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Chromatographic Fingerprint and Chemometric Approach for Quality Control of Tongkat Ali (*Eurycoma longifolia*)

Nor Nasriah Zaini[1], Mardiana Saaid[1], Hafizan Juahir[2] and Rozita Osman[1]*

[1]Faculty of Applied Sciences, Universiti Teknologi MARA (UiTM), 40450 Shah Alam, Selangor, Malaysia
[2]East Coast Environmental Research Institute, Universiti Sultan Zainal Abidin (UNISZA), 21300 Kuala Terengganu, Malaysia

ABSTRACT

Tongkat Ali (*Eurycoma longifolia*) is one of the most popular tropical herbal plants as it is believed to enhance virility and sexual prowess. This study looked examined chromatographic fingerprint of Tongkat Ali roots and its products generated using online solid phase-extraction liquid chromatography (SPE-LC) combined with chemometric approaches. The aim was to determine its quality. Pressurised liquid extraction (PLE) technique was used prior to online SPE-LC using polystyrene divinyl benzene (PSDVB) and C18 columns. Seventeen Tongkat Ali roots and 10 products (capsules) were analysed. Chromatographic dataset was subjected to chemometric techniques, namely cluster analysis (CA), discriminant analysis (DA) and principal component analysis (PCA) using 37 selected peaks. The samples were grouped into three clusters based on their quality. The PCA resulted in 11 latent factors describing 90.8% of the whole variance. Pattern matching analysis showed no significant difference ($p>0.05$) between the roots and products within the same CA grouping. The findings showed the combination of chromatographic fingerprint and chemometric techniques provided comprehensive evaluation for efficient quality control of Tongkat Ali formulation.

Keywords: Chemometric, chromatographic fingerprint, cluster analysis, discriminant analysis, eurycomanone, online SPE-LC, principal component analysis

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E-mail addresses:
nasriah_zaini@yahoo.com (Nor Nasriah Zaini),
mardiana6951@salam.uitm.edu.my (Mardiana Saaid),
hafizanjuahir@unisza.edu.my (Hafizan Juahir),
rozit471@salam.uitm.edu.my (Rozita Osman)
*Corresponding Author

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INTRODUCTION

Recently, quality control of herbal medicines has become a major concern in consumer protection (Bhat & Karim, 2010). Tongkat Ali is one of the most well-known traditional herbs and it is attracting researchers' interest due to its medicinal values (Mohamad et al., 2013). Tongkat Ali has active chemical

components such as quassinoids which contribute to its bitter taste. The five quassinoids markers are eurycomanone, eurycomanol, eurycomanol-2-O-β-D-glucopyranoside, 13,21-dihydroeurycomanone, and 13α (21)-epoxyeurycomanone (Teh et al., 2011).

Currently, marker compounds are used in quality control and authentication of herbal products (Wang et al., 2015; Li et al., 2010). However, the use of a single compound may not be able to evaluate the quality consistency of herbal products (Li et al., 2010). Thus, it is important to provide a more comprehensive chemical profile of a sample for quality control purposes. Chromatographic fingerprint technology has been accepted by leading organisations such as the Federal Drug Administration (FDA), World Health Organization (WHO) and the British Herbal Medicine Association (Zaini et al., 2016).

In this study, online SPE-LC method was used in obtaining fast and comprehensive chromatographic fingerprint of Tongkat Ali. The chromatographic dataset was subjected to chemometric techniques by means of multivariate statistical analysis. Unsupervised pattern recognition techniques, principal component analysis (PCA) and cluster analysis (CA) were utilised for data visualisation by observing the relationship between samples and variables with no predetermined class. Supervised pattern recognition, discriminant analysis (DA) and pattern matching analysis were employed in supporting the clusters obtained by CA.

METHOD

Online Solid Phase Extraction-Liquid Chromatography (SPE-LC)

A Dionex Ultimate 3000 Liquid Chromatography system equipped with diode array detector (DAD) was used for online SPE-LC analysis, performed utilising polystyrene divinyl benzene (PSDVB) and C18 column by two pumps (right and left) operated simultaneously. Solvents carried by right pump were acetonitrile, methanol and ultrapure water, while left pump were 5% ultrapure water and 95% Methanesulfonic acid (MSA). The identification of eurycomanone and eurycomanol were performed using Q Exactive Plus Liquid Chromatography-Mass Spectrometer (Thermo Fisher Scientific). For root samples, extraction was done using pressurised liquid extraction (Osman et al., 2016) prior to online SPE-LC.

Chemometric Analysis

The chemometric analysis was done using XLSTAT Software (XLSTAT, 2015, Addinsoft, New York, NY, USA) for statistical analysis. A total 37 peaks were chosen and their areas were used as variables.

Cluster Analysis (CA). CA is used for classification of variables into clusters with high similarities within the class and high dissimilarity between different classes.

Discriminant Analysis (DA). Subsequently, DA was applied to confirm the results of the CA analysis. DA specifies the variables that discriminate between two or more clusters obtained from CA (Goncalves et al., 2006). DA was performed to the raw data in standard, stepwise forward and stepwise backward modes.

Principal Component Analysis (PCA). PCA provides information on the most significant factors that explain the total dataset by excluding the less meaningful factors with a minimum loss of original information (Goncalves et al., 2006). The PCA helps to determine in what respect one sample is different from another and which variables contribute most to this difference. It also helps to find out which variables contribute most to this difference, and whether those variables contribute in the same way (positively correlated) or are inversely correlated (Saim et al., 2009).

Pattern Matching. Pattern matching was conducted by plotting 2D-chromatograms. T-statistics was used to evaluate whether two groups differ from one another for a tested variable.

RESULTS AND DISCUSSION

Chromatographic Fingerprint

Chromatographic fingerprints of four representative Tongkat Ali root samples from different sources in Malaysia and four of its products (Figure 1) were obtained using SPE-LC (Zaini et al., 2016). The main quassinoids of Tongkat Ali are eurycomanone and eurycomanol (Hajjouli et al., 2014), eluted at retention times of 5.6 and 5.7 minutes respectively. The chromatographic fingerprint clearly showed similarities and dissimilarities among the samples.

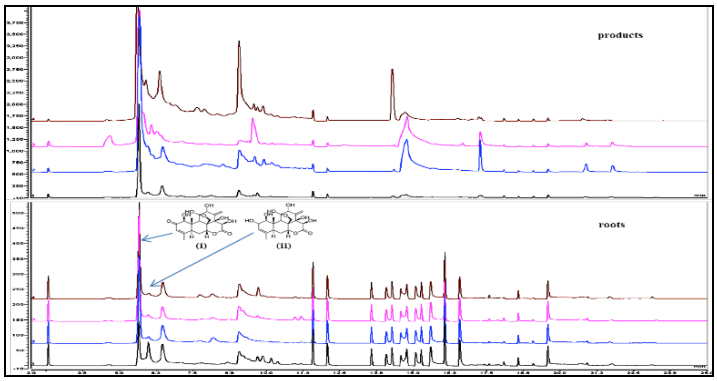

Figure 1. Chromatographic fingerprint selected Tongkat Ali roots and products: (i) eurycomanone; and (ii) eurycomanol

Chemometric Analysis

In this study, 17 Tongkat Ali root samples (R1 to R17) from four states in Malaysia - R1 (Sarawak), R2 to R8 (Pahang), R9 to R13 (Kedah) and R14-R17 (Perak) - and 10 products (capsules) from various manufacturers were analysed. In order to perform chemometric analysis, 37 variables based of reproducible peak areas were selected.

Cluster Analysis (CA). The dendrogram (Figure 2) clearly showed that all samples were formed into three clusters. All root samples from Kedah were clustered in cluster II. In addition, five

root samples from Pahang and three from Perak were clustered in cluster III. However, two samples from Pahang were separated and clustered into cluster I, suggesting their dissimilarity in chemical constituents. The composition of Tongkat Ali may vary depending on cultivation conditions, maturity, soil properties, harvesting age, storage, and processing temperature (Li et al., 2010).

The clustering of products in the same group with the roots of Tongkat Ali may suggest that they were from the same source. As the products are formulated using Tongkat Ali extract, the extraction solvent used may affect the fingerprint composition (Locatelli et al., 2012). The CA provides initial evaluation based on dissimilarity between samples but it does not show details of these differences. Thus, CA should be confirmed by DA and PCA.

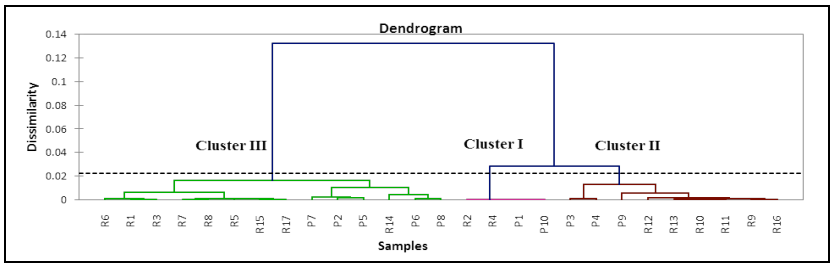

Figure 2. Dendrogram showing the cluster of Tongkat Ali roots and products

Discriminant Analysis (DA). In DA, the three clusters (CI, CII and CIII) obtained from CA in addition to sources or locations of Tongkat Ali were used as dependent variables. Peak areas of chromatographic fingerprints were the independent variables. The DA showed that each group differed from the others in terms of different compositions (Al-Odaini et al., 2012). The results from standard, stepwise forward and stepwise backward modes gave 100% correct classification based on the confusion matrix of the estimation sample (Figure 3).

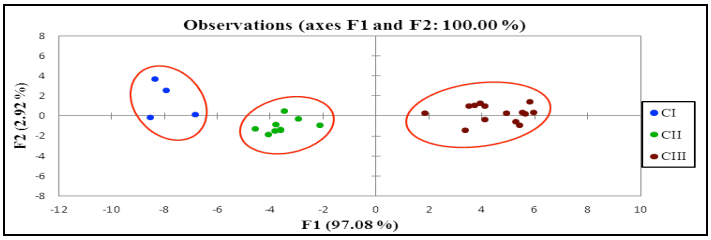

Figure 3. Plot of discriminant functions showing three clusters of Tongkat Ali roots

Stepwise backward mode yielded 100% correctly assigned with 19 discriminant peaks or variables whereas stepwise forward mode contributed 100% with only one discriminant peak. Stepwise forward mode yielded little difference in matching for each sample compared

with the stepwise backward mode. The discriminant variable was determined by the value of p-value. Variable with p-value of <0.05 was considered as discriminant variable. Although some compounds have p-values of >0.05, they are still able to discriminate Tongkat Ali. Therefore, result obtained by stepwise backward mode was chosen, suggesting all 19 discriminant variables (Table 1) as significant in discriminating the quality of Tongkat Ali whereas the remaining 18 peaks did not correlate significantly in discriminating the Tongkat Ali. As shown in Table 1, A10 has the highest discriminant capacity (F) followed by A27 probably because the compounds have high variations of peak areas between clusters (Al-Odaini et al., 2011).

The Wilks' Lambda value test gives Lambda value of 0.054 and p<0.0001. The null hypothesis stated that the mean vectors of the three clusters (roots and products) are equal. The alternative hypothesis, alongside, stated that at least one of the mean vectors is different from another. Since the computed p-value is lower than the significance level of alpha = 0.05 (at 95% confidence level), one should reject the null hypothesis and accept the alternative hypothesis. The risk to reject the null hypothesis while it is true is lower than 0.01%. Thus, the three clusters are indeed different from one another.

Table 1
Wilks' lambda and F test of group means

Variable	Lambda	F	p-value
A3	0.832	2.116	0.146
A6	0.958	0.459	0.638
A7	0.864	1.656	0.215
A8	0.781	2.951	0.074
A10	0.457	12.461	0.000
A12	0.691	4.691	0.021
A14	0.893	1.255	0.306
A15	0.855	1.784	0.193
A16	0.805	2.541	0.103
A21	0.886	1.358	0.279
A25	0.960	0.440	0.650
A26	0.838	2.033	0.156
A27	0.590	7.308	0.004
A28	0.770	3.138	0.064
A31	0.788	2.821	0.082
A32	0.722	4.041	0.033
A33	0.952	0.531	0.596
A34	0.755	3.408	0.052
A35	0.829	2.166	0.140

Principal Component Analysis. In this study, the peak areas of 37 selected peaks were treated as variables. The principal components (PCs) generated by PCA are sometimes not readily

interpreted (Al-Odaini et al., 2011). Varimax rotations applied on the PCs with eigenvalues more than 1 are considered significant (Baharudin et al., 2014) in order to obtain new groups of variables called varimax factors (VFs).

The first two PCs (PC1 and PC2) were selected to provide the highest variation of data objects (31.15% and 9.30% of the variation). Figure 4 showed that the Tongkat Ali roots and products were distinctively separated. The result suggested that roots and products have their own unique chemical compositions.

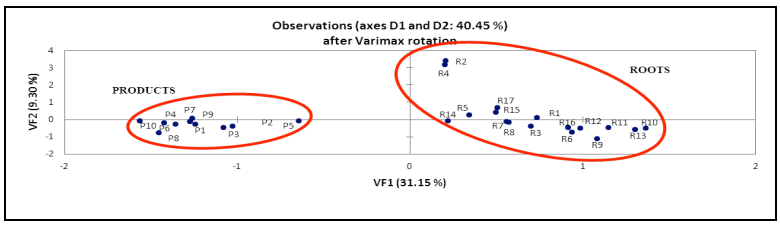

Figure 4. PCA plot of Tongkat Ali

Based on PCA analysis results, only the first 11 PCs with eigenvalue > 1 were considered to perform varimax rotations. The parameter loading for the 11 components from PCA of the data set after varimax rotation is given in Table 2. The results explain approximately 90.8% of the total variances, where a correlation greater than 0.75 is considered "strong"; 0.50-0.75, "moderate"; and 0.30-0.50 as "weak" significant factor loading. The higher the loading of the variable (either positive or negative), the more that variable contributes to the variatio accounted for the particular varifactors (Baharuddin et al., 2014). Table 2 shows factor loadings of PCA, the first VF explained total variances of 31.15 % with 9 strong and 9 moderate factor loadings. The VF2 contributed 9.30% of data variability. The third factor described 6.17% of the variance in the dataset. VF4, VF5 and VF6 accounted for 7.46%, 5.25% and 4.38% of the total variances respectively. VF7 and VF8 exhibited moderate loadings for eurycomanol and eurycomanone, the major quassinoids in Tongkat Ali by 8.08% and 4.74% of the total variance respectively. VF9, VF10, VF11 demonstrated 4.78%, 5.29% and 4.16% respectively.

Table 2
Factor loadings of PCA

Peaks	VF1	VF2	VF3	VF4	VF5	VF6	VF7	VF8	VF9	VF10	VF11
A1	-0.0559	0.1851	0.1629	0.2429	0.4189	0.0921	-0.3546	-0.6374	-0.1140	-0.1312	-0.0943
A2	0.2518	0.1959	0.0133	0.0547	0.1717	0.0409	0.7990	-0.1161	-0.3104	0.0055	-0.0721
A3	0.5736	0.1312	-0.1392	0.2513	-0.0202	0.2125	0.4088	-0.0823	0.1400	-0.1737	0.2979
A4	-0.1058	-0.0367	-0.0105	-0.8932	0.0471	0.0791	-0.0564	-0.2897	-0.0395	-0.0973	0.0039
A5	0.6013	-0.1841	0.1464	0.1921	0.0747	0.1205	-0.1873	-0.0697	0.1360	-0.0654	-0.6005
A6	-0.5939	0.2451	-0.0799	0.2395	0.1549	0.2340	0.1012	-0.0019	0.1124	0.1231	0.5856
A7	-0.2401	-0.0387	-0.0691	0.1644	0.0180	0.2493	0.6995	-0.1903	0.2995	-0.0490	0.2014
A8	0.1841	-0.2554	-0.1705	0.0358	-0.7123	0.1223	-0.2068	0.0433	0.1344	0.3249	-0.1478
A9	0.0280	0.0311	0.9656	0.0414	0.0136	-0.0739	-0.0444	-0.0176	0.0564	0.0035	-0.1361
A10	0.5466	-0.4900	-0.1548	0.0966	0.1185	0.2093	-0.1330	-0.0130	0.1353	-0.2266	0.5009
A11	0.1166	0.9340	-0.1059	0.0133	0.0474	-0.0264	0.2387	0.0028	0.0740	0.0026	0.1410
A12	0.8124	0.1527	0.2146	0.2751	0.1561	0.2761	0.0683	-0.0685	0.0391	-0.0476	0.1007
A13	-0.2030	0.1369	0.1633	0.1124	-0.8834	0.0019	-0.1251	-0.0990	0.0193	-0.1055	0.0251
A14	-0.3219	-0.0534	0.0923	0.0554	0.0786	-0.8262	-0.0026	-0.1723	0.0479	0.0230	-0.0443
A15	0.5058	0.0025	-0.1935	0.1254	0.2499	0.0850	0.2868	0.0621	0.3986	-0.4032	0.0585
A16	0.1153	0.1725	-0.0395	0.0193	0.0873	-0.0781	0.9359	0.0387	0.0602	-0.0526	0.0283
A17	0.0812	0.6544	0.6733	0.0605	-0.1119	0.0568	0.0031	-0.1149	0.1628	-0.0773	-0.0295
A18	0.1533	0.9330	0.1560	0.0642	-0.0391	0.0749	-0.0236	-0.0721	-0.0515	-0.0079	0.0098
A19	0.4409	0.0706	-0.1070	0.2915	-0.0118	-0.5805	-0.1034	0.4437	-0.0252	0.0082	-0.0217
A20	0.9648	0.1444	-0.0239	0.0521	0.0032	0.1162	0.0202	0.0448	-0.0245	-0.0063	-0.0422
A21	-0.8519	-0.1984	-0.0734	0.2112	0.0098	0.2791	-0.0363	0.2074	0.0326	0.1044	0.1208
A22	0.9137	0.0131	-0.0218	0.1873	-0.1555	-0.0836	0.0688	0.1385	0.0171	0.1863	-0.0707

Table 2 *(continue)*

A23	0.9478	0.0469	-0.0020	0.1705	-0.0254	-0.0076	-0.0009	0.0741	-0.1365	-0.0057	-0.0618
A24	0.9723	0.1068	0.0421	0.1154	0.0556	0.0610	0.0373	0.0639	-0.0468	0.0022	-0.0136
A25	0.0739	-0.0811	-0.0243	0.0722	0.2282	0.1506	-0.2751	0.8164	-0.0514	0.0732	-0.0025
A26	0.7330	0.1786	-0.0378	0.0099	0.0128	-0.0749	0.3685	0.1274	0.0665	-0.1054	0.4184
A27	0.9033	-0.0521	-0.0049	0.1106	0.0700	0.1042	-0.0716	0.1107	-0.0795	0.1467	-0.1358
A28	0.8903	-0.0763	0.1320	0.0631	0.0628	0.0330	0.1711	0.0739	-0.2336	0.2426	-0.0321
A29	0.2920	0.6308	-0.2165	0.1089	0.2163	-0.0548	0.2923	-0.0554	0.2921	-0.3485	-0.0970
A30	0.3632	-0.1568	0.6715	0.0888	-0.0708	0.0514	-0.0844	-0.0632	-0.4689	-0.2315	0.1795
A31	0.5832	0.0452	-0.1874	-0.0672	-0.0035	0.0267	-0.0907	0.1265	0.0016	0.7205	-0.0706
A32	-0.8568	-0.2240	-0.1044	-0.2800	-0.1767	-0.0864	-0.0933	0.2013	0.0669	0.0832	-0.0372
A33	0.2207	-0.1127	-0.0652	0.0847	0.1229	0.0270	0.0158	0.0102	-0.8921	-0.0516	-0.0409
A34	0.6654	-0.1733	-0.0846	-0.0963	0.0441	-0.0078	0.0605	0.1024	0.2142	0.6131	0.0531
A35	-0.5441	0.0636	-0.0656	-0.7288	0.1119	0.0150	-0.0104	0.1701	0.1468	-0.0450	-0.0480
A36	-0.2757	-0.1398	-0.0934	-0.8302	0.0096	0.0217	-0.1010	0.2316	0.0629	0.2537	-0.0209
A37	-0.2447	-0.1875	-0.0636	0.1242	-0.2906	-0.2963	-0.1827	0.0600	0.0794	0.4980	0.2972
Eigenvalue	12.2650	4.7826	3.3002	2.4682	2.3612	1.7287	1.6942	1.4975	1.2784	1.1337	1.0706
Variability %	31.1496	9.2995	6.1749	7.4550	5.2509	4.3818	8.0785	4.7386	4.7754	5.2906	4.1622
Cumulative %	31.1496	40.4491	46.6240	54.0790	59.3299	63.7118	71.7902	76.5288	81.3042	86.5949	90.7571

Note: Strong loadings (> 0.75) are shown in bold; moderate loading (0.5-0.75) in italic bold; weak loading (< 0.50)

Pattern Matching. Pattern matching is used in comparing the percentage similarities among the roots of Tongkat Ali and its products. Peak areas of 58 peaks were used as variables. Figure 5 (a) shows 2D chromatogram of Tongkat Ali root and product within the same cluster (R6 and P8 of cluster III) while Figure 5 (b) shows the 2D chromatogram of Tongkat Ali root and product of different clusters (R6 of cluster III, P1 of cluster I). At 95% confidence level, the computed p-value is greater than the significance level alpha = 0.05 for both groups (within same cluster and of different clusters). However, within the same cluster, a high percentage similarity (92.6 %) was obtained compared with that of different clusters (88.0 %).

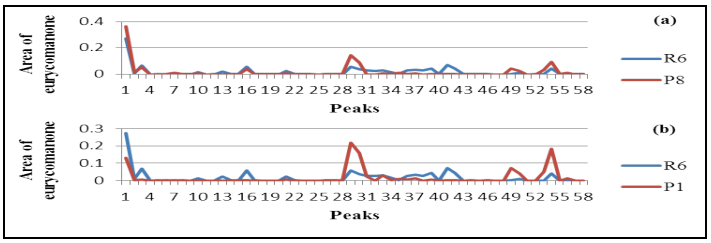

Figure 5. 2D chromatograms of representative Tongkat Ali roots and products: (a) within same cluster; and (b) of different clusters

CONCLUSIONS

A fast, reliable and comprehensive chromatographic fingerprint of Tongkat Ali root was obtained using an online SPE-LC method. Chemometric techniques were applied on Tongkat Ali datasets to show the relationship between variables. The CA was useful in showing similarities among the roots and products forming three clusters. DA confirmed the results of CA and yielded 100% correct assignation with 19 discriminant compounds while PCA is able to differentiate between roots and products, resulted in 11 varifactors with a total variance of 90.8 %. For pattern matching, the root and product within the same cluster have high percentage of similarity, 92.6 %. The results showed that chromatographic fingerprint of Tongkat Ali obtained using online SPE-LC combined with chemometrics could be a promising approach for quality control of herbal formulation.

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