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Discriminating *Ficus deltoidea* var. *bornensis* from Different Localities by HPTLC and FTIR Fingerprinting

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ABSTRACT

A nondestructive, efficient, and accurate fingerprinting method using Fourier transform infrared spectroscopy (FTIR) has been developed and optimized for the investigation and demonstration of the variance in chemical characteristics among extracts of *Ficus deltoidea Jack var. bornensis* from different but closely situated origins. The capacity of attenuated total reflectance (ATR) to differentiate these samples were studied using methanol and water extracts which were preliminarily screened using HPTLC with vitexin and isovitexin being used as markers for authentication. The mobile phase used was ethyl acetate: formic acid (0.1%): methanol at ratio of 5:5:2 (v/v/v) and the profile showed that methanol extracts had higher affinity for the markers. The FTIR spectra indicated that there was no obvious difference in spectroscopic pattern for either extracts when comparing samples from different localities but the absorption intensities of some peaks were different. Multivariate statistical analyses of PCA and HCA showed that both these techniques were capable of identifying the most similar as well as most differing samples and the identification depended on the type of extract. Overall, FTIR fingerprinting has the potential to be a fast and reliable analytical methodology for the discrimination between variants of plant from closely situated locations and hence chemically similar samples.

INTRODUCTION

Ficus deltoidea (FD) is mistletoe fig and locally known as mas cotek. It is mainly identified by its golden dots on the surfaces of the leaves (Hakiman *et al.*, 2012). FD has a variety of therapeutic potential such as to treat headache, high blood pressure and diabetes (Musa, 2006 and Adam *et al.*, 2010). Studies have shown that FD has biological activities such as antioxidant and possesses neuroprotective effect (Hakiman and Maziah, 2009 and Dzolin *et al.*, 2010). Sulaiman et al. (2008) reported that the aqueous extract of FD leaves has antinociceptive activity whereas other studies mentioned FD has also anticancer (Norrizah *et al.*, 2012), antiulcerogenic (Farsi *et al.*, 2011), anti-inflammatory (Abdullah *et al.*, 2009) and antibacterial activity (Alimon *et al.*, 2012; Jamal *et al.*, 2011). Various chemical components of FD have been reported with at least 25 flavonoids being identified with the major constituents being flavan-3-ol monomers, proanthocyanidins, and C-linked flavone glycosides (Omar et al, 2011 and Abdullah *et al.*, 2009). As reported by Choo et al. (2012), the C-linked glycoside flavones, vitexin and isovitexin possess alpha glucosidase inhibitory activity. There are various varieties in this species and initially, Corner (1969) classified FD into thirteen varieties which are FD var. *deltoidea*, var. *angustifolia*, var. *arenaria*, var. *bilobata*, var. *bornensis*, var. *intermedia*, var. *kinabaluensis*, var. *kunstleri*, var. *lutescens*, var. *motleyana*, var. *oligoneura*, var. *peltata* and var. *trengganuensis*. However, other researchers have grouped some of the varieties and Musa et al. (2005) have mentioned that FD consist of at least six varieties namely *bilobata*, *angustifolia*, *intermedia*, *kustleri*, *motleyana* and *trengganuensis*. FD var. *bornensis* was selected to be proceeded in chemical analysis in order to distinguish several localities of this variety as FD var. *bornensis* is generally distributed around Borneo in lowland and mountain, up to 1500 m alt (Corner, 1969).

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Thus, the aims of this study were to profile and fingerprint FD var. *bornensis* from six different but closely situated localities using two types of solvent extracts. This was carried out by using two techniques. The first was High Performance Thin Layer Chromatography (HPTLC) with two markers, vitexin and isovitexin, being used for authentication.

The second approach was by Fourier-transform infrared (FTIR) for metabolite fingerprinting. Chemometric techniques, namely principal component analysis (PCA) and hierarchical cluster analysis (HCA) were employed for the analysis of FTIR data. The finding from discrimination analyses of fingerprints using two different extracts will be beneficial in identifying the differences or the similarities of various localities for the same variety of plant.

EXPERIMENTAL

Chemicals and reagents

The pure standards of vitexin and isovitexin were purchased from Fluka-Sigma. The solvents consisting of methanol, ethyl acetate, and formic acid were from Merck, Damstadt, Germany. HPTLC plates precoated with silica gel F254 (layer thickness 0.2 mm) was purchased from Merck.

Plant Materials

Plant materials consisting of the leaves of FD var. *bornensis* was collected from six locations in Sarawak, of which five locations were around Santubong, Sarawak, East Malaysia. The five locations from which the variety bornensis were collected are identified as B1, specimen No: FD253 (Location: N01^{o}41'04.5" E11^{o}19'34.8"); B2, specimen No: FD260 (Location: N01^{o}41'04.4" E11^{o}19'34.8"); B3 specimen No: FD264 (Location: N01^{o}41'04.6" E11^{o}19'34.8"); B4, specimen No: FD265 (Location: N01^{o}41'04.7" E11^{o}19'34.8"); B5, specimen No: FD266 (Location: N01^{o}41'05.1" E11^{o}19'34.3"); and B6, specimen No: FD256 (Location: N01^{o}23'59.8'' E110^{o}19'11.5"). Each sample was identified by Prof. Nashriyah Mat, University Sultan Zainal Abidin (UniSZA), Terengganu, Malaysia and deposited at UniSZA's herbarium.

Sample extraction

The methanol extracts were prepared by macerating one gram of powdered sample with 19 ml methanol for three days. For water extracts, one gram powdered material was extracted at 100^{o}C with distilled water for one hour. The extracts were filtered using Whatman filter paper and then the filtrates were dried using rotary evaporator and kept in -20^{o}C prior to analysis.

Preparation of sample

Both the methanol and water extracts of FD var. *bornensis* from various locations were reconstituted in methanol at concentration of 20 mg/ml, respectively. The volume of application for sample extracts were 10 µl for each spot.

HPTLC-Fingerprinting Analysis

The HPTLC-fingerprinting analysis was carried out by spotting the above extracts and standard solutions on HPTLC plates precoated with silica gel F_{254} (layer thickness 0.2 mm) (Merck) using Camag Linomat 5 model. Standard markers, isovitexin and vitexin were prepared at concentrations of 100 µg/ml in methanol, respectively and were spotted on HPTLC plates at 10 µl.

The samples consisting of reconstituted extracts and standard markers were streaked in the form of narrow bands of length 8.0 mm, 9 mm from bottom edge, 17 mm from margin, 12.7 mm apart at a constant rate of 50 nl/s using nitrogen aspirator. Several solvent systems were screened initially for vitexin and isovitexin. The selection of solvent system was based on the separation of marker compounds and the optimum mobile phase was ethyl acetate: formic acid (0.1%): methanol at ratio of 5:5:2 (v/v/v). The migration distance was 8 cm. The densitometric analysis of separated components was carried out using Camag TLC scanner 3 (Camag, Switzerland) in the absorbance mode at 340 nm.

The bands were scanned using deuterium and tungsten lamps with scanning speed of 20 mm/s and macroslit dimension of 8.00 x 0.2 mm. Integration of chromatograms was performed using the Camag TLC scanner system and winCATS software. The developed plate was visualised under 254 nm wavelength and UV spectrum analysis was carried out at 340 nm.

Instrumentation and FTIR measurements

FTIR spectra were obtained using Perkin Elmer Spectrum 400 Infrared spectroscope coupled with an air-cooled Deuterated Triglycine Sulphate (DTGS) detector. Attenuated Total Reflectance (ATR) scan technique was used directly for all samples. The infrared measurements were made at resolution 4 cm^{-1} with 16 inferograms co-added before Fourier transformation. The data were recorded at mid-IR range of 600-4000cm^{-1}.

Data analysis for Spectral Fingerprinting

Each FTIR spectrum was baseline corrected using Spectrum (PerkinElmer, Inc.) software to minimize the differences between spectra due to baseline shifts. The most intense band of peak absorbance was normalized and the spectra were then exported as Spectrum. SP file and imported into the multivariate statistical software program The Unscrambler (CAMO, Trondheim, Norway).

Analysis was based on a 36 x 3311 data matrix assembled so that each row corresponded to a sample and each column represented the spectra data at a given wavelength. The PCA and HCA were performed to discriminate the metabolites of FD var. *bornensis* from different locations based on either methanol or water extracts. The square Euclidean distance was employed to establish clusters in HCA and calculate dissimilarity coefficients, respectively.

RESULTS AND DISCUSSION

In this work, methanol and water extracts of FD *var. bornensis* from six locations were analyzed. Methanol and water extracts of all locations were analyzed by HPTLC and FTIR.

Qualitative analysis using HPTLC techniques with marker compounds

The TLC profiles of methanol and water extracts of FD var. *bornensis* from six locations are shown in Fig.1. The TLC profiles of both extracts were carried out by using solvent system of ethyl acetate: formic acid (0.1%): methanol at ratio of 5: 5: 2 (v/v/v) and the mobile phase shows the clear separation of chemical constituents without any tailing. Two compounds were used as markers and these are vitexin and isovitexin (Fig. 2). The chromatograms of FD var. *bornensis* show two bands of vitexin and isovitexin with relative R_f values of 0.49 and 0.41, respectively and the intensities of both markers vary according to the types of extracts as well as locations.

The HPTLC profiles of methanol extracts (Fig 1a) when compared to water extracts (Fig 1c) show that the methanol extracts have greater ability than water extracts to extract both vitexin as well as isovitexin and this is confirmed by the HPTLC densitometry (Fig.1b and 1d). The concentration of markers varies with locations with the B5 having the highest concentration of vitexin whereas isovitexin is highly present in B3.

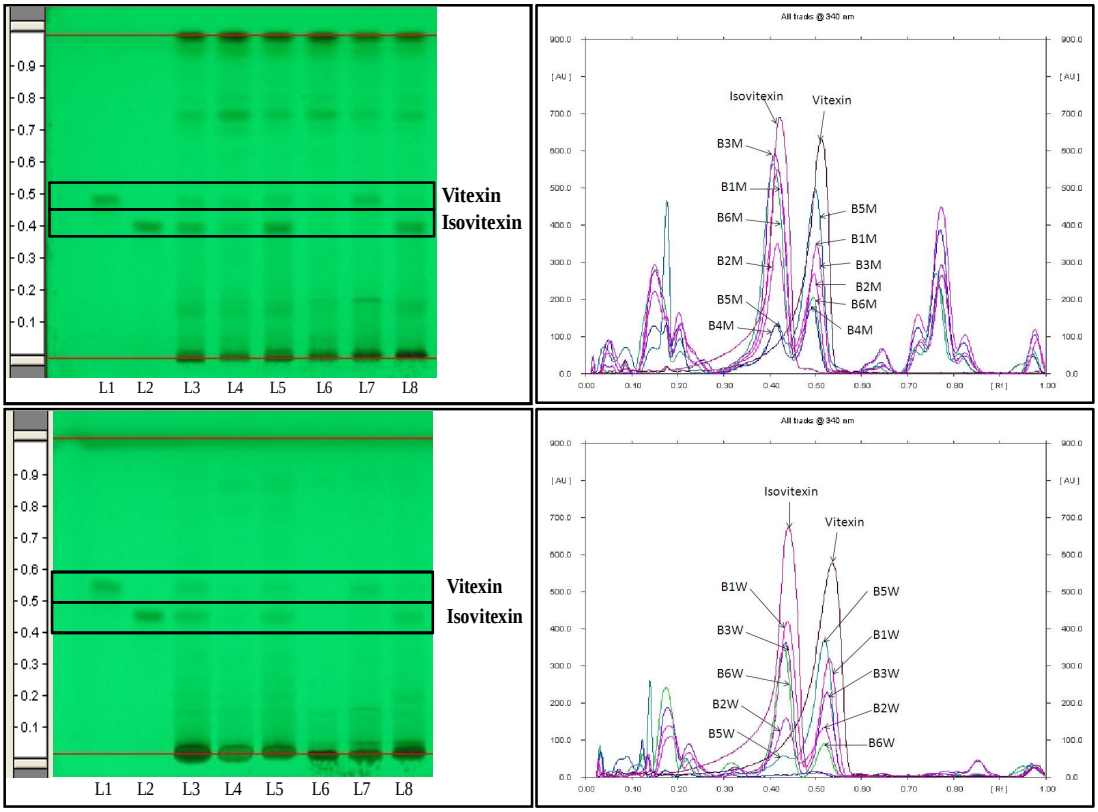


Fig. 1: HPTLC profile of FD var. bornensis from various locations.
The chromatogram of (a) methanol and (c) water extract from various locations (L3 to L8) and marker compounds (vitexin and isovitexin) observed under UV 254. L1: vitexin; L2: isovitexin; L3 to L8: samples B1 to B6. The 2D-densitogram of (c) methanol and (d) water extract showing vitexin and isovitexin with Rf of 0.49 and 0.41.

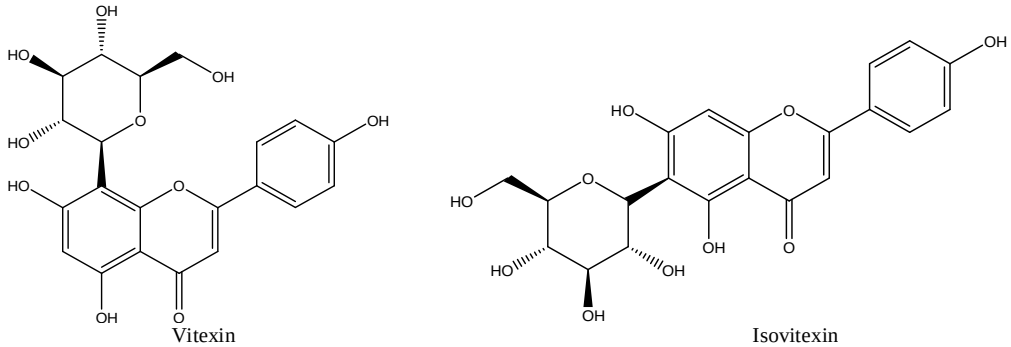


Fig. 2: Standard markers of vitexin and isovitexin.

FTIR Analysis

In the present study, ATR-FTIR was used to discriminate FD var. *bornensis* according locations to locations and this was achieved by using two different extracts. Typical FTIR spectra for different localities of FD var. *bornensis* in methanol and water extracts are shown in Fig. 3.

In general, the spectra of all samples look very similar with the number of peaks generally more or less the same for a particular solvent extract but varying in the intensity. In general, methanol and water extracts had different fingerprints. This data is in agreement with the HPTLC fingerprints where more bands observed in methanol extracts compared to water. FTIR peaks are attributed to stretching and bending vibrations that characterize the functional groups.

For methanol extract, there are eleven significant peaks, which is similar in all localities. These peaks were at 3550-3200 cm^{-1} which can be assigned as intermolecular hydrogen bonded hydroxyl group, 2930-2850 cm^{-1} being assigned as asymmetry or symmetry stretching vibration in methylene C-H, 1750-1735cm^{-1} being assigned as C=O stretching vibration of saturated aliphatic ester, 1660-1610 cm^{-1} being assigned as C=C stretching vibration of vinyl ether, 1570-1515 cm^{-1} assigned as C-N-H bending and stretching vibration of secondary acyclic,1450 cm^{-1} attributed as asymmetrical bending vibration of C-H bonds while at 1375 cm^{-1} assigned as the symmetrical bending vibration of C-H bonds, 1275-1200 cm^{-1} attributed as asymmetrical C-O-C stretching vibration, 1075-1020 cm^{-1} attributed as symmetry C-O-C stretching vibration, 900-675 cm^{-1} assigned as out-of-plane C-H bending vibration of a ring hydrogen atom and 700-610 cm^{-1} assigned as C-H bending vibration of alkynes.

Meanwhile, for the water extract, there were eight obviously similar peaks of this variety from different localities in the spectra which were at 3550-3200 cm^{-1}, 1600-1585 cm^{-1} and 1500-1400 cm^{-1} assigned as C-C stretching within the ring, 1570-1515 cm^{-1}, 1275-1200 cm^{-1}, 1075-1020 cm^{-1}, 900-675 cm^{-1} and 700-610 cm^{-1} for which the assignments have been mentioned in methanol spectra.

The significant differences between methanol and water extracts were the absence of peaks at 2930-2850 cm^{-1}, 1750-1735 cm^{-1}, 1660-1610 cm^{-1}, 1450 cm^{-1} and 1375 cm^{-1}in water extracts and occurrence of peaks at 1600-1585 and 1500-1400 cm^{-1}in water extracts but absent in methanol extract. It can be summarized that from a qualitative point of view, the main differences between samples were observed in the region between 1800-1300 $cm^{-}1$.

Discriminant Analyses

The PCA on FTIR spectra was initially carried out to differentiate six different localities of FD var. *bornensis* based on either methanol or water extracts.

The first PCA carried out on the methanol extracts showed that the first two principal components (PC) accounted for 91% of the total variance and provided separation of the evaluated samples into four groups as shown in Fig. 4 at top-left. The second PCA carried out on the spectral data of the water extract, shows that the first two PC accounted for 97% of total variance and provided separation of the sample into three groups as shown in Fig 4 at bottom-left.

The used of only two PC (PC1 and PC2) on the 2D-scatter plot show that those six localities of methanol extracts cluster into four groups. B2M and B5M were clustered into two separate groups on their own whereas the four other samples were clustered into two groups of which the first consisted of B1M and B3M. The second group of two samples close together were B4M and B6M.

While for the water extract, the samples were clustered into three separate groups. B4W and B6W were plotted far apart as two separate groups whereas B1-3W as well as B5W was clustered together, overlapping closely and forming the third group. However, 3D-scatter plot had assisted in order to determine a better discrimination among the extracts for the different localities as shown in Fig 4 at right.

The methanol extracts in 3D plot in Fig 4 at top-right are clustered into five groups of which first four groups consist of B2M, B4M, B5M and B6M respectively whereas the fifth group consists of B1M and B3M. This differs from 3D plot for the water extracts in Fig 4 at bottom-right in which there are 4 groups. The three separate groups consist individually of the samples of B4W, B5W and B6W, whereas the fourth group consists of B1-3W.

To verify the dissimilarity between localities and to single out some classes, HCA was applied to set of variables employed for PCA and the resulting dendrograms are shown in Fig. 5.

The HCA analysis on methanol extracts confirmed the close grouping of B1M and B3M indicated earlier by PCA as well as the different individual clusters of B4M, B6M, B5M and B2M. However, HCA showed that B4M and B5M were quite similar to B6M whereas B2M was the most differing sample, which is also similar to the results of PCA. As for the water extracts, HCA confirmed the close chemical relationship of B1W and B2W with B5W being the next closest group among the remaining ones. The close chemical relationship shown by the proximity of B3W with B1W as well as B2W as seen in the PC plots are not shown in HCA. However, HCA plot confirms the results more emphasized in 2D PC plot that the most differing sample among the water extracts is B6W.

It has to be noted that HCA summarizes the whole spectral data in a plot showing the relationship between different samples whereas the 3D PC plot captures only part of the data to enable easier visualization. The FTIR spectral data from locations quite close geographically shows that the PCA and HCA techniques are capable of differentiating these samples using either methanol or water extracts.

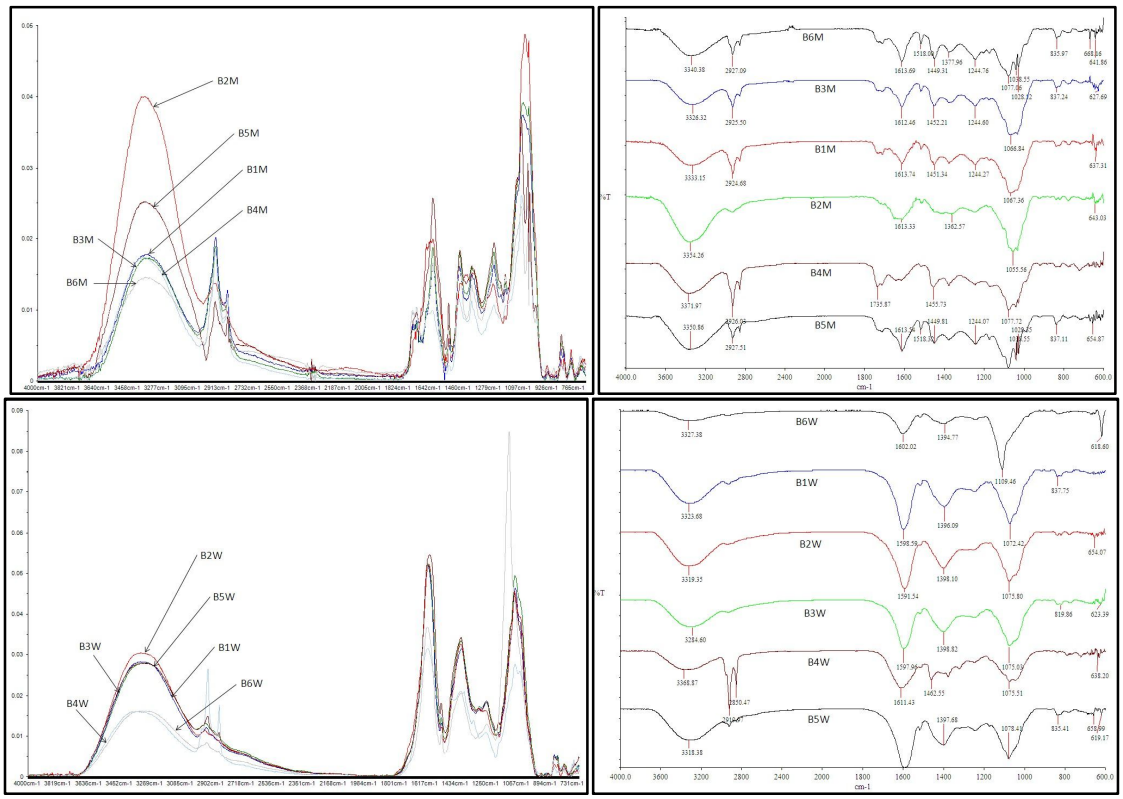


Fig. 3: FT-IR spectra for different localities of FD var. *bornensis*. FTIR spectra of overlay (on left) and split (on right) for methanol extract (on top) and water extract (at bottom) for locations B1 to B6.

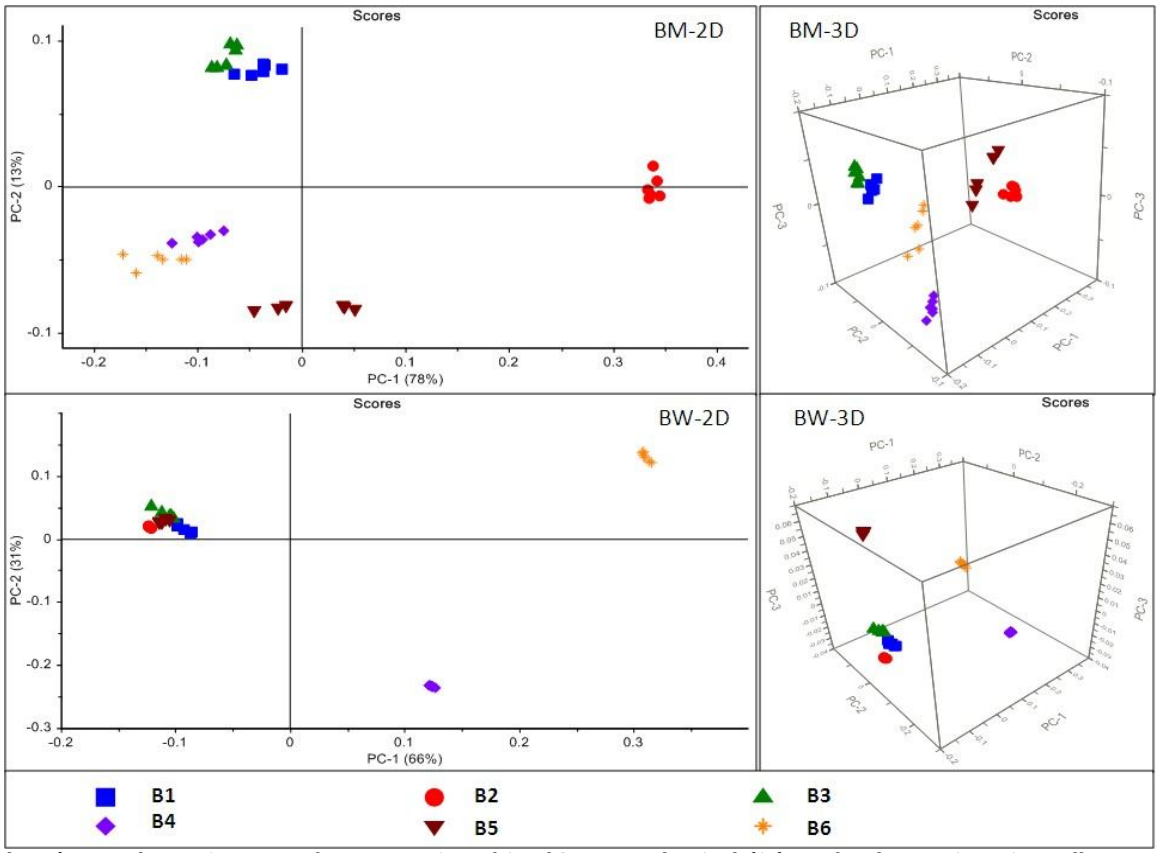
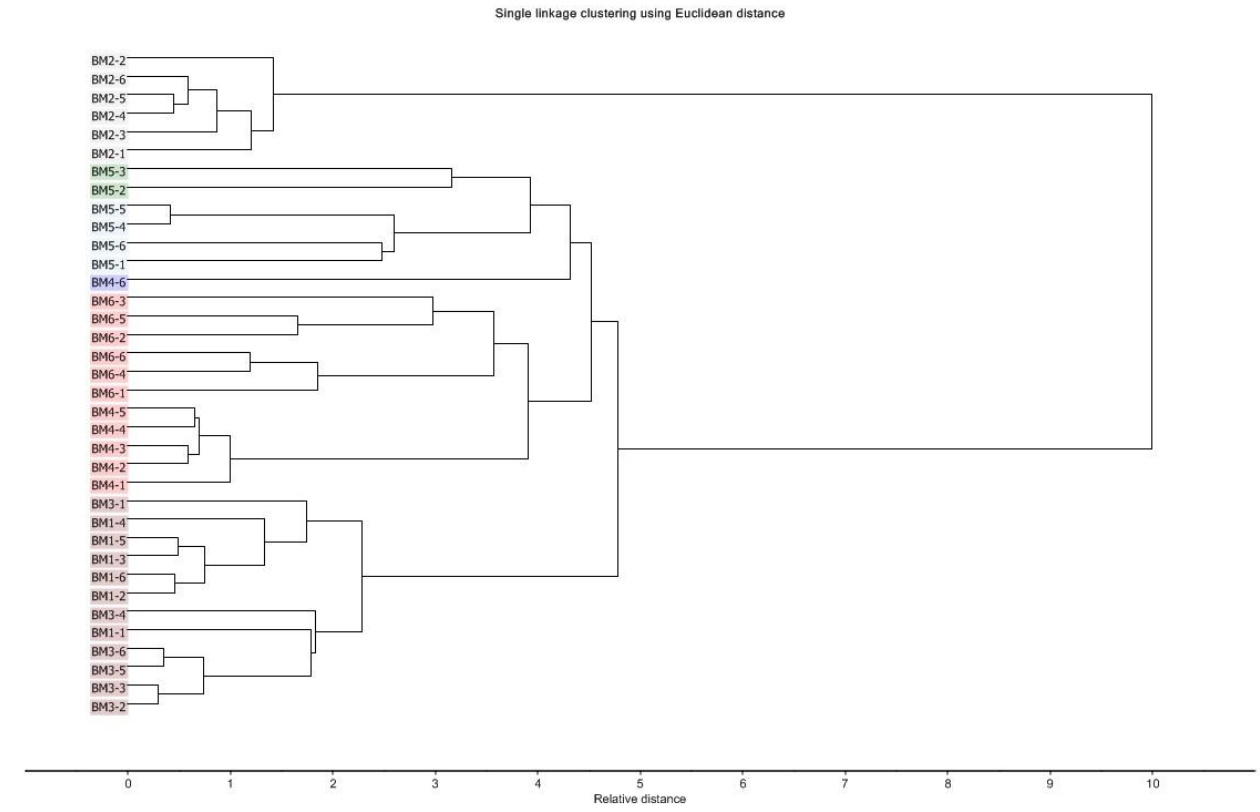


Fig. 4: PC plots of FD var. *bornensis* extracts, The 2D-scatter (on right) and 3D-scatter plots (on left) for methanol extract (on top) as well as water extract (at bottom) for locations B1 to B6.



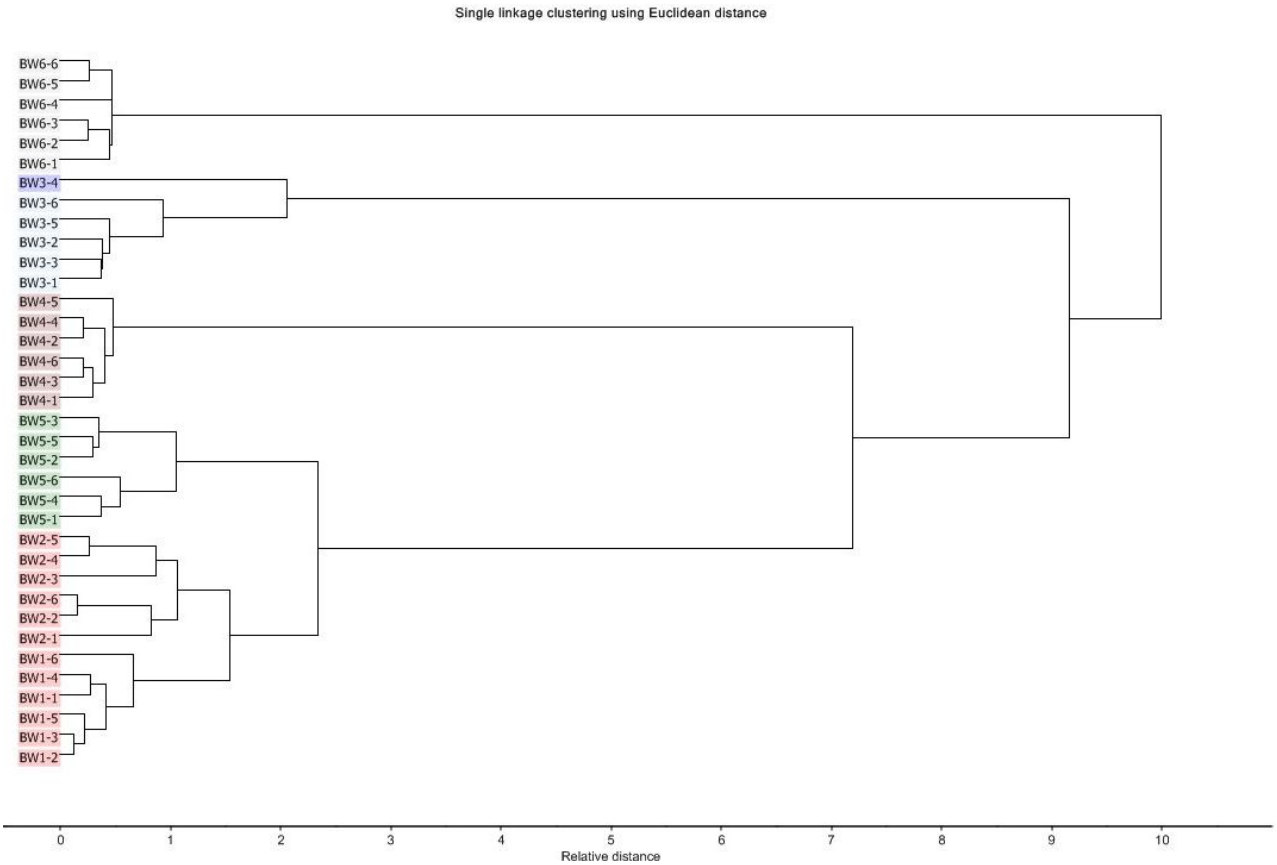


Fig. 5. HCA dendograms of (a) methanol and (b) water extract of FD var. *bornensis* of six different locations in Sarawak.

CONCLUSION

The use of HPTLC in this work provides simple, accurate, flexible and inexpensive separation technique for multiple samples at a time. This technique was employed to assist the identification of the same variety of FD from different localities as well as comparing the composition of two markers, namely vitexin and isovitexin. The study of different plant extracts by using two markers, namely isovitexin and vitexin showed that the different solvents have different affinity for the compounds.

The FTIR study shows the discrimination between localities for FD var. *bornensis* based on two different solvents respectively, and results demonstrate that identification of most similar samples as well as the most differing ones can be established easily by using either chemometric analyses of PCA or HCA.

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