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DIFFERENTIATION OF CHEMICAL PROFILES OF *EURYCOMA LONGIFOLIA*, *POLYALTHIA BULLATA* AND *PRISMATOMERIS TETRANDRA* USING 1D & 2D FTIR

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Tongkat Ali, or *Eurycoma longifolia*, is one of the valuable medicinal substances that is in high demand, not only in Malaysia, but from all over the world because of its benefits and uses. Based on its use in traditional Malay medicine, tongkat ali root can improve men's vitality and health. Due to the benefits of its use in some biological activities such as antitumor, antimalarial, antibacterial, antidiabetic, antihypertensive, osteoprotective and ergogenic, the demand for the supply of tongkat ali raw materials has increased tremendously. There are several types of tongkat ali in the Malaysian market including tongkat ali kuning (*E. longifolia*), tongkat ali hitam (*Polyalthia bullata*) and tongkat ali merah (*Prismatomeris tetrandra*). However, information related to the chemical profile and benefits of using tongkat ali merah dan tongkat ali hitam is very limited. Therefore, this study was conducted to obtain the chemical fingerprint profile of the three tongkat ali to ensure the authenticity of the raw materials used. Accurate validation of raw materials is essential to ensure product consistency, safety and efficacy, and ultimately build consumer trust. Analysis involved Fourier transform infrared spectroscopy (FT-IR), second derivative IR spectroscopy, and two-dimensional correlation infrared spectroscopy (2D-IR) with thermal interference. The study found the roots of *E. longifolia, P. bullata* and *P. tetrandra* can be differentiated by their unique peaks in the secondary derivative spectra. While, the 2D-correlation FTIR based on thermal perturbation emerges as a powerful and distinctive analytical tool, providing a precise fingerprint for differentiating the roots of *E. longifolia, P. bullata* and *P. tetrandra* with unparalleled accuracy and reliability.

Keywords: *Eurycoma longifolia, Polyalthia bullata, Prismatomeris tetrandra,* FT-IR, 2D-IR correlation spectroscopy

INTRODUCTION

The family Simaroubaceae contains many bitter taste plants which includes the genus of *Eurycoma*. It is a very small genus of trees, native to Southeast Asia such as Myanmar, Thailand and Indo-China. *Eurycoma longifolia* is used to cure lumbago and indigestion. It is used as a power tonic after delivery, and use for treatment of fever, jaundice, cachexia, and dropsy. *E. longifolia* is one of the most popular folk medicines for its aphrodisiac effects and treatment of intermittent fever (malaria). Decoctions of *E. longifolia* leaves are used for washing itches, while its fruits are used in curing dysentery. Its bark is mostly used as a vermifuge, while the taproots are used to treat high blood pressure, and the root bark is used for the treatment of diarrhea and fever (Shaheed et al. 2016). Previous articles have reported on the presents of quassinoids, quassinoids diterpenoids, eurycomaoside, tirucallane-type triterpenes, squalene derivatives, biphenylneolignans, eurycolactone, laurycolactone and eurycomalactone from its roots (Adiana & Zunoliza 2018).

Polyalthia bullata is frequently referred to as tongkat ali hitam or pasak bumi hitam due to the deeper hue of its roots and bark. *P. bullata* belongs to the genus *Polyalthia* and family Annonaceae. The plant can grow up to 2–3 metres, primarily in the lowlands of Peninsular Malaysia and the primary and secondary forests of Sabah (a state of Malaysia located within Borneo island). *P. bullata* is another aphrodisiac herb historically utilised predominantly in Malaysia. The root extract of *P. bullata* is often ingested as a decoction by most consumers and is readily accessible on the global market. It is also taken to increase sexual desire and as a general tonic for males (Fatinah et al. 2023).

Prismatomeris tetrandra or locally known

as tongkat haji samad or tongkat ali merah belongs to genus *Prismatomeris* and the family Rubiaceae, distributed in Sri Lanka, Myanmar, Vietnam, Peninsular Malaysia, Philippines and Indonesia. *Prismatomeris tetrandra* is a shrub or small tree up to 7 m tall; young branches with 2 prominent longitudinal ridges. It has dark grey brown colored bark. The roots of *P. tetrandra* were used in traditional Chinese medicine to treat leukocythemia, gum bleeding, hepatitis, and anemia. (Wan Mohd Nuzul Hakimi et al. 2019).

Fourier transform infrared (FT-IR) spectroscopy is one of the most used methods to identify the chemical constituents and elucidate the structures of compounds, and thus has been used as one of the requisite methods to identify medicines in the Pharmacopoeia of many countries. Owing to the fingerprint characters and extensive applicability to the samples, FT-IR has played an important role in pharmaceutical analysis in recent years (Tan et al. 2010). As long as the samples are different in components or contents, the differences will be embodied in their FT-IR spectra. The combined three steps of spectral analysis; FT-IR, second derivative IR spectroscopy and the two-dimensional infrared (2D-IR) correlation spectroscopy, can not only identify the major components in medicinal materials and their extracts but also able to find the tiny differences among similar samples (Hong et al. 2006). Likewise, the 2D-IR correlation spectroscopy is also used extensively in various research fields (Noda 1989). The 2D-IR correlation spectra will enhance the spectral resolution by spreading peaks over the second dimension, and simplify the complex spectra consisting of many overlapped peaks, as well as identify various inter- and intra-molecular interactions through selective correlation of IR peaks.

The use and duplication of local names that are often used among the community in Malaysia causes confusion in the identification of the identity of the plants used. This causes the actual benefit of the plant as a medicinal substance is not as expected. Furthermore, almost all raw materials come in dried or powdered form which necessitates identification by a chemical approach using chromatographic or spectroscopic procedures and chemical reactions. As we know that the chemical constituents in the medicinal materials are the substantial foundation of curative effect and the compositions are influenced by many factors, it is therefore necessary to establish a reliable and convenient analytical method to distinguish the three types of tongkat ali roots which is tongkat ali kuning (*E. longifolia*), tongkat ali hitam (*P. bullata*) and tongkat ali merah (*P. tetrandra*). This study aims to address this gap by developing and validating a reliable and straightforward method using FT-IR spectroscopy, including both 1D and 2D techniques, to ensure accurate identification and differentiation of these herbal materials. This technique can be developed as a new approach and simple operational procedures for easy identification or differentiation of herbs.

MATERIAL AND METHODS

Plant materials

Samples were collected from three sites namely Batu Kurau, Perak (*E. longifolia*), Pahang (*P. tetrandra*) and bukit Hari, Selangor (*P. bullata*). All three species have been confirmed by the botanists with specimen number; TA-BK-001 (*E. longifolia*), FRI45576 (*P. bullata*) and FRI54359 (*P. tetrandra*).

Equipment

IR spectra were recorded on a Spectrum 100 Fourier transform-infrared (FT-IR) spectrometer (Perkin Elmer, CA, USA), equipped with a mid-infrared deuterated triglycine sulphate (DTGS) detector. The spectra were obtained in the frequency range of 4000–450 cm^{-1} with a resolution of 4 cm^{-1} and with a total accumulation of 16 scans. Portable programmable temperature controller (4000 series™ High Stability Temperature Contoller, Specac, Ltd.) was used in the range of 50–120 °C.

Procedures

The dried roots were ground and sieved with 150 µm mesh. Exactly 2 mg of each sample was mixed with 100 mg of potassium bromide (KBr) powder and the mixture was further ground and pressed into a 13 mm diameter disc. 1D FT-IR spectra were recorded from a total of 16 scans

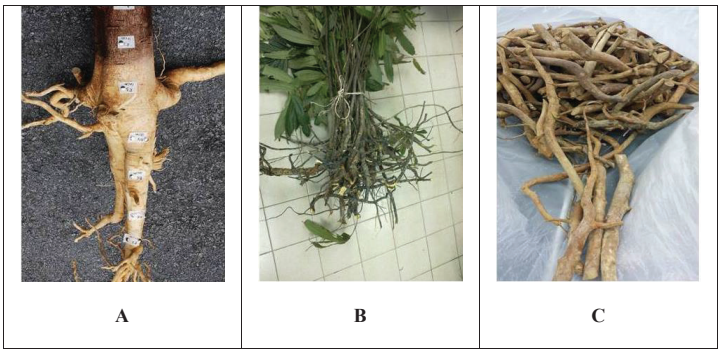

Figure 1 Roots of *E. longifolia* (A), *P. bullata* (B) and *P. tetrandra* (C)

in the 4000–450 cm^{-1} range with resolution of 4 cm^{-1}. The second derivative IR spectra were obtained by using Savitzky-Golay filter through 13-point smoothing. Savitzky-Golay smoothing aimed for minimum distortion by least squares fitting a cubic polynomial. For the measurement of 2D-IR spectra, each sample disc was put in the sample pool connected with a temperature controller. The dynamic 2D-IR spectra were collected at different temperatures from 50 to 120 °C at interval of 10 °C. 2D-IR correlation spectra were acquired by treatment of the series of temperature-dependant dynamic spectra with 2D-IR correlation analysis employing Softdoc software developed by Tsinghua University (Beijing, China). Each sample was analysed in triplicates.

RESULTS AND DISCUSSION

1D FT-IR spectral analysis

IR spectroscopy is a rapid and simple methodology which is non-destructive for the analytes. The entire chemical property of a sample can be revealed and shown in the IR spectrum for unique characterization (Fauziah et al. 2012). The comparison of IR spectra for the roots of *E. longifolia*, *P. bullata* and *P. tetrandra* is shown in Figure 2a. The 1D IR spectrum showed a total overlap of each absorption spectrum of various components. Generally, the three spectra showed similar IR absorption peaks stating that their chemical properties were not distinctively different. Nevertheless, some variations in term of peak positions and intensities could be observed among the samples in the expanded region of 1800–900 cm^{-1} (Figure 2b). The presence of peaks at 2855 cm^{-1} and 1076 cm^{-1} and a distinctive and sharp absorption peak at 1032 cm^{-1} in *P. bullata* clearly differentiated *P. bullata* from the other two species. While the presence of a peak at 1051 cm^{-1} in *P. tetrandra* also clearly differentiated it from the other two species. As shown in Figure 2a, the strongest absorption peak can be seen at 3364-3374 cm^{-1} assignable to the O-H stretching vibration. Another strong peak occurred at 1032-1035 cm^{-1} due mainly to the absorption of conjugated carbonyl group (C=O). The peaks at 2923-2926 cm^{-1} and 2855 cm^{-1} were due to the stretching vibration of C-H in saturated hydrocarbons. A previous study by Adiana and Zunoliza (2018) and Liling et al. 2021, also reported the presence of a strong peak at ~3400 cm^{-1} which belongs to the stretching

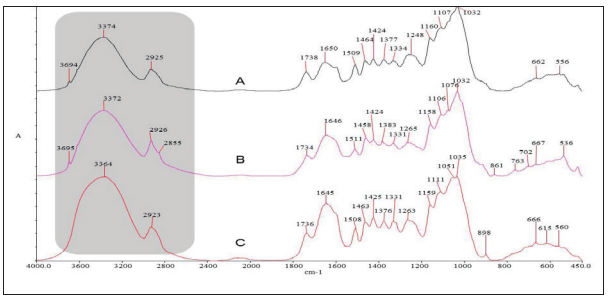

Figure 2a IR spectra of the roots of *E. longifolia* (A), *P. bullata* (B) and *P. tetrandra* (C)

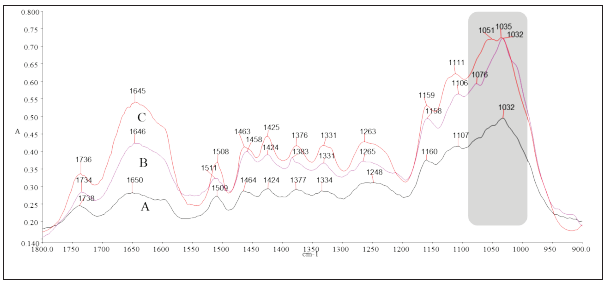

Figure 2b Expanded IR spectra in the region of of 1800–900 cm^{-1} for the roots of *E. longifolia* (A), *P. bullata* (B) and *P. tetrandra* (C)

vibration of the O-H group, the peak at ~2930 cm^{-1} is assigned to the stretching vibration of the saturated hydrocarbon group and a light absorption peak at 1155–1000 cm^{-1} mainly due to the C-O stretching vibration, which displays the absorption characteristics of glycosides. In addition, the peaks at ~1730 cm^{-1} are due to C=O stretching vibrations in compounds that include carbonyl groups. These data show that different types of carbonyl compounds with different quantities exist in the roots of *E. longifolia*. IR absorption of the above functional groups is also present for *P. bullata* and *P. tetrandra* but with different intensities. The IR absorption of different functional groups for the three species is tabulated in Table 1. Each band represents an overall overlap of some characteristic absorption peaks of functional groups in the sample.

Table 1 Preliminary assignment of IR spectra of *E. longifolia*, *P. bullata* and *P. tetrandra*

Base group and vibration mode	Main attribution	Wavenumber (cm^{-1})		
		E. longifolia	*P. bullata*	*P. tetrandra*
ν(O–H)	Hydroxyl	3374	3372	3364
ν_{as}(C–H)	Methylene	2925	2926	2923
ν_{s}(C–H)	Methylene	1738	2855	-
ν(C=O)	Ester, carbonyl, ketone	1650	1734	1736
ν(C=C)	Aromatic benzene ring	1509	1646	1645
ν(ar)	Flavonoids, aromatic ring	1424	1511	1508
δ(C–OH)	Methyl, flavonoids	1464	1434, 1458	1425, 1463
δ(C–OH)	Hydroxyl	1334, 1377	1331, 1383	1331, 1376
ν(C=O)	Phenolic hydroxyl	1248	1265	1263
δ(C–O)	Lipid, tertiary	1160	1158	1159
δ(C–OH)	alcohol groups			
δ(C–H)	Methyl or phenyl	1107	1106	1111
ν_{s}(C–O)	=C–O–C of aromatics	1032	1032, 1076	1035, 1051
γ(C–H)	End methylene	-	861	898
γ(C–H)	Benzene ring	556, 662	536, 667, 702, 763	560, 615, 666

ν= stretching or vibration, ν_{as} = asymmetrical, ν_{s} = symmetrical, ν_{rf} = ring frame,
δ = in plane deformation, ar = aromatic, ha = heteroaromatic

Second derivative spectral analysis

Second derivative IR spectra, the second step of multi-step IR macro-fingerprinting could enhance the spectral resolution by amplifying tiny differences in the IR spectrum (Fauziah et al. 2012, Tan et al. 2010). Some overlapped absorption peaks can be resolved by using the second derivative spectral analysis. Figures 3a and 3b show the second derivative spectra for the roots of *E. longifolia*, *P. bullata* and *P. tetrandra*. The spectral data in the region 1550–1180 cm^{-1} and 1180–850 cm^{-1} showed many dissimilarities between the three species. Some broad peaks in the IR spectrum included many second derivative peaks especially in the region of 1550–1180 cm^{-1}. Both of the regions showed that *E. longifolia* exhibited more peaks as compared to *P. bullata* and *P. tetrandra*. In the 1D-FTIR (Figure 2a), region of 1550–1180 cm^{-1} showed only six absorption peaks for every species. When the ID-FTIR was derived to second derivative (Figure 3a), more characteristic differences were featured in the spectrum. There was no evidence of characteristic peaks at 1400 cm^{-1}, 1356 cm^{-1}, 1345 cm^{-1}, 1303 cm^{-1}, 1281 cm^{-1}, 1247 cm^{-1}, 1236 cm^{-1}, 1204 cm^{-1} and 1193 cm^{-1} for the roots of *P. bullata* and *P. tetrandra* as compared to the root of *E. longifolia* while a sharp absorption peak appeared at 1384 cm^{-1} in the root of *P. bullata*. Another dissimilarity was observed in the intensity of absorption peaks between the three species. The peaks at 1317 cm^{-1} appeared in higher intensities for the root of *P. tetrandra* as compared to the other two species (Figure 3a). The spectra of *P. bullata* and *P. tetrandra* exhibited more or less absorption peaks except the peaks at 1007 cm^{-1} and 938 cm^{-1} which did not appear in the spectrum of *P. tetrandra*. In addition, the spectrum of *E. longifolia* showed the presence of peaks at 1150 cm^{-1}, 930cm^{-1}, 920 cm^{-1}, 888 cm^{-1} and 877 cm^{-1} which were not observed in the spectra of *P. bullata* and *P. tetrandra* (Figure 3b). Generally, the roots of *E. longifolia*, *P. bullata* and *P. tetrandra* can be distinguished from each other through the typical peaks in the secondary derivative spectra.

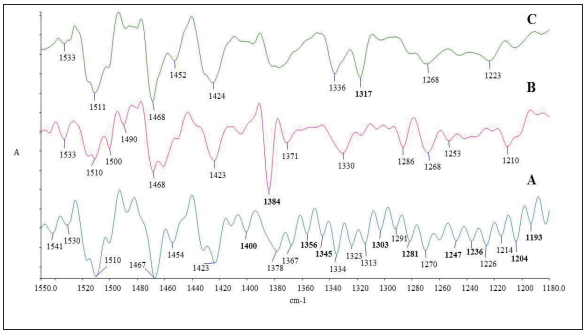

Figure 3a Second derivative IR spectra in the range of 1180–1550 cm^{-1} for the roots of *E. longifolia* (A), *P. bullata* (B) and *P. tetrandra* (C)

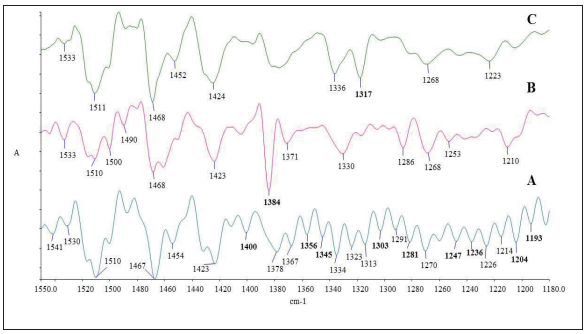

Figure 3b Second derivative IR spectra in the range of 850-1180cm^{-1} for the roots of *E. longifolia* (A), *P. bullata* (B) and *P. tetrandra* (C)

Two-dimensional correlation IR spectral analysis

The 2D correlation IR analysis is the study of spectrum through the vibration with external perturbation and is able to enhance the resolution of the spectra which cannot be acquired from the 1D FT-IR and second derivative IR spectra. Therefore, 2D correlation IR spectroscopy based on thermal perturbation reveals molecular vibrating behavior of relative group of molecules during the temperature perturbation (Fauziah et al. 2012, Tan et al. 2010). The 2D correlation synchronous and asynchronous plot is acquired from forward Fourier transform of dynamic spectrum at spectral variable and conjugate of the other Fourier transform of dynamic spectrum and calculated over the interval of thermal perturbation (Tan et al. 2010). In synchronous spectrum, the auto-peaks on the diagonal line show the self-correlativity and susceptibility of some normal vibration of functional groups with the increasing temperature. The cross-

peak located at the off-diagonal position reveals the relativity of intensity variations of a pair of group vibrations corresponding to their frequencies (Fauziah et al. 2012, Tan et al. 2010). Cross-peaks appear when the dynamic variations of the IR spectrum at two different wavenumbers are correlated or anti-correlated to each other. The positive cross-peak indicates the intensity variation from the two wave numbers with thermal perturbation which proceed in the same direction. In contrast, the negative cross peak means that the intensity variation are in opposite directions (the intensity of one wave number is increasing and the other one is decreasing). The more coincidence the reorientation direction is, the stronger the intensity of cross peaks will be. The absence of any cross peak indicates the lack of chemical coupling or interaction among various functional groups (Li et al. 2004).

2D-correlation infrared spectral analysis with thermal perturbation has been utilised for identification and classification of traditional medicine. It serves as a distinct fingerprint for the complex mixture of chemical components in herbs. In order to obtain enhanced spectral resolution, we carried out the synchronous 2D-IR spectroscopy under thermal perturbation from 50 °C to 120 °C. The synchronous 2D-IR spectroscopy analysis further differentiated the roots of *E. longifolia*, *P. bullata* and *P. tetrandra*. Figure 4 presents the contour plot, auto peaks and the mesh plot for the three species in the range of 800–1000 cm^{-1}. Comparison based on the positions of auto-peaks as the third step of identification, revealed the presence of the strongest auto-peak at 1649 cm^{-1} for *E. longifolia* and *P. bullata* and 1651 cm^{-1} for *P. tetrandra*. There were nine obvious auto-peaks for *E. longifolia* while five obvious auto-peaks were observed in *P. bullata* and *P. tetrandra* (Table 2). However, there were dissimilarities in the signal intensities for each peak. The 2D correlation IR synchronous spectra showed a strong cross-peak at (1501 cm^{-1}, 1198 cm^{-1}) for *E. longifolia* which was not present in *P. bullata* and *P. tetrandra*. Therefore, the 2D-correlation FTIR base on thermal perturbation is distinctive and can be used as a fingerprint for differentiating the roots of *E. longifolia*, *P. bullata* and *P. tetrandra*.

Table 2 Characteristic auto-peaks in the range of 1800–1000 cm^{-1} for the roots of *E. longifolia*, *P. bullata* and *P. tetrandra*

Peak number	Wavenumber (cm^{-1})		
	E. longifolia	*P. bullata*	*P. tetrandra*
1	1068	-	-
2	1098	1100	1100
3	1148	1138	-
4	-	1190	-
5	1209	1219	1211
6	1311	-	-
7	1351	-	1309
8	1408	-	-
9	1500	-	1488
10	1649	1649	1651

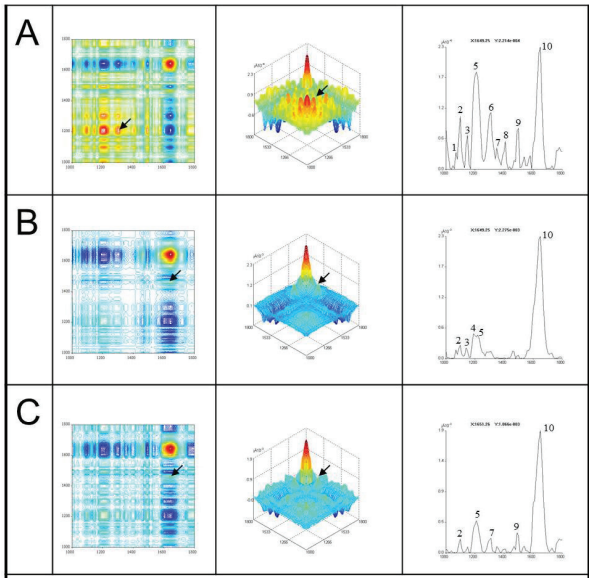

Figure 4 Synchronous 2D IR contour plot (left), synchronous 2D IR auto-peaks (middle) and synchronous 2D IR mesh plot (right) in the range of 1800-1000 cm^{-1} for *E. longifolia* (A), *P. bullata* (B) and *P. tetrandra* (C). Cross-peaks are indicated by the arrows

CONCLUSION

This study demonstrated that while the 1D IR spectral features of *E. longifolia*, *P. bullata*, and *P. tetrandra* exhibited subtle variations, their second derivative spectra revealed more pronounced differences. The 2D correlation IR spectral analysis under thermal perturbation further elucidated their similarities and discrepancies. These findings underscore the efficacy of combining FT-IR with second derivative IR spectroscopy and 2D correlation IR spectroscopy as a robust, non-destructive tool for the rigorous quality control of herbal materials. Nonetheless, to ensure absolute and unequivocal identification, morphological analysis should precede this advanced spectroscopic approach.

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