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Discrimination of *Syzygium Polyanthum* (Wight) Walp. Cultivars Based On ATR-FTIR Spectroscopy

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Fourier transform infrared spectroscopy (FTIR) was utilised to discriminate *Syzygium polyanthum* cultivars (Serai Kayu and Serai Kayu Hutan) due to its effectiveness in discriminating plants species based on their chemical constituents. The ATR-FTIR mid infrared region (4000-400 cm^{-1}) of the IR spectrum was applied to the ethanolic and aqueous leaves extracts of Serai Kayu and Serai Kayu Hutan, to identify various fingerprints and discriminate them using multivariate analysis. Much phytochemical similarity was found between the two cultivars through the Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA). There was also found variation between intraspecies among same cultivars of Serai Kayu and Serai Kayu Hutan. The findings obtained from this study are very important for taxonomic identification, in which to be served as a guide for quality control in natural product development.

Keywords: ATR-FTIR; Serai Kayu; Serai Kayu Hutan; Discrimination

INTRODUCTION

The science of assigning, naming and describing of plants species to a group or class has been considered as plant taxonomy (Govindaraghavan et al., 2012; Zervakis et al., 2012). In order to understand the evolutionary relationship that exists among plants, Morpho-anatomical features are considered classical but also need are the chemical and molecular identification in other to place the plants properly to particular taxa. Based on the molecular and chemical identification of the plants, taxonomists have identified the biomarkers which helped and facilitated in naming or assigning a particular plant to a Taxa (Gao et al., 2012; Zimmermann & Kohler, 2014). However with regard to taxonomic identification of the plants species there is still wide disagreement in respect to the grouping of the plants (Naturalness of the plants) as a result of wide variation of the chemical and molecular content of the plant even within the similar species of plant (Zervakis et al., 2012; Khairudin et al., 2014). The results obtained from chemical studies of the plants will provide fundamental proof of confirmation for the characterization and identification of the plants.

Fourier Transform Infrared Spectroscopy (FTIR) is known to be fast, easy with high resolution and based on the vibration, to analyse functional groups and bonds that are highly polar (Samelis et al., 2011; Lagunin et al., 2014; Morgenstern et al., 2014; Song et al., 2014; Ul Hassan et al., 2014; Lang et al., 2017). The technique provides biological profiles with overlapping signals from most of the compounds available from the analyzed cells (Kim et al., 2004). The biological

profiles or biochemical profiles of the Fourier Transform Infrared generated from the analysed cells, are sets of data with a very high density which must be interpreted or analysed through multivariate analysis, the technique or the concept that has been used in microbial discrimination of closely related species (Kim et al., 2004; Mularczyk et al., 2012; Khairudin et al., 2014).

In ethnopharmacology, quality control is compulsory and vital to confirm the quality and authenticity of the plants for the development of modern medicine or herbal supplements (Keidel et al., 2010). One of the greatest sets back in the development of herbal product is quality assurance as a result of large variation in the chemical constituents of the plants (Keidel et al., 2010; Khairudin et al., 2014; Zhang et al., 2018). The discrepancies are as a result of many factors ranging from the time of collection (harvesting), environmental factor (climate), the age of the plants, soil and storage condition. Therefore, quality control with the identification of few compounds is insufficient for validation (Kim et al., 2004; Keidel et al., 2010; Gao et al., 2012; Nikzad et al., 2017).

Development of herbal product or modern medicine lies completely with authenticity and safety of the plants materials (Gao et al., 2012; Khairudin et al., 2014; Al-Tameme et al., 2015). Therefore, technique or concept must be adopted in other to identify and discriminate the real plant needed to overcome challenges of adulterations and safety of consumers (Gao et al., 2012; Gad et al., 2013). The presently available methods used for quality assurance are subjective to many challenges (Naumann et al., 2010; Gad et al., 2013). The conventional method of classification and identification of plants from the dried materials by means of morphological and anatomical (Macroscopic and Microscopic) characteristics or features requires an extensive labour, time consuming and specific or prior knowledge of the concept. Molecular identification and description of the plants is very expensive, time consuming and also required sophisticated machines to be able to interpret the result. Biochemical or molecular identification is difficult or complex as a result of sample preparation or processing with full of uncertainty of the end result such as the quality of the sequence purified or the quantity and quality of the extracted DNA which is the building block of molecular studies (Gao et al., 2012). For the purpose of improvement in Herbal supplement development and pharmacological research, the chemical constituents of all medicinal plants must be evaluated to be able to have reliability and repeatability of the process (Sultana et al., 2011; Khairudin et al., 2014).

Myrtaceae is of significant importance throughout the globe as it contributed economically, because many members of the family are edible and are domestically cultivated as food or as ornamental plants (Moneruzzaman et al., 20011). *Syzygium* has been considered as the largest genus in the family Myrtaceae, having approximately one hundred and twenty (120) species found almost all over the Asia (Abdulrahman et al., 2018b). *Syzygium* is of significant importance biologically; for its ability to cure diverse diseases (Abdulrahman et al., 2018a). Being among the largest genera of flowering plants, it also plays a significant role in the rainforest ecosystem. *Syzygium polyanthum* is locally known as Serai Kayu, Serai Kayu Hutan, Salam, Daun and Kelat among the people of Peninsular Malaysia (Abdulrahman et al., 2018a). Whilst in Indonesia, it is named as Indonesian laurel or Bay leaf. *S. polyanthum* is a plant known for its medicinal value among the Malay ethnic group for curing diverse ailments such as Diabetes, Malaria, Postpartum, Hypertension, Diarrhea, and Endometriosis (Abdulrahman et al., 2018a). The aim of this study was to utilise data set from Fourier Transform Infrared (FTIR) with the aid of multivariate analysis to discriminate Serai Kayu and Serai Kayu Hutan in order to be able to classify them phylogenetically.

MATERIALS AND METHODS

Collection and Preparation of the Sample Material

Two cultivars of *Syzygium polyanthum* (Serai Kayu and Serai Kayu Hutan) were collected from the wild in Besut, Terengganu, Peninsular Malaysia, and fully confirmed by a Botanist at the Universiti Sultan Zainal Abidin (UniSZA), after which the identified herbarium specimen was further taken to the Universiti Kebangsaan Malaysia (UKM) and the University of Malaya (UM) for further identification, and finally deposited at the Herbarium of UniSZA, UKM and UM respectively. The leaves were excised from the plants and dried under shade at room temperature and kept in clean place in order to avoid contamination before extraction was carried out.

Extraction

The dry samples were ground using the grinding machine until it becomes a coarse powder. The powder forms were weighed. Ethanolic and aqueous extraction was carried out using Soxhlet. Evaporation machine was used (Heidolph Germany) in order to obtain crude compound from both the ethanolic and aqueous extracts. The obtained crude extracts was kept in the dryer (FDD-720) at 40˚C to complete drying (Wang et al., 2010).

FTIR Spectra Measurement

The spectra from the FTIR were recorded from the IRPREATIGE-21 model Malaysia. The five extracted leaf sample of Serai Kayu and Serai Kayu Hutan were each with three technical replicates, and the spectra were scanned on at mid-IR with spectral range of 4000-400 cm^{-1} and the FTIR spectra were obtained by using the Perkin Elmer Spectrum 400 Infrared spectroscopy (Malaysia) coupled with an air cooled Deuterated Triglycine Sulphate (DTGS) detector. ATR (Attenuated Total Reflectance) scan method was directly employed for all samples at 16 scans and 4cm^{-1} resolutions. Each of the spectrums of the FTIR was baseline corrected in order to minimise the spectra difference from the shifts of the baseline and exported to ASCII file. Analysis of the spectra was carried out based on the existing literature and the spectrum was further subjected to multivariate analysis where PCA and HCA were carried out by using the SIMCA-P (V.14.1 Umetrics Sweden) software to discriminate Serai Kayu and Serai Kayu Hutan (Mohd et al., 2014; Zimmermann & Kohler, 2014; Lang et al., 2017).

RESULTS

Voucher numbers of the collected specimen were given in the deposited herbarium of Universiti Sultan Zainal Abidin (UniSZA), Universiti Kebangsaan Malaysia (UKM) and Universiti Malaya (UM) (Table 1a). Fourier Transform Infrared (FTIR) spectra of the ethanolic and aqueous extracts of the five biological replicates of Serai Kayu and Serai Kayu Hutan was generated. The overlay of the ethanolic and aqueous extracts of the five biological replicate each with three technical replicates (Fig. 1 and 2) The peaks of the ethanolic extracts were sharp at 812 cm^{-1} which represent C-O stretching (phenyl) and the spectral region of 892, 1000, 1200, 13100, 1500, 2800, 3000 and 3500 cm^{-1} that represent the fingerprints of C-C, C-O (Deoxyribose), Phosphodiester region, P=O stretching (Phosphodiester), N=O stretching of aromatic ring in lignin, protein, O–H stretching of CH_2 in fatty acids, O-H stretching water and O-H bonds respectively (Fig. 1 and Table 1). While in the case of aqueous extracts the peaks were sharp at 1000, 13100, 1370, 1600 and 3500 cm^{-1} which represent Phosphodiester region, protein, lignin, stretching (lipids) and O-H bonds respectively (Fig. 2 and Table 1). Spectra filter model was obtained from the ethanolic data with X matrix with highest variation (R^2X (cum). 0.997) and the highest predictive power (fitness of the model) (Q^2 (cum).0.987) (Fig. 3) while the data set from the aqueous sample with the best spectra filter model was obtained with X matrix, highest variation (R^2X (cum).0.993) and with highest predictive power (fitness of the model) (Q^2 (cum).0.981) (Fig. 4). The relationship (similarities and discrimination) established from the score plot of the ethanolic extracts of the Serai Kayu and Serai Kayu Hutan has resulted from the following fingerprints along the positive loading line of the PC1; 1654.92, 1560.41, 1508.33, 1490.97, 1473.62, 486.062 and 418.553 cm^{-1} and 1651.07, 1539.2 and 1504.48 cm^{-1} from the negative loading fingerprints respectively (Fig 5 and 6). While in respect to the aqueous extracts the relationship was established based on the following fingerprints 1755.52, 1730.15, 1354.03, 1311.59, 1201.65, and 1058.92 and 2926.01, 2850.79, 1616.35, 1570.06 and 447.485 cm^{-1} along the PC1 and PC2 respectively (Fig .7 and 8). Hierarchical Cluster Analysis (HCA) was constructed from the ethanolic spectra data which resulted into two major clades, with first major clade was divided into three subclades, containing different cultivars depending on the clade. The major clade was further divided into six clade also containing different cultivars (Fig. 9). Similar pattern was also recorded from the HCA of aqueous leaves extract (Fig.10). The dendrogram divided into two major clades. The first major clade contained only Serai Kayu Hutan and the second major clade was divided into two subclades with the first subclades divided into two mini clades.

DISCUSSION

Fourier Transform Infrared spectroscopy (FTIR) is a method with an excellent performance in terms of analyzing phylogenetic relationships among flowering plants. The concept of FTIR combined with chemometrics, show potential as rapid methods to discriminate between plants of the same cultivar or different species as it has

been previously reported by Ul Hassan et al, (2014) in taxonomic identification of *Solanum nigarum* and *Solanum giganteum.* Similarly, Lang et al., (2017) in their study of identifying plant species to family level have reported FTIR Posses the potential of taxonomically identifying plants to family level from their branches and has the potential to apply to all plants for taxonomic discrimination.

Table 1a: Voucher numbers of deposited specimen in respective Herbarium

S/N	SPECIMEN	HERBARIUM NO
1	***Syzygium polyanthum* (Serai Kayu)**	Unisza 00395
2	***Syzygium polyanthum* (Serai Kayu Hutan)**	Unisza 00396
5	***Syzygium polyanthum* (Serai Kayu)**	UM-KLU49443
6	***Syzygium polyanthum* (Serai Kayu Hutan)**	UM-KLU49444
7	***Syzygium polyanthum* (Serai Kayu)**	UKMB 40352
8	***Syzygium polyanthum* (Serai Kayu Hutan)**	UKMB 40353

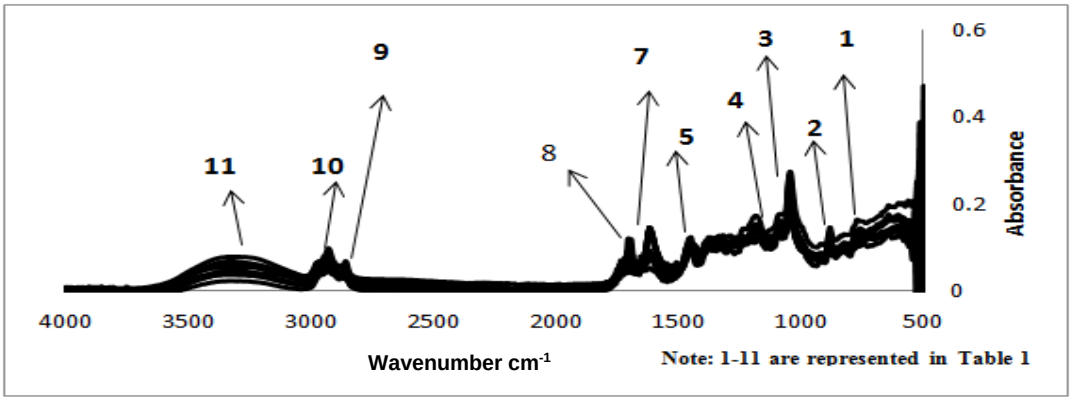

Figure. 1: Overlay spectra of ethanolic leaves extracts *Syzygium polyanthum* (Serai Kayu and Serai Kayu Hutan)

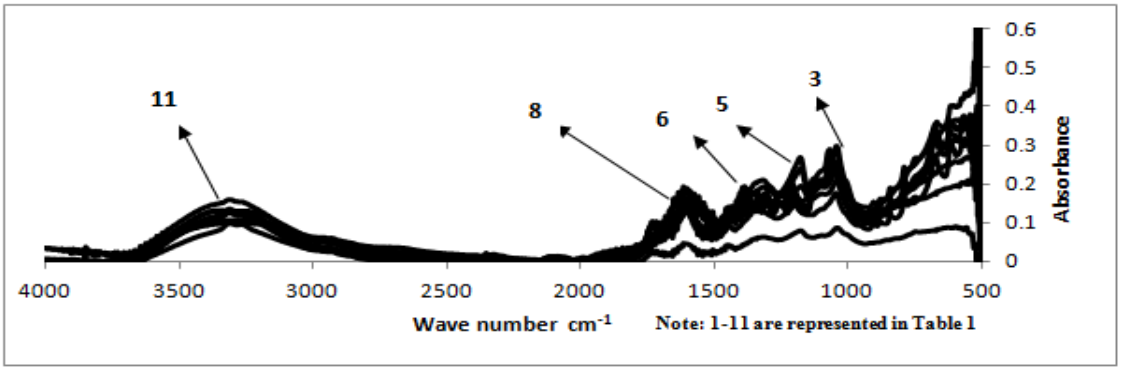

Figure. 2: Overlay spectra of aqueous leaves extracts of *Syzygium polyanthum* (Serai Kayu and Serai Kayu Hutan)

Table 1: Characteristics of absorption bands of Serai Kayu and Serai Kayu Hutan (*Syzygium polyanthum)*

S/N	Wave number cm^{-1}	Fingerprints
1	800	C-O stretching (phenyl)
2	892	C-C, C-O (Deoxyribose)
3	1000	Phosphodiester region
4	1200	P=O stretching of Phosphodiester
5	1310	Protein
6	1370	Lignin
7	1500	N=O stretching of aromatic ring in lignin
8	1600	Stretching (lipids)
9	2800	C–H stretching of CH_2 in fatty acids
10	3000	O-H stretching water
11	3500	O-H bonds

Note: S/N = Serial Number of the overlay ethanolic and aqueous extracts

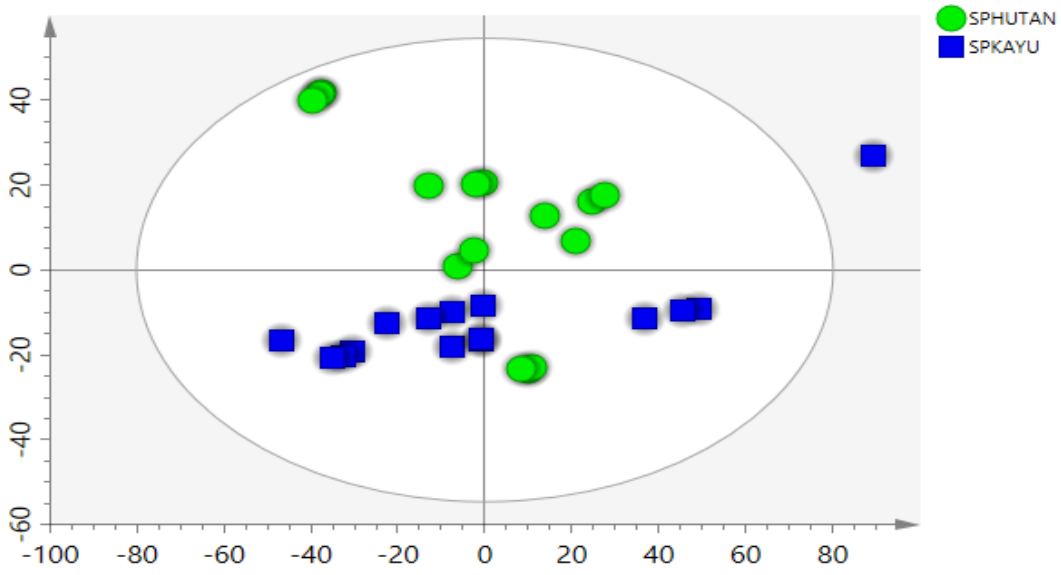

Figure. 3: PCA score plot of ATR-FTIR spectroscopy data of ethanolic leaves extracts of *Syzygium polyanthum* (Serai Kayu and Serai Kayu Hutan)

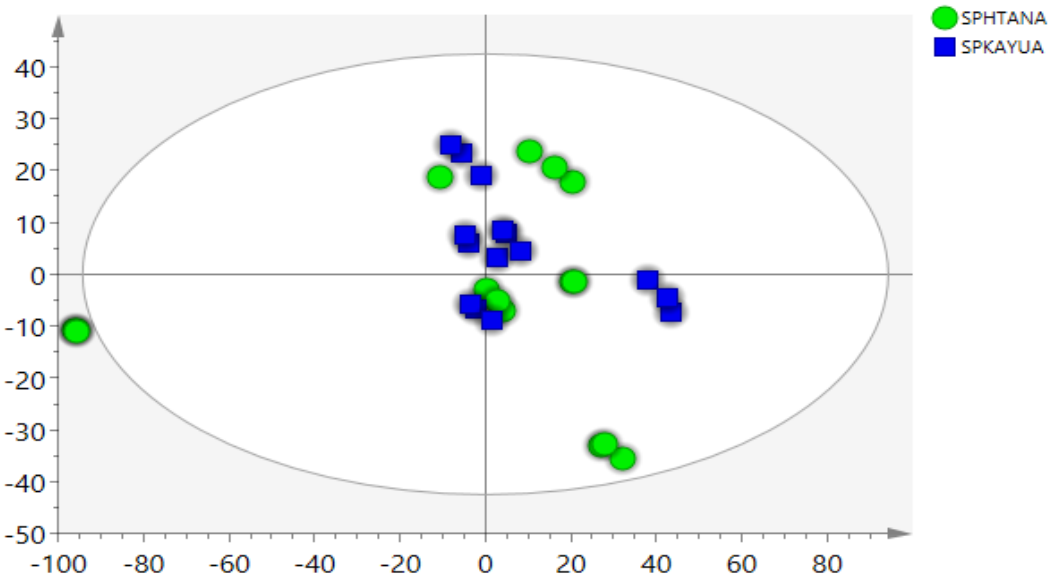

Figure. 4: PCA score plot of ATR-FTIR spectroscopy data of aqueous leaves extracts of *Syzygium polyanthum* (Serai Kayu and Serai Kayu Hutan)

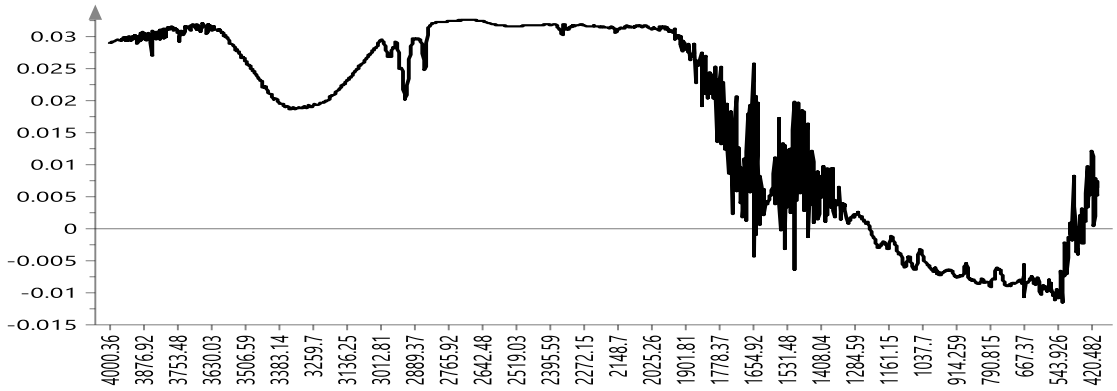

Figure. 5: Loading line plot from ATR-FTIR spectroscopy of ethanolic leaves extracts showing fingerprints responsible for the discrimination and relationship within the PC1 of Serai Kayu and Serai Kayu Hutan (*Syzygium polyanthum*)

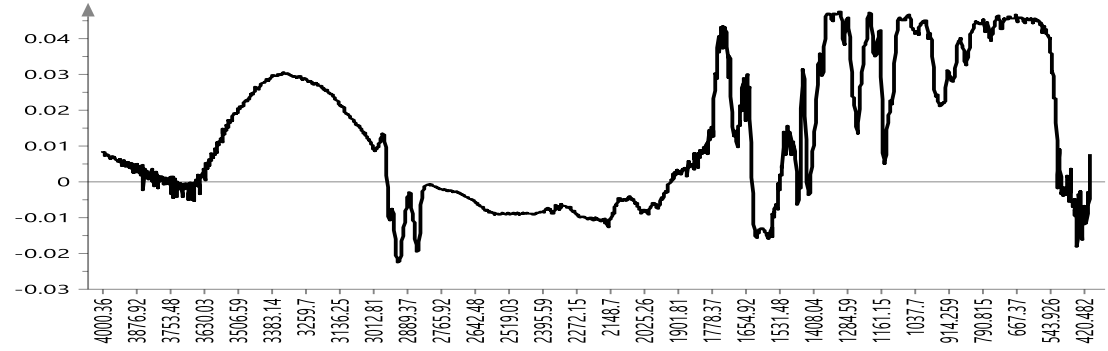

Figure. 6: Loading line plot from ATR-FTIR spectroscopy of ethanolic leaves extracts showing fingerprints responsible for the discrimination and relationship within the PC2 of Serai Kayu and Serai Kayu Hutan (*Syzygium polyanthum*)

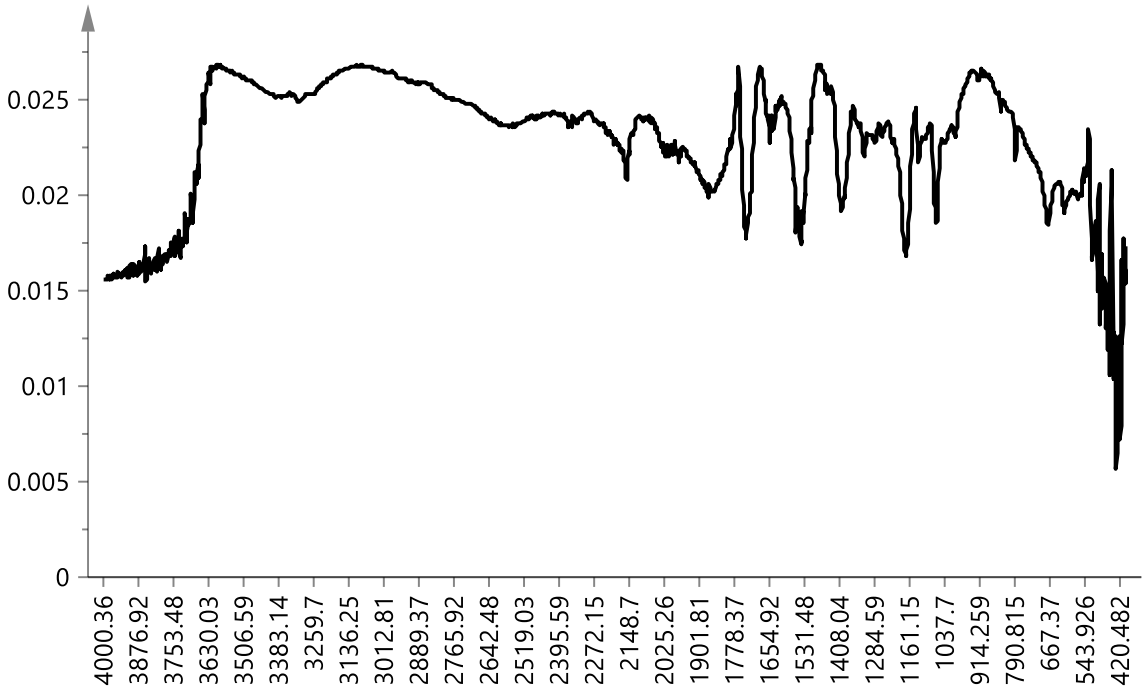

Figure. 7: Loading line plot from ATR-FTIR spectroscopy of aqueous leaves extracts showing fingerprints responsible for the discrimination and relationship within the PC1 of Serai Kayu and Serai Kayu Hutan (*Syzygium polyanthum*)

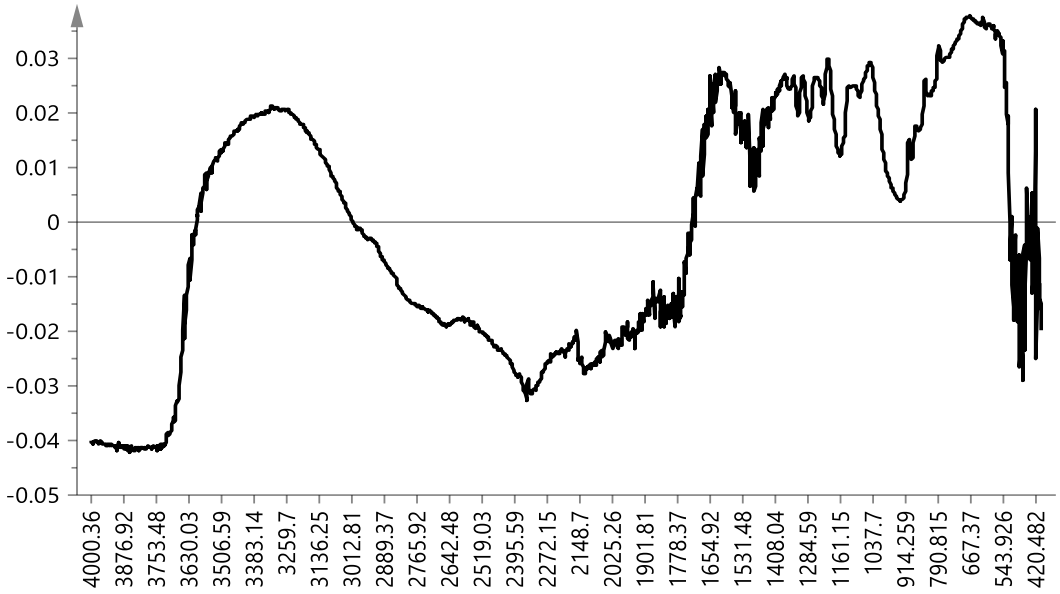

Figure. 8: Loading line plot from ATR-FTIR spectroscopy of aqueous leaves extracts showing fingerprints responsible for the discrimination and relationship within the PC2 of Serai Kayu and Serai Kayu Hutan (*Syzygium polyanthum*)

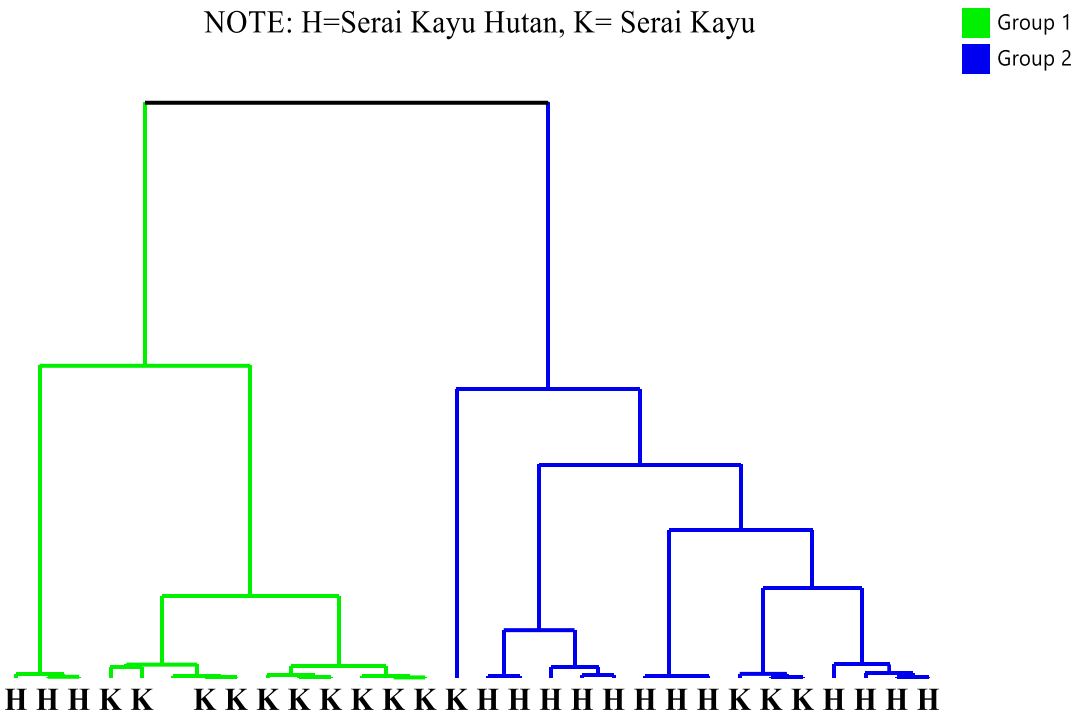

Figure. 9: Dendrogram of the HCA of the ethanolic leaves extracts showing the discrimination and relationship between Serai Kayu and Serai Kayu hutan (*Syzygium polyanthum*).

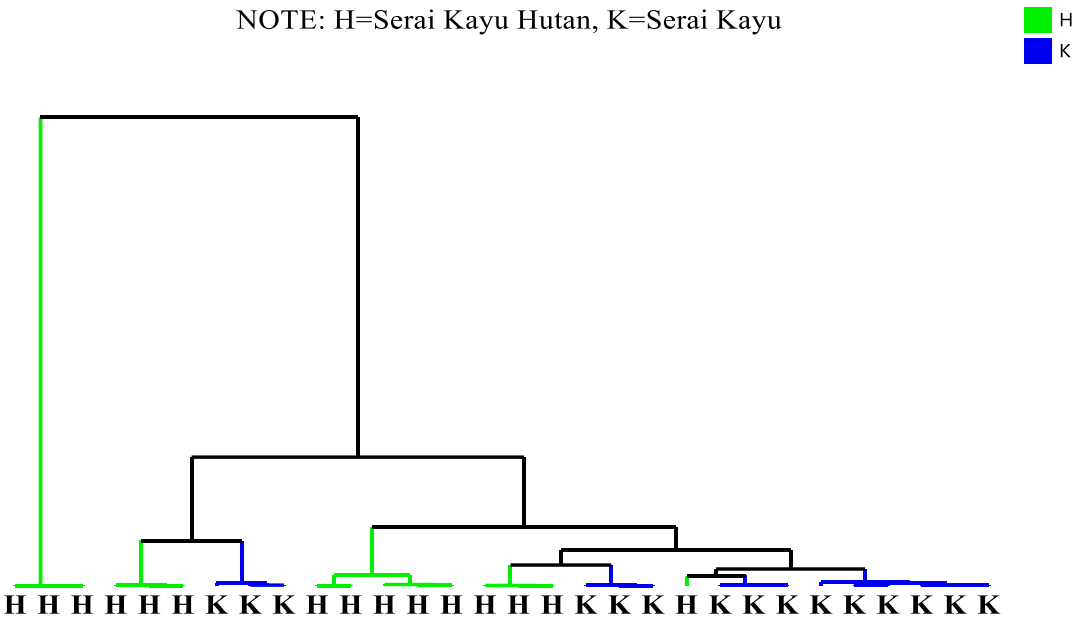

Figure. 10: Dendrogram of the HCA of the aqueous leaves extracts showing the discrimination and relationship between Serai Kayu and Serai Kayu Hutan (*Syzygium polyanthum*).

Ten spectra were obtained from the ethanolic and aqueous extracts of all the ten samples of Serai Kayu and Serai Kayu Hutan collected in all the localities. The peaks of the ethanolic extracts were sharp at 812 cm^{-1} which represent C-O stretching (phenyl) and the spectral region of 892, 1000, 1200, 13100, 1500, 2800, 3000 and 3500 cm^{-1} that represent the fingerprints of C-C, C-O (Deoxyribose), Phosphodiester region, P=O stretching (Phosphodiester), N=O stretching of aromatic ring in lignin, protein, O–H stretching of CH_2 in fatty acids, O-H stretching water and O-H bonds respectively (Fig. 1 and Table 2). While in the case of aqueous extracts the peaks were sharp at 1000, 13100, 1370, 1600 and 3500 cm^{-1} which represent Phosphodiester region, protein, lignin, stretching (lipids) and O-H bonds respectively (Fig. 2 and Table 2). A broad region was observed to have a significant difference between ethanolic and aqueous extracts at the region of the spectra of 3500-3000 cm^{-1} peaks, and the peaks and some fingerprints were not seen in aqueous extracts that was due to the fact of the polarities of the compounds to be able to be extracted from the ethanolic extracts. The results show no significant difference from the fingerprints of ethanolic extracts of Serai Kayu and Serai Kayu Hutan, same as in the aqueous extracts. The finding supported the results of Khairudin et al., (2014) where no significant difference was reported in their spectral bands of direct discriminations of some plant populations using FTIR spectroscopy. The studies also in agreement with Mohd et al., (2014) in their studies to discriminate seven varieties *Ficus deltoidea* using FTIR dataset coupled with chemometrics where they report no significance was observed from the fingerprint of the methanolic and aqueous extracts of both the seven varieties. The study disagreed with Keidel et al., (2010) on their study to discriminate Green arabica and Robusta (coffee beans).

The complete datasets of the ethanolic sample of (*Syzygium polyanthum)* Serai Kayu and Serai Kayu Hutan were analysed using the Principal Component Analysis (PCA). The best spectra filter model was obtained with X matrix with highest variation (R^2X (cum). 0.997) and the highest predictive power (fitness of the model) (Q^2 (cum).0.987) while the data set from the aqueous sample with best spectra filter model was obtained with X matrix, highest variation (R^2X (cum).0.993) and with highest predictive power (fitness of the model) (Q^2 (cum).0.981). The fitness of the model revealed how good the data of both the ethanolic and aqueous extracts is to further carry the analysis of the PCA and HCA model. Score plots (scattered) of the two PCs (PC1 and PC2) of the ethanolic extracts of the Serai Kayu and Serai Kayu Hutan dataset was constructed and evaluated to account for 99.7% variation R^2X (cum). Along the PC2 axes, eight main clusters were constructed in the second PC. In the first PC along the PC1, one strong outlier can be observed from Serai Kayu and also variation within the same sample (intraspecies variation). Two groups are constructed from Serai Kayu and one group from Serai Kayu Hutan along the X axes of the PC1 with variation accounting for 49.6% (R^2X.0.496) (Fig. 3). The fitness and the predictive model obtained in the following study supported the findings of Maree & Viljoen, (2011) were the obtained a similar pattern of fitness and the predictive model (FT-MID: R^2X (cum): 0.956, Q^2 (cum) =0.952. FT-MID: R^2X (cum): 0.753, Q^2 (cum) =0.731). Principal Component Analysis (PCA) suggested that there were large phytochemical similarities between the two cultivars, and intra variation also existed between species of the same cultivar whereby no clear separation can be seen along the PC1 and bit separation was seen in the PC2 (Fig. 3). The findings were also in agreement with Song et al. (2014) in discriminating closely related *Brassica rapa* species. Also concur with the findings of Mohd et al., (2014) on discrimination of *ficus deltoidea* jack in Peninsular Malaysia where they report variation (intra and inter) among similar species of the same cultivar.

Score plots (scattered) of the 2 PCs (PC1 and PC2) of the aqueous extracts dataset were constructed with and evaluated. Along the PC1 both the Serai Kayu and Serai Kayu Hutan were seen overlapping each other no clear separation was observed (Fig. 4). In the second PC along the PC2 one strong outlier can be observed from Serai Kayu Hutan and also variation within the same sample (intra species variation). Also, no clear separation was seen but overlapped along the PC1 with variation accounting for 68.8% (R^2X.0.688). This is in agreement with Mohd et al., (2014) in their methanolic and aqueous studies of seven varieties of *F.deltoidea*. The score plot of FTIR spectroscopy data from the ethanolic and aqueous extracts exhibit a strong relationship between the two cultivars. This is further confirmed the morphology and anatomical studies carried out on *Syzygium polyanthum* cultivars (Serai Kayu and Serai Kayu Hutan) by Abdulrahman et al. (2018b), further validate the

findings of Song et al., (2014) on discrimination of cabbage progenies from their parents and Khairudin et al, (2014) where they used FTIR to establish relations that exists between plant species. Also is in agreement with Mohd et al., (2014) where they conclude a good clustering was observed in their studies. But the study completely disagrees with Naumann et al., (2010) where they report clear clustering on discrimination of Oats and Peanuts root using FTIR datasets coupled with chemometrics. Morgenstern et al., (2014) in their studies of *Myrciaria jaboticaba* and *Myrciaria cauliflora* to discrimination them from their fruit using near-infrared spectroscopy where they also report clear clustering from the fruits of the two species.

The similarities and discrimination of the score plot from the ethanolic extracts of the Serai Kayu and Serai Kayu Hutan has resulted from the following fingerprints along the positive loading line plot of the PC1; 1654.92, 1560.41, 1508.33, 1490.97, 1473.62, 486.062 and 418.553 cm^{-1} and the fingerprint within the negative loading line plot are 1651.07, 1539.2 and 1504.48 cm^{-1} . These were responsible for the intra and interspecies variations of the cultivars that fall within the region of PC1 (Fig.5 and 6) respectively. Likewise, the relationship established from the score plot of the aqueous extracts was as a result of the following fingerprints; 1755.52, 1730.15, 1354.03, 1311.59, 1201.65, and 1058.92 and 2926.01, 2850.79, 1616.35, 1570.06 and 447.485 cm^{-1} along the PC1 and PC2 respectively (Fig .7 and 8).

To further confirm their relationships and dissimilarity of the two cultivars, the dataset from the ethanolic and aqueous leaves extract were used to construct Hierarchical Cluster Analysis (HCA) in order to figure them into distinct classes (Fig. 9 and 10). The dendrogram from the HCA of the ethanolic extracts (Fig. 9) was further confirmed by the Relationship (similarities and dissimilarities) that has already been established by the PCA (Fig. 3). These resulted them into two major clades, with first major clade was divided into three subclades, the first clade having species of Serai Kayu hutan, second clades contained only Serai Kayu and the last clade contain Serai Kayu only. Another major clade was further divided into six clades, with first clade containing only Serai Kayu, the second clade containing Serai Kayu Hutan, third clade Serai Kayu Hutan, fourth clade Serai Kayu Hutan, fifth clade Serai Kayu and the last clade containing Serai Kayu Hutan. The HCA from the ethanolic extracts has further supported the result of the ethanolic PCA where a variation existed between same cultivars and further established a strong relationship existed between Serai Kayu and Serai Kayu Hutan based on their chemical constituents.

Similar pattern from the score plot was also observed with the HCA of aqueous leaves extract (Fig.10). The dendrogram divided into two major clades. The first major clade contained only Serai Kayu Hutan and the second major clade was divided into two subclades with the first subclades divided into two mini clades. The first mini clade contained Serai Kayu Hutan while the second mini clade contained Serai Kayu. The second subclade further divided into two major clades, with the first clade divided into two mini subclades. The first and the second mini clades contained only Serai Kayu Hutan. The second subclade divides into two subclades, with the first mini clades contained both Serai Kayu Hutan and Serai Kayu and also the second mini clade also contains both Serai Kayu and Serai Kayu Hutan. The results further proved that no clear separation was observed from the PCA. The results from both the ethanolic and aqueous extracts of Serai Kayu and Serai Kayu Hutan were also in agreement with Kim et al., (2004) in their studies on taxonomic identification of flowering plants by using multivariate analysis coupled with FTIR datasets. These results were in agreements with the findings of Gao et al., (2012), where they report phylogenetic relationships of *ulam* using FTIR dataset. The findings were in disagreement with Schulz et al., (2005) on their studies of characterization of essential oil by using IR and Raman spectroscopy were they obtained clear discrimination of essential oil distilled in plants collected from Turkey from Principal Component Analysis before they are subjected to Hierarchical Cluster Analysis. The results of the studies from the FTIR datasets coupled with Chemometrics have provided an efficient way of discriminating plant species of closely related and further supported the findings of Mularczyk et al., (2012) were they find FTIR coupled with chemometrics as a great tool for taxonomic identification of plants to species level.

CONCLUSION

The studies have fully discriminated Serai Kayu and Serai Kayu Hutan based on their relationships and dissimilarity, which also provided that; there is intra variation within the same species collected at the same location. PCA provided a good explanation on the discrimination

and further proved by HCA through the dendrogram created from the SIMCA software. Analysing of the FTIR dataset coupled with chemometrics will be of great importance for the taxonomic identification of plant species based on; intra or inter variations. In conclusion, the results would be of great value for quality determination of the raw materials of Serai Kayu and Serai Kayu Hutan in order to avoid adulteration of the plant.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

All authors contributed equally in all parts of this study.

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