328	0.627	0.216
14.63	-0.064	-0.113	-0.975	-0.118	-0.079	0.055	-0.098
14.71	-0.064	-0.113	-0.975	-0.118	-0.079	0.055	-0.098
14.78	-0.361	-0.232	0.323	-0.248	-0.352	0.663	-0.296
14.8	-0.110	0.870	0.094	-0.105	-0.070	0.011	0.454
14.87	0.761	-0.165	-0.164	0.423	0.343	0.128	0.230
14.97	-0.064	-0.113	-0.975	-0.118	-0.079	0.055	-0.098
15.10 (squalene)	0.541	-0.253	-0.692	0.017	-0.157	0.160	-0.338
15.21	0.967	-0.111	0.130	-0.121	-0.087	0.082	-0.081
15.29	0.672	0.243	-0.411	0.489	-0.175	0.101	-0.201
15.43	0.397	-0.055	0.075	0.092	0.141	0.898	-0.007
Eigenvalues	40.799	26.436	24.377	21.681	19.921	17.023	14.763
Variability (%)	17.720	15.415	11.598	17.169	11.119	12.253	14.725
Cumulative %	17.720	33.134	44.733	61.902	73.022	85.275	100.000

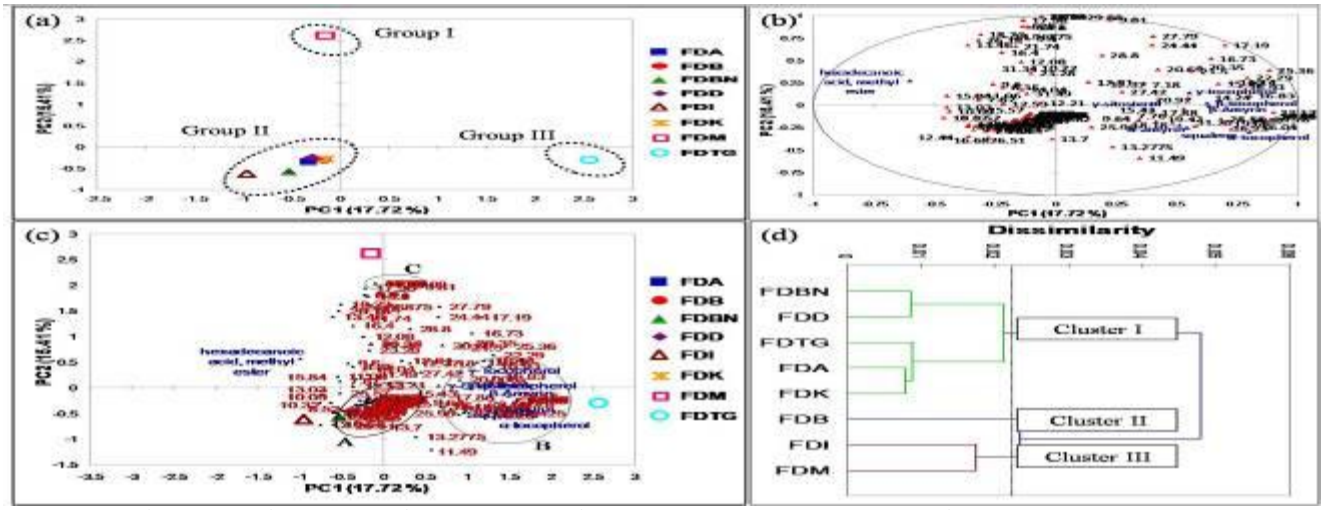


Fig. 3: Data set from GC-MS fingerprinting of methanol extract of eight FD varieties; (a) PCA scores plot after varimax rotation, (b) PCA-loading plot, (c) PCA-biplot and (d) HCA dendrogram. In (c), a group of variables overlapped together with FDA, FDB, FDD and FDK in (A), known peaks grouped together in (B), and unknown overlapping peaks variables in (C). FD varieties; FDA: FD var. *angustifolia*; FDB: FD var. *bilobata*; FDBN: FD var. *borneensis*; FDD: FD var. *deltoidea*; FDI: FD var. *intermedia*; FDK: FD var. *kunstleri*; FDM: FD var. *motleyana*; FDTG: FD var. *trengganuensis.*

3.3. Similarity Characteristic of FD varieties

Fig. 3(d) shows the HCA of methanol extract of eight FD varieties based on separation of volatiles compounds. HCA of GC-MS data was differed from clustering in PCA score plot. FDTG was clustered with FDA and FDK in HCA instead of clustered alone in PCA. Moreover, they were also clustered into one subclade with FDBN and FDD. FDB clustered alone in Cluster II indicates that it having specific chemical compositions that vary it from other varieties.

3.4. Determination of the Most Significant Factor for FD varieties

GC-MS data set for methanol extract of FD varieties was further analyzed by DA. The label of cluster in Fig. 4(a) was based on: G-MAP = GC-MS-cluster for Methanol extract for All Peaks of compounds. Discriminant analysis was resulted two groups FD varieties from three clusters obtained in HCA as shown in discrimination function plot (Fig. 4a). Clusters of G-MAP1 and G-MAP3 were observed accumulate together in one point forming a new

cluster (black dotted circle). The new cluster of black dotted line revealed that they were having the similar characteristics of variables (Fig. 4b). The standard, forward stepwise and backward stepwise modes of DA yielded 100% assigning corresponding correlation using five variables (unidentified compounds at retention time of 8.85, 14.80, 16.25, 16.55 and 27.06) (Table 3 and 4). Therefore, DA results based on all peaks compounds suggest that these five variables are the significant variables in discriminating methanol extracts of FD varieties into two clusters. Based on the observation of the percentage area of those five significant peak compounds mentioned earlier, unidentified compounds at retention time of 16.55 and 27.06 possess high percentage of peak area in FDB, which might contribute for G-MAP2 cluster.

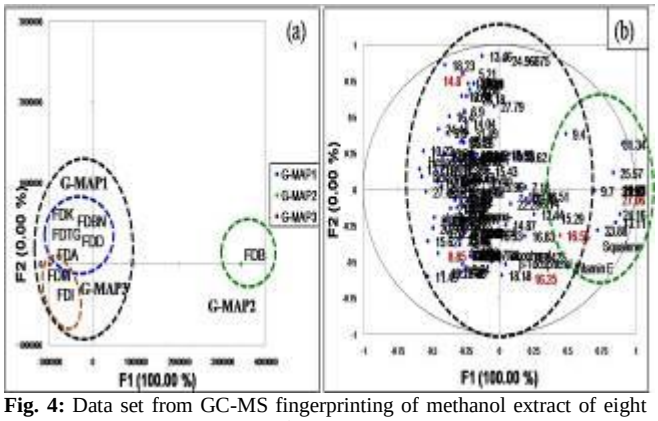


Fig. 4: Data set from GC-MS fingerprinting of methanol extract of eight FD varieties; (a) discrimination function plot and (b) its variables correlation plots. FD varieties; FDA: FD var. *angustifolia*; FDB: FD var. *bilobata*; FDBN: FD var. *borneensis*; FDD: FD var. *deltoidea*; FDI: FD var. *intermedia*; FDK: FD var. *kunstleri*; FDM: FD var. *motleyana*; FDTG: FD var. *trengganuensis*. G-MAP= GC-MS-cluster for Methanol extract for All Peaks of compounds.

This finding was supported by the previous studies as GC-MS have been utilized as a tool for discrimination purpose of plant samples. Chemometric coupled with GC-MS analysis have been discriminated Schizonepeta tenuifolia Briq, based on its essential oil identification for quality control, geographical origins and extraction method [36]. On the other hand, recently, two species of Notopterygium (traditional Chinese medicine); Notopterygium incisum Ting ex H. T. Chang (NI) and Notopterygium franchetii H. de Boiss (NF) have been distinguished based on their volatile compounds using GC/MS-PCA/HCA, which three volatile compounds; 1R-alpha-pinene, beta-pinene and 4-isopropyl-1-methyl-1, 4-cyclohexadiene had found to give great contribution to the discrimination [37]. The authors also claimed that as the finding on the differences of this two species (NI and NF) in the chemical compositions had facilitate quality control of Qianghuo, thus the comparison study on biological and pharmacological activities should be proceed to ensure their quality and efficacy. In term of similarity finding, in [38] have reported that five species (U. duckei, U. floribunda, U. refescens, U. stipitata and U. guatterioides) of the Amazonian Unonopsis have grouped into four cluster based on GC-MS-chemometric analysis of the leaf essential oils. From their finding, U. floribunda and U. refescens was clustered in one group in HCA and PCA which indicate high chemical similarity between them and α-guaiene, α-calacorene as well as widdrol were found as their possible chemical markers. While U. duckei, U. stipitata and U. guatterioides are significantly different from each other and U. duckei yet possess dissimilarity between samples from different location. GC-MS coupled with chemometric analysis from their study could be an important tool for chemotaxonomy evaluation approach as Unonopsis genus having confusion regarding their taxonomic classifications.

4. Conclusion

The use of Gas Chromatography-Mass Spectrometry (GC-MS) in this study provides a reliable technique to profile volatile compounds in eight FD varieties. As for this study, GC-MS fingerprinting coupled with chemometric analysis also found can discriminate FD varieties based on untargeted volatile compounds which could be used for their quality control. This finding indicates that 22 volatile compounds that commonly occur in FD varieties do not contribute to the discrimination and FDB revealed to have specific volatile compounds that distinguish it from other varieties. Those unidentified compounds assigned as significant discriminant of FD varieties could be further investigated in term of identification and characterization of those compounds. With the good chemical profile, GC-MS analysis could be improved by developing and comparing the current profiles with that of reference standards and thereby validated and quantified in order to see the relationship with the quantification data. The developed chemometric profiles from GC-MS fingerprinting derived from this study provides useful and promising information for authentication and quality control of FD varieties as well as can be used as a template for other plants with varietal level based on their overall and untargeted volatile compounds for quality control purpose.

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