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GC-MS based metabolomics and multivariate statistical analysis of *Wedelia trilobata* extracts for the identification of potential phytochemical properties

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Abstract

Wedelia trilobata is known as a noxious weed with potential pharmaceutical properties that can be used to treat inflammation and bacterial infections. Despite its impacts and potentials, the characterization of *W. trilobata*'s metabolite profiles using metabolomics approach has never been described. In this study, we used a non-targeted gas chromatography mass spectrometer (GC-MS) and multivariate statistical analysis (MVA) to determine the metabolic content of *W. trilobata*. Metabolite extraction was carried out using solvents of methanol/water, methanol/chloroform/water, ethanol and water. Unsupervised principle component analysis (PCA) and partial least square discriminant analysis (PLSDA) were applied to evaluate grouping trends between the different solvents extracts. Upon evaluation of four different extraction solvents systems, ethanol was found to have good extraction efficiency based on metabolites contribution and separation trend observed in PCA and PLSDA. Variable importance in projection (VIP) scores revealed that separation between solvents extract were largely contributed by monosaccharides and diterpenes of resin acids of 13-*cis*-retinoid acid and isopimaric acid. High abundance of resin acids in *W. trilobata* suggested potential allelopathy properties that can have beneficial herbicides. This study presents a simple non-targeted metabolomics approach to determine the metabolite differences in *W. trilobata*. The findings can be used to further optimise metabolite extraction from *W. trilobata*.

Keywords: Wedelia trilobata, metabolomics, GC-MS, allelopathy

Introduction

Plants are considered as sources of the most complex individual mixtures (Hall et al., 2002). Plants contain a plethora of phytochemicals that are useful to prevent and treat human diseases. In order to explain its medicinal value, the bioactive compounds found in plants require comprehensive identification and characterization. This process is an ongoing challenge, which requires unbiased extraction methods to extract metabolites and sophisticated analytical platforms to detect and identify metabolites (Wolfender et al., 2015). Analytical strategies with application to natural extracts in particular have been used to provide an overview of the chemical profiles, a field often referred to as metabolomics. Metabolomics approach offers a holistic overview of the biochemical processes through the study of interaction, changes and levels of low molecular weight metabolites. The information may also help to understand the plant's biological aspects, such as growth and development, responses to external stresses, defence and productivity (Desai and Alexander, 2013; Misra et al., 2014). Gas chromatography (GC) coupled with mass spectrometry (MS) is preferable for quantitative and qualitative analysis of metabolites especially from plant extracts (Wong et al., 2015). GC-MS is useful to gain an overview of metabolite profiles as the tool can identify up to a hundred primary metabolites in simple plant extracts (Obata et al., 2013; Lee et al., 2014). Furthermore, the availability of a large GC-MS electron impact (EI) mass spectral library has allowed better and effective identification of metabolites (Musharraf et al., 2013). Apart from the analytical platform, sample preparation strategy plays an important role in metabolomics. An optimal extraction method is a major factor that affects the array of metabolite profiling (t'Kindt et al., 2009; Ying et al., 2009; Musharraf et al., 2013). Metabolites are extracted using organic solvents such as methanol (Lapornik et al., 2005), ethanol (Amzad Hossain and Shah, 2015) and water coupled with conventional extraction methods such as soxhlet, reflux or solvent precipitation, followed by lyophilization and subsequently analysed using analytical platforms. A combination of different organic solvents allows better extraction of hydrophilic metabolites (Lin et al., 2007). The efficiency of solvent and method used to extract metabolites are also dependent on the crude samples and thus may affect the total metabolic content. On the other hand, the application of temperature may also aid in the extraction efficiencies of metabolites (t'Kindt et al., 2009). *Wedelia trilobata* is a member of the *Asteraceae* family that is widely distributed in many wet tropical areas. *W. trilobata* is considered as a noxious weed and fast-growing mat-forming perennial herb. The plant is known for its beautiful yellow flower and has been widely used as decorative groundcover in gardens, paths and in public areas (Huang et al., 2006). Like other genera of *Wedelia*, *W. trilobata* has been widely used in the folk medicine of many countries to treat a variety of diseases, such as headaches, fevers, and infections. *W. trilobata* has been reported to contain potential bioactive compounds, such as diterpenoids, sesquiterpene lactone and phytosteriods, which exert important biological activities towards cancer, microbial infection and inflammation (Balekar et al., 2012a; Balekar et al., 2012b; Keerthiga et al., 2012; Ren et al., 2015). Although previous studies for the phytochemical analysis of

W. trilobata have been reported, comprehensive metabolites analysis using GC-MS based metabolomics has never been reported. Since *W. trilobata* exhibited various medicinal properties, this study further evaluated the metabolic content of *W. trilobata* using non-targeted metabolite profiling approach and GC-MS analysis. The study aims to determine the metabolite composition of different solvents extracts of *W. trilobata* by using GC-MS. Briefly, extraction using different solvents was carried out and compared using principal component analysis (PCA) and partial least squares discriminant analysis (PLSDA). PCA and PLSDA were used to determine phenotypic variation and relationships between metabolite contents. Finally, the integration of metabolomics and chemometric revealed bioactive metabolites that have important implications towards the survival of *W. trilobata*.

Results

Multivariate statistical analysis of metabolite profiling

Metabolic content of *W. trilobata*, extracted with methanol/water (A), ethanol (B) methanol/chloroform/water (C), and water (D) were analysed using GC-MS for the first time. GC-MS analysis of the solvents extract led to the tentative identification of 63 compounds with significance ($p \leq 0.05$) (Table 1). Metabolites were tentatively identified by comparing MS spectra with National Institute of Standards and Technology (NIST) library with cut off probability of 800. In order to assess the influence of the solvent extraction on overall metabolites, the data matrices were subjected to principal component analysis (PCA) and partial least square discriminant analysis (PLSDA). Unsupervised PCA provides an unbiased and minimum loss of multivariate information without specifying the different sample types. Initially, PCA score plot separates samples based on their metabolite profiles. The PCA score plot may also be used to screen outliers. The PCA loading plot indicates the discernment of metabolites that contributes to the differences among samples on the score plot. Fig. 1A describes the PCA score plot of different solvents extractions. The separation between solvents system was achieved using the first two principal components (PCs) (PC1 versus PC2) with a total variance of 96.5%. PC1 was the highest variance (95.2%) while PC2 was the greatest variance (1.3%) in subspace perpendicular to PC1. For further consideration of model quality, R^2X, R^2Y and Q^2 (cumulative) values were used. R^2 explains the degree of agreement between the derived model and the data. Q^2 determines the predictive ability of the derived model. The overall pattern demonstrates clear separation between the solvents extract with R^2X and Q^2 (cumulative) value of 96.5% and 94.2% respectively. The R^2X and Q^2 (cumulative) values close to 1 suggest a better fitting and more accurate model (Yamamoto et al., 2012). The PCA loading plot in Fig. 1B shows that PC1 was dominated largely by galactose. PC2 was dominated by monosaccharides and resin acids of 13-*cis*-retinoid acid and isopimaric acid. From the score of PCA, clear separation between solvents was achieved. A showed the highest reproducibility, followed by D, C and B. A was mainly influenced by resin acids whereas B was affected largely by D-galactose. Meanwhile, C was affected by the number of metabolites. Separation of D was driven by 2-butanal.

Supervised PLSDA

Subsequently, PLSDA was carried out to further classify the metabolic trajectories associated with different solvents extraction. PLSDA is a supervised analysis with a more abundant and accurate information in regards to the discrimination and biomarker selection. Compared to PCA, PLSDA offers variable importance in projection (VIP) values and S-plot to find out which metabolites have the most significant influence on the group. Specifically, metabolites with higher VIP value are regarded as potential biomarkers. Generally, PCA selects axes that best represent the data cluster whereas PLSDA selects axes that maximize discrimination between classes (Robel and Kristo 2008). The PLSDA model illustrated in Fig. 2 shows the first two latent variables (LVs) (LV1 versus LV2) to explain the separation between solvents extract. A total variance of 95.4% (R^2X) was achieved with R^2Y and Q^2 (cumulative) value exceeding 50% (R^2Y = 93.4%, Q^2 (cumulative) = 80.7%). PLSDA revealed a clustering trend between A and D. On the contrary, score plot of PCA showed clear separation between solvents. Comparison between the score plot of PCA and PLSDA showed B was separated along PC2 in PCA and along LV2 in PLSDA. Loading plot of PLSDA confirmed that the separation of B was mainly influenced by galactose. Meanwhile, the clustering trend of A and D was likely to be influenced by water extract, which is contributed largely by ribose and fructose. The interpretation of PLSDA model was further carried out using VIP scores. Fig. 3 shows metabolites with highest VIP scores according to solvents extract. As shown in Fig. 3, the VIP scores showed distinguishable peaks that differentiate each solvent system. The highest VIP scores was observed in B and C (D-galactose VIP = 4.5). Interestingly, the resin acids, 13-*cis*-retinoic acid and isopimaric acid showed high VIP scores in B but observation on the loading plot (Fig. 2a and 2b) revealed that both compounds greatly influenced the separation of A. D-Galactose showed the highest VIP scores in all solvents extracts and greatly influenced B. Effects of monosaccharides (glucose, mannose, and fructose) were predominantly in D followed by A and C. It can be concluded that B showed consistent separation but with less influence of metabolites. The main metabolites that influenced B were organic acids of malic acid and ribonic acid. D showed good separation in PCA score plot but the discrimination was influenced mainly by monosaccharides. VIP scores of metabolites that influenced A were relatively the same as D whereas C showed the lowest VIP scores of metabolites.

Discussion

Global metabolite profiling using different solvent extractions and GC-MS

For global metabolomics approach, the combination of solvents such as methanol/water or methanol/chloroform/water is favoured (Rowan, 2011; Rohloff, 2015). Combination of polar (water) and nonpolar (organic solvent) increases the metabolite coverage and could also reduce variations that can occur when trying to combine metabolite information from separate samples (Tambellini et al., 2013; Mushtaq et al., 2014). Briefly, methanol was used because methanol extract is more active than other extracts and is known to have good extraction efficiency for various samples (Darah et al., 2013). Ethanol was employed as previous reports revealed antimicrobial (Shankar and Thomas, 2014), wound healing (Balekar et al., 2012a) and anti-inflammatory activities (Govindappa et al., 2011) from the ethanolic extract of *W. trilobata*. Water, on the other hand, is recognised as a universal solvent and has been used to extract plant samples. Since each extraction solvent has its own chemical and physical

Table 1. Chemical composition of *W. trilobata* leaves, detected using GC-MS. Tentative identification was carried out using NIST library spectral.

rT (min)	Retention Indices (RI)	Formula	*p*-value (≤0.05)	Name	KEGG
8.1	-	$C_2H_2Cl_2$	<0.0001	Vinylidene cyanide	
8.14	-	$C_{12}H_{22}OSi$	<0.0001	3-Methyl-3-vinyl-1-cyclopropene	
8.19	-	$C_3H_6ClNO_2$	<0.0001	1-Chloro-2-nitropropane	
8.32	-	$C_2H_3ClO_2$	<0.0001	Chloracetic anhydride	
8.37	-	C_7H_8O	<0.0001	Hexahydro-1ah-1-oxadicyclopropa[cd,gh]pentalene	
8.43	-	$C_6H_5NO_2$	<0.0001	2-Pyridinecarboxylic acid	C10164
14.68	-	$C_8H_{11}NO$	<0.0001	Phenylethanolamine triTMS	
14.68	-	$C_5H_9NO_3$	<0.0001	5 Aminolevulinic acid tri-TMS	
14.98	-	$C_{19}H_{26}O_2Si$	<0.0001	2-(4-Methoxyphenyl)-2-(4-trimethoxysilyloxy)propane	
15.05	-	$C_{12}H_{32}O_3Si_3$	<0.0001	Trimethylsilyl ether of glycerol	C00116
16.53	-	$C_{12}H_{30}O_4Si_3$	<0.0001	Propanoic acid, 2,3-bis[(trimethylsilyl)oxy]-, trimethylsilyl ester	
17.33	844	$C_{10}H_{20}O_4Si_2$	<0.0001	(E)-2-Butenedioic acid, bis(trimethylsilyl) ester	
20.16	972	$C_{13}H_{30}O_5Si_3$	<0.0001	Malic acid, O-(trimethylsilyl)-, bis(trimethylsilyl)ester	C00149
20.19	974	$C_{13}H_{30}O_5Si_3$	<0.0001	Butanedioic acid, [(trimethylsilyl)oxy]-, bis(trimethylsilyl) ester	C04067
20.19	974	$C_9H_{20}O_4Si_2$	<0.0001	Propanedioic acid, bis(trimethylsilyl) ester	C00383
21.26	1130	$C_{13}H_{34}O_3Si_3$	<0.0001	Propanetriol, 2-methyl-, tris-O-(trimethylsilyl)-	
21.52	1140	$C_{16}H_{40}O_5Si_4$	<0.0001	2,3,4-Trihydroxybutyric acid tetrakis(trimethylsilyl) deriv.	
22.8	1319	$C_{16}H_{42}O_4Si_4$	0.0002	(R,S)-3,8-Dioxa-2,9-disiladecane, 2,2,9,9-tetramethyl-5,6-bis[(trimethylsilyl)oxy]	C00503
24.08	1407	$C_{20}H_{52}O_5Si_5$	0.0345	L-Arabitol, pentakis(trimethylsilyl) ether	C01904
24.14	1409	$C_{18}H_{45}NO_5Si_4$	<0.0001	(S)-Butanal, 2,3,4-tris[(trimethylsilyl)oxy]-3-[[(trimethylsilyl)oxy]methyl]-, O-methyloxime	C07329
24.68	1424	$C_{20}H_{39}NO_5$	<0.0001	L-Norvaline, N-(2-methoxyethoxycarbonyl)-, undecyl ester	C01826
25.37	1443	$C_{22}H_{52}O_7Si_5$	<0.0001	D-Glycero-L-manno-Heptonic acid, 2,3,5,6,7-penta-O-(trimethylsilyl)-, γ-lactone	
25.48	1446	$C_{17}H_{42}O_5Si_4$	<0.0001	Arabinofuranose, 1,2,3,5-tetrakis-O-(trimethylsilyl)-	C06115
25.5	1447	$C_{20}H_{50}O_6Si_5$	<0.0001	Ribonic acid, 2,3,4,5-tetrakis-O-(trimethylsilyl)-, trimethylsilyl ester	C01685
25.91	1510	$C_{20}H_{48}O_6Si_4$	<0.0001	β-d-Galactofuranoside, ethyl 2,3,5,6-tetrakis-O-(trimethylsilyl)-	
26.02	1512	$C_{21}H_{52}O_6Si_5$	<0.0001	L-Sorbopyranose, 1,2,3,4,5-pentakis-O-(trimethylsilyl)-	C08356
26.14	1515	$C_{19}H_{42}O_5Si_4$	<0.0001	Shikimate	
26.23	1517	$C_{12}H_{22}O_7Si$	<0.0001	1,2,3-Propanetricarboxylic acid, 2-[(trimethylsilyl)oxy]-, tris(trimethylsilyl) ester	C00158
26.28	1519	$C_{16}H_{38}O_5Si_3$	<0.0001	β-Galactopyranoside, methyl 6-deoxy-2,3,4-tris-O-(trimethylsilyl)-	
26.38	1521	$C_{18}H_{45}NO_5Si_4$	<0.0001	D-Ribose, 2,3,4,5-tetrakis-O-(trimethylsilyl)-, O-methyloxime	C00121
26.54	1525	$C_{21}H_{52}O_6Si_5$	0.0001	D-Fructose, 1,3,4,5,6-pentakis-O-(trimethylsilyl)-	C00095
26.68	1528	$C_{21}H_{50}O_7Si_5$	<0.0001	2-Keto-l-gluconic acid, penta(O-trimethylsilyl)-	C06473
27.45	1609	$C_{22}H_{55}NO_6Si_5$	0.0017	(E)-D-Glucose, 2,3,4,5,6-pentakis-O-(trimethylsilyl)-, o-methyloxyme	C00031
27.66	1613	$C_{22}H_{55}NO_6Si_5$	<0.0001	(Z)-D-Glucose, 2,3,4,5,6-pentakis-O-(trimethylsilyl)-, o-methyloxyme	C00031
27.87	1618	$C_{22}H_{55}NO_6Si_5$	0.0033	(E)-D-Galactose, 2,3,4,5,6-pentakis-O-(trimethylsilyl)-, o-methyloxyme	C00124
27.93	1619	$C_{20}H_{52}O_5Si_5$	<0.0001	Ribitol, 1,2,3,4,5-pentakis-O-(trimethylsilyl)-	C00474
28.16	1624	$C_{22}H_{55}NO_6Si_5$	<0.0001	(Z)-D-Mannose, 2,3,4,5,6-pentakis-O-(trimethylsilyl)-, o-methyloxyme	C00275
28.31	1627	$C_{22}H_{55}NO_6Si_5$	<0.0001	(Z)-D-Galactose, 2,3,4,5,6-pentakis-O-(trimethylsilyl)-, o-methyloxyme	C00124
28.82	1704	$C_{17}H_{42}O_5Si_4$	<0.0001	Lyxose, tetra-(trimethylsilyl)-ether	C00476
29.64	1720	$C_{18}H_{45}NO_5Si_4$	<0.0001	D-Xylose, 2,3,4,5-tetrakis-O-(trimethylsilyl)-, O-methyloxime	C00181
29.91	1725	$C_{18}H_{44}O_5Si_4$	0.0105	6-Deoxy-2,3,4,5-tetrakis-O-(trimethylsilyl)hexose	
30.3	1804	$C_{19}H_{40}O_2Si$	<0.0001	Hexadecanoic acid, trimethylsilyl ester	C00249
30.41	1806	$C_{17}H_{42}O_5Si_4$	<0.0001	Arabinose, 2,3,4,5-tetrakis-O-(trimethylsilyl)-	C00259
32.86	2400	$C_{17}H_{42}O_5Si_4$	<0.0001	D-Xylopyranose, 1,2,3,4-tetrakis-O-(trimethylsilyl)-	
33.11	2403	$C_{21}H_{44}O_2Si$	<0.0001	Octadecanoic acid, trimethylsilyl ester	
33.38	2406	$C_{23}H_{36}O_2Si$	<0.0001	13-cis-Retinoic acid, trimethylsilyl ester	C07058
33.69	2410	$C_{19}H_{46}O_6Si_4$	<0.0001	Glycoside, α-methyl-trtrakis-O-(trimethylsilyl)-	
33.7	2411	$C_{21}H_{52}O_6Si_5$	<0.0001	β-D-Galactopyranoside, methyl 2,3,4,6-tetrakis-O-(trimethylsilyl)-	C03619
34.35	2502	$C_{19}H_{46}O_6Si_4$	<0.0001	α-D-Mannopyranoside, methyl 2,3,4,6-tetrakis-O-(trimethylsilyl)-	
34.39	2503	$C_{23}H_{38}O_2Si$	<0.0001	Isopimaric acid TMS	C09118
37.37	2707	$C_{21}H_{52}O_6Si_5$	<0.0001	D-Glucopyranose, 1,2,3,4,6-pentakis-O-(trimethylsilyl)-	
37.78	2711	$C_{21}H_{52}O_6Si_5$	<0.0001	α-D-Mannopyranose, 1,2,3,4,6-pentakis-O-(trimethylsilyl)-	
39.27	3102	$C_9H_{18}O_5$	<0.0001	L-Mannopyranose, 6-deoxy-1,2,3,4-tetrakis-O-(trimethylsilyl)-	
39.92	3100	$C_{21}H_{44}$	<0.0001	2-Methyleicosane	
39.93	3107	$C_{21}H_{52}O_6Si_5$	<0.0001	α-D-Galactopyranose, 1,2,3,4,6-pentakis-O-(trimethylsilyl)-	
40.84	3306	$C_{21}H_{52}O_6Si_5$	<0.0001	Gulose, 2,3,4,5,6-pentakis-O-(trimethylsilyl)-	C15923
41.87	3405	$C_{21}H_{52}O_6Si_5$	<0.0001	Talose, 2,3,4,5,6-pentakis-O-(trimethylsilyl)-	C06467
42.13	3500	$C_{14}H_{30}$	<0.0001	2-Methyltridecane	
42.57	3503	$C_{20}H_{42}$	<0.0001	2-Methylnonadecane	
43.23	3507	$C_{17}H_{38}O_7Si_3$	<0.0001	Dimethyl 2,3,4-tris-O-(trimethylsilyl)hexopyranosiduronate	
45.71	-	$C_{11}H_{10}O_6$	<0.0001	1,2,4-Benzenetricarboxylic acid, 1,2-dimethyl ester	
45.95	-	$C_{24}H_{36}O_2Si_2$	<0.0001	4-Methyl-2,4-bis(4'-trimethylsilyloxyphenyl)pentene-1	
46.37	-	$C_{12}H_{18}O_2Si$	<0.0001	5-Methyl-2-trimethylsilyloxy-acetophenone	

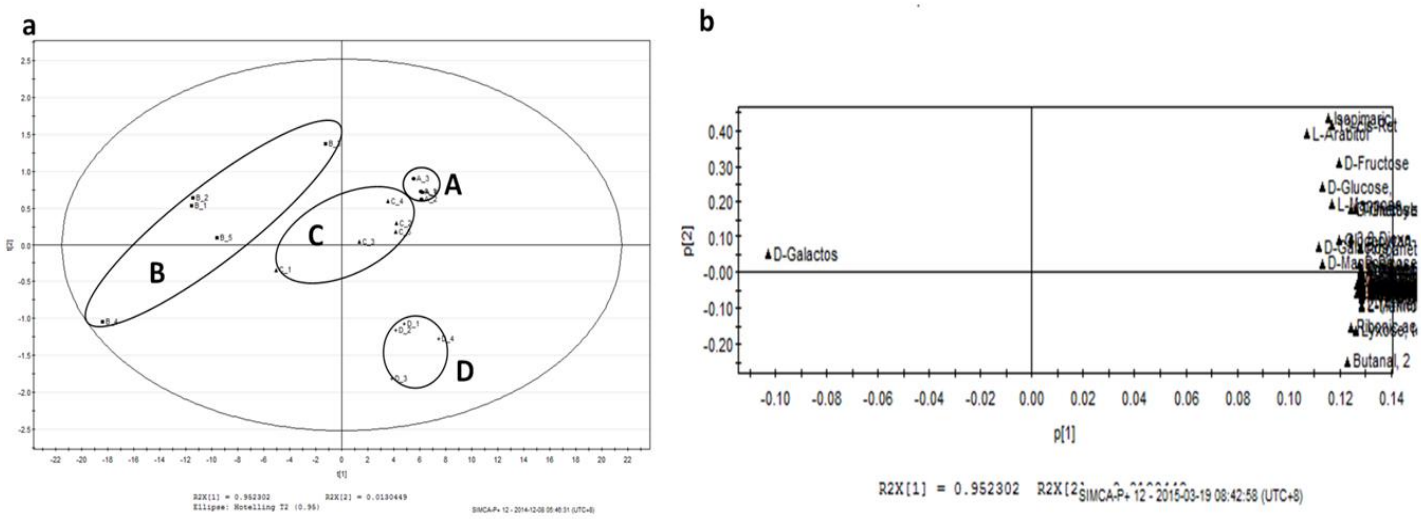

Fig 1. PCA of *W. trilobata*, analysed using GC-MS. Score (a) and loading plots (b) were generated using the first two PCs (PC1 versus PC2). Extraction was carried out using methanol/water (A), ethanol (B), methanol/chloroform/water (C) and water (D).

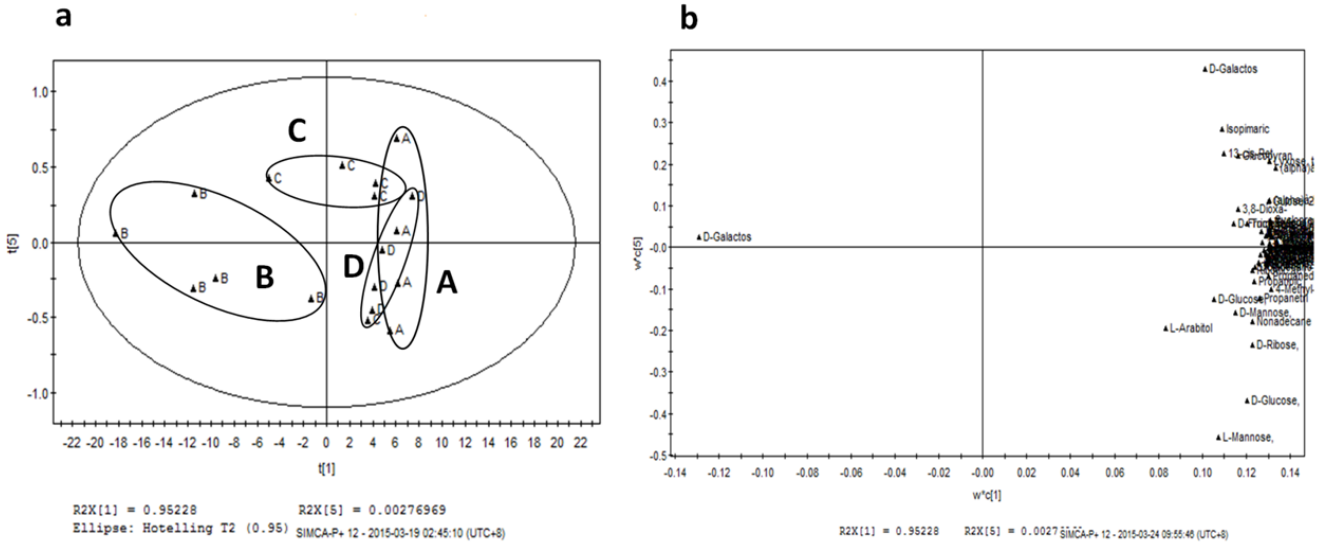

Fig 2. PLSDA of *W. trilobata*, analysed using GC-MS. Score (a) and loading plots (b) were generated using the first two LVs (LV1 versus LV2). Extraction was carried out using methanol/water (A), ethanol (B), methanol/chloroform/water (C) and water (D).

properties, evaluation of different solvent systems can be carried out based on MVA of PCA and PLSDA and/or peak intensity of known metabolites (chromatogram). Overall, the phytochemical constituents of *W. trilobata* using GC-MS showed limited classes of metabolite were detected. Metabolite profiles revealed that predominant peaks belonged to monosaccharides and organic acids (Supplementary S1). From previous studies, *W. trilobata* was reported to contain alkaloids, polyphenols, flavonoids, and terpenoids and could be easily extracted using ethanol and methanol (Balekar et al., 2012a). However, these secondary metabolites are usually detected using liquid chromatography (LC) because LC can determine metabolite with high molecular weight without the need for prior derivatization. In addition, LC based metabolite profiling of *W. trilobata* has not been described. Since LC-based plant metabolomics may increase the metabolite coverage, comprehensive metabolite profile of *W. trilobata* using LC could potentially yield a large number of secondary metabolites when compared to GC. On the other hand, apart from ethanol and methanol, extraction using hexane and ethyl acetate has also been described with *W. trilobata* but showed negligible results for antioxidant and antimicrobial activities (Prakash et al., 2012). Based on the GC-MS profiles, we managed to detect two resin acids (13-*cis*-retinoic acid and isopimaric acid) belonging to the diterpenes that had not been reported in *W. trilobata* before. A comparison between the GC-MS total ion chromatogram from the different solvents showed high abundance of resin acids in A, B and C. With the exception of the D (water), the resin acids were detected in all solvent extracts (Supplementary S1). Previously, GC-MS and Fourier transform infrared spectrometry (FT-IR) analysis for the fraction of methanolic extracts of *W. trilobata* have been reported (Kavya et al., 2014). The results showed presence of retinol acetate and pimaric acid but no further discussion was provided. In addition, determination of sesquiterpene lactones in *W. trilobata* using gas chromatography/gas chromatography–mass spectrometry (GC/GC–MS) has also been reported (Li et al., 2012).

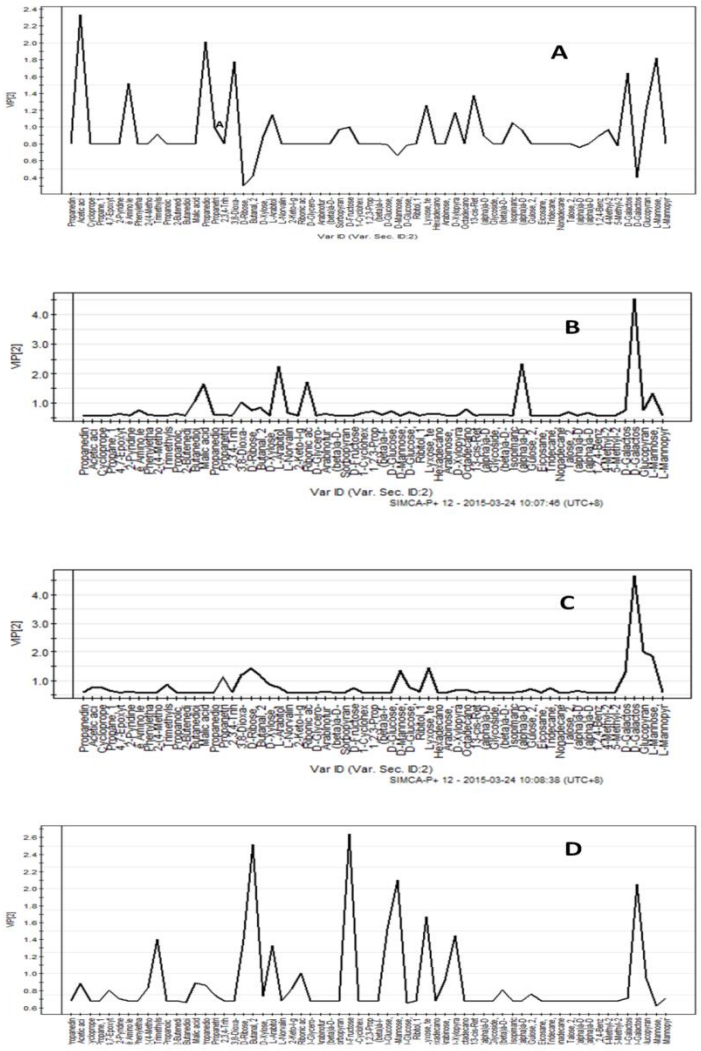

Fig 3. Variable importance in projection (VIP) scores of metabolite profiles contributes to separation in PLSDA. Extraction was carried out using methanol/water (A), ethanol (B), methanol/chloroform/water (C) and water (D).

Phytochemical constituents of W. trilobata

The analysis of carbohydrate content in biological systems is of great interest and importance. Sugars can be used as a biomarker or tracker to understand biological mechanism because they are generally safe and highly abundant. Furthermore, due to their polar nature and large sizes, sugars require conversion into volatile derivatives for GC-MS analysis. As shown in Table 1, the number of sugar isomers was observed. A majority of the sugars detected belongs to monosaccharides of pentose and hexose. Apart from sugars, GC-MS successfully detected 13-*cis*-retinoic acid and isopimaric acid. Both compounds are classified as resin acids that are known to have alleopathic effects that also act as a defence mechanism against herbivory insect and fungal invasion (Furstenberg-Hagg et al., 2013). For example, it was reported that ethanol extract of *W. trilobata* showed positive activity against Diamondback moth (Junhirun et al., 2012). At the same time, the allelochemicals of *W. trilobata* may cause negative effects to important agricultural crops such as rice and wheat. It was reported that irrigating rice with *W. trilobata* extracts had negative effects on the growth, biomass and physiological parameter of rice (Nie et al., 2005). The negative effects of W. trilobata extract have also been reported on rice seed. It was observed that treated rice seed with *W. trilobata* extract had higher membrane permeability, lower respiratory and vitality (Nie et al., 2005).

Materials and Methods

Chemical and reagents

All chemicals used were analytical grade. Methoxyamine hydrochloride (MeOX) and N-methyl-N-trimethylsilyl trifluoroacetamide (MSTFA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Alkane standard of C8-C20 and C21-C40 were also purchased from Sigma-Aldrich (St. Louis, MO, USA). Solvents like chloroform, ethanol, and methanol (MeOH) were obtained from Merck (Germany).

Plant materials

Young leaves (aerial part) of *Wedelia trilobata* were collected from the National Science Centre (PSN) (Bukit Kiara, Kuala Lumpur). The plant was grown side by side in the field of PSN. Fresh plant leaves were harvested in the morning session and packed in polyethylene bags. Collected sample was kept at -80ºC prior to extraction.

Metabolite extraction

Fresh plant leaves were immediately frozen in liquid N_2 and ground with a mortar and pestle. An approximate 200 mg of pooled homogenized plant material was weighed in an Eppendorf tube. Each extraction was performed in five biological replicates on the same pool of plant leaves. The extraction solvent combinations comprised of 300µL liquid for each extraction, spiked with ribitol as internal standard (25µg/mL). The extraction method was previously described by t'Kindt et al. (2009) with modification. Briefly, plant material was extracted with cold methanol/water ($MeOH/H_2O$ (80/20 v/v)), Methanol/chloroform/water ($MeOH/CHCI_3/H_2O$ 20/60/20 (v/v)), cold ethanol (EtOH (100%)) and cold water (dH_2O) in a Thermomixer for 15 min (1250 rpm, 70°C). All extracts were sonicated for 5 min and centrifuged at 3000 rpm at 4°C for 5 min. The supernatant was then collected and lyophilized. Derivatization was carried out using 40µL of O-methoxyamine hydrochloride (20 mg mL^{-1}) solution in anhydrous pyridine to the dried extracts. The resultant mixture was mixed vigorously and incubated for 30 min at 60°C in a heating block. Subsequently, 40 µL of N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA) was added and the extracts were incubated at 60°C for further 60 min (Azizan et al., 2012).

GC-MS parameter

Samples were analysed using Perkin Elmer Clarus 600 Turbo Mass GC-MS (Perkin Elmer, USA) coupled to quadrupole type MS operated at 70eV. An aliquot of 1µL was injected into Elite 5MS (5% phenyl 95% dimethylpolysiloxane, 30 m X 0.25 mm ID X 0.25 µm) column. The helium carrier gas was set to a flow rate of 1mL/min and the initial oven temperature was set to 70°C. The temperature was increased at 1ºC/min to 76°C and then at 6ºC/min to 300°C. The total run time was 50.33 min. The full scan mode was acquired at a mass range of 50-500 m/z, with a solvent delay of 7 min. The injection and ion source (EI) temperature were adjusted

to 200°C and 250ºC. Samples were injected in ratio mode (50:1).

Calculation of retention indices (RI)

Retention indices (RI) were calculated using *n*-alkanes (C8-C20, C21-C40) (Sigma-Aldrich, St. Louis, MO, USA) as reference compound (Bianchi et al., 2007). Briefyl, the *n*-alkanes was prepared in hexane (25:75) and analysed using the same GC-MS parameter as sample.

Data processing and statistical analysis

Raw GC-MS data were processed using Turbo Mass software (Perkin Elmer, USA) to obtain data table with compound name, retention time, peak height and peak area percentage. Identification was carried out using NIST (National Institute of Standards and Technology) mass spectral library (2005) with cut off of 800. The data table was then imported to MetaboAnalyst 2.0 server (http://www.metaboanalyst.ca) for further integral normalization and statistical analysis (Xia et al., 2012). In MetaboAnalyst, data table was normalized using ribitol as an internal standard, and subjected to log transformation. Analysis of variance (ANOVA) was used to determine metabolites with significance level of $p \leq 0.05$. Finally, normalized and validated data table was exported to SIMCA-P version 12 (Umetrics AB, Umea, Sweden) for PCA and PLSDA analysis.

Conclusion

GC-MS based metabolomics approach was used to evaluate the metabolic content of *W. trilobata* extract. Unbiased PCA revealed clear metabolic compositional differences in extraction solvents of methanol/water (A), ethanol (B) methanol/chloroform/water (C), and water (D). VIP score of PLSDA revealed the contribution of monosaccharides and resin acids towards the separation of different solvents. It was demonstrated that the solvent used for metabolite extraction affects the metabolic content. The finding provides basic information to optimise *W. trilobata* extraction for further metabolic characterization.

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Competing interests

The authors declare that they have no competing interests.

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