SHORT REPORT

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LC-MS Based Metabolomics Analysis to Identify Potential Allelochemicals in *Wedelia trilobata*

Kamalrul Azlan Azizan[*1], Sarah Ibrahim[1], Nurul Haizun Abdul Ghani[2] and Mohammad Firdaus Nawawi[2]

[1]*Metabolomics Research Laboratory, Institute of Systems Biology (INBIOSIS), Universiti Kebangsaan Malaysia (UKM), 43600, Bangi, Selangor, Malaysia*
[2]*Nationa Science Centre (PSN), Persiaran Bukit Kiara, 50662, Kuala Lumpur, Malaysia*

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Abstract: *Wedelia trilobata* is a noxious invasive weed that has been widely cultivated as a decorative and groundcover plant. The plant has been reported to contain diverse bioactive compounds with a broad spectrum of biological activities including allelochemicals. Allelochemicals contribute to allelopathy interactions that suppress the growth and development of nearby plants. Several studies have reported the allelopathic potential of *W. trilobata* and its negative effects to crop plants. However, relatively little is known about the allelochemicals' composition and how allelochemicals contribute to the allelopathic behavior of this plant. In order to prove allelopathy, the identification of the causative allelochemicals is required. The identification of potential allelochemicals that serve as biomarkers could be useful for assessing allelopathy interactions. In this study, a liquid chromatography (LC) based metabolomics approach was applied to find biomarkers with allelopathic effects from W. trilobata. Ethanol and water were used to extract metabolites from the leaves of *W. trilobata* and analyzed using liquid chromatography coupled with quadrupole high-resolution time of flight mass spectrometry (LC-QTOF-MS). Using multivariate statistical analysis (MVA), we identified eight Rt-*m/z* pairs as candidate marker compounds for assessing allelopathy interactions of W. trilobata. The results highlight the application of metabolomics for understanding of the role of allelochemicals in allelopathy interactions of *W. trilobata*.

Keywords: Allelopathy; Metabolomics; LC-QTOF-MS; *Wedelia trilobata*.

1. Plant Source

Wedelia trilobata is a notoriously invasive weed species that has been introduced as a decorative and groundcover plant in gardens, paths and public areas [1,2]. The plant is a member of the family Asteraceae (sunflower family) and can rapidly spread through vegetative propagation [3]. The leaves, stem and flower extracts of this plant are reported to contain therapeutic effects and are used in traditional medicine to treat inflammation and swelling, rheumatism and arthritic painful joints [4-7]. The leaves of *W. trilobata* were collected in early morning from the herbarium field of the National Science Centre (PSN), Bukit Kiara, Selangor, Malaysia.

2. Previous Studies

Specifically, several studies have highlighted the allelopathic potential of *W. trilobata* and its impact to farmlands, forests, and germination of some crop species [8-11]. In spite of all the negative

[*] Corresponding author: E-Mail: kamalrulazlan@ukm.edu.my

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effects, relatively little is known about the allelochemicals composition in *W. trilobata* and how allelochemicals contribute to the allelopathic behavior of this plant. To date, only sesquiterpene lactones have been recognized to contribute to the allelopathic effects of *W. trilobata* [12].

3. Present Study

3.1 Analysis of metabolic pattern using multivariate statistical analysis (MVA)

In this study, a minimal of four biological and four technical replicates of the samples were used and analysed in random sequences. This is to ensure that any experimental trends observed were directly associated with the sample and not due to any changes in the instrument's performance over time [35]. Metabolite profiling of *W. trilobata* was firstly carried out using HPLC-PDA for parameter optimization and subsequently analyzed using reverse phase LC-QTOF-MS. Evidently, comprehensive multivariate statistical analysis of unsupervised principal component analysis (PCA) was performed to evaluate the capability of LC-QTOF-MS based metabolomics approach in distinguishing between solvent extractions.

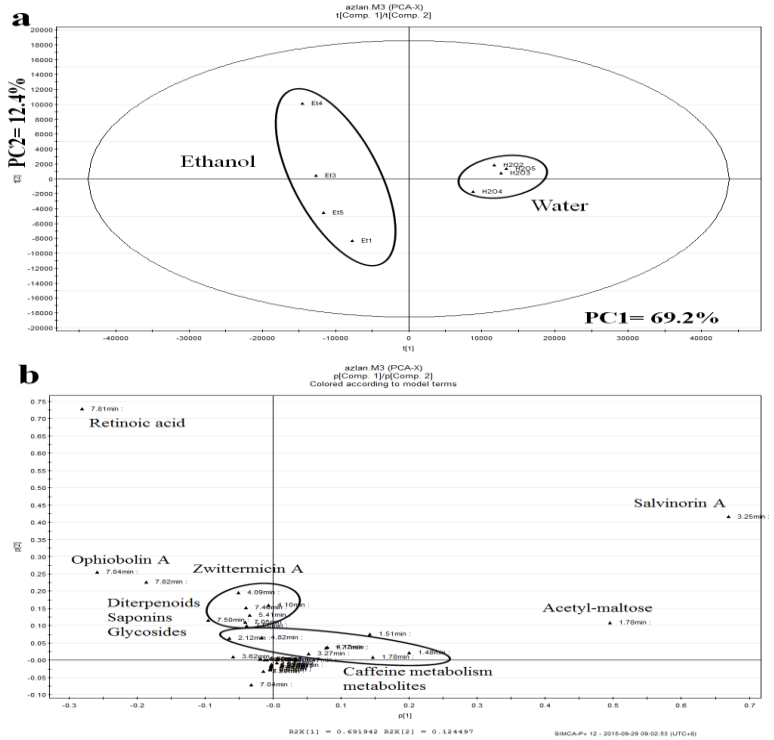

Figure 1. Principal component analysis (PCA) of *W. trilobata* leaf ethanol and water extracts.

Figure 1a describes the first two-component model (PC1 versus PC2) with total variance (R^2) of 81.6% and model predictive values (Q^2) of 45.5%. Namely, the separation shown in the PCA score plot was contributed by solvent extraction. As shown in figure 1a, the ethanol extract replicates are largely spread in the left quadrant of the PCA score plot whereas the water extract replicates are clustered closely in the right quadrant of the score plot. Meanwhile, loading plot in figure 1b indicates that most of the Rt-*m/z* pairs are scattered from the upper left to the center of the quadrant and to the right quadrant of the loading plot. Specifically, Rt-*m/z* pairs obtained from the water extract are mainly separated along PC1. Rt-*m/z* pairs obtained from the water extract are separated along PC2.

Generally, Rt-*m/z* pairs that are furthest away from the origin contributed to the separation between the groups and can be considered as the differentiating marker compound. As shown in figure 1b, Rt-*m/z* pairs that discriminate the ethanol extract are putatively identified as retinoic acid and Ophiobolin A, while putatively identified metabolites of Salvinorin A and acetyl-maltose are mostly linked to the water extract.

Namely, the metabolite capacities of the extracts have a strong relationship with the solvent employed, mainly due to the different metabolites with different polarities. Water is considered more polar than ethanol but is less efficient to extract non-polar compounds/metabolites compared to ethanol. Solvents with intermediate polarity such as ethanol are favorable as many bioactive secondary metabolites are more efficiently extracted than with water. Subsequently, in order to explore the most characteristic marker compounds from ethanol and water extract, partial least square discriminant analysis (PLSDA) was performed to find a potential characteristic marker compound for each extraction.

In this study, the candidate marker compounds were checked for their contribution to the variation and correlation within the data set by using variable important for projection (VIP) scores of PLSDA [35]. It is suggested that the metabolites with VIP exceeding 1 (VIP>1) greatly influence the discrimination pattern in PLSA model. By using VIP and S-plot of PLSDA, six Rt-*m/z* with VIP exceeding 1 (VIP>1) were identified as candidate marker compounds of *W. trilobata*.

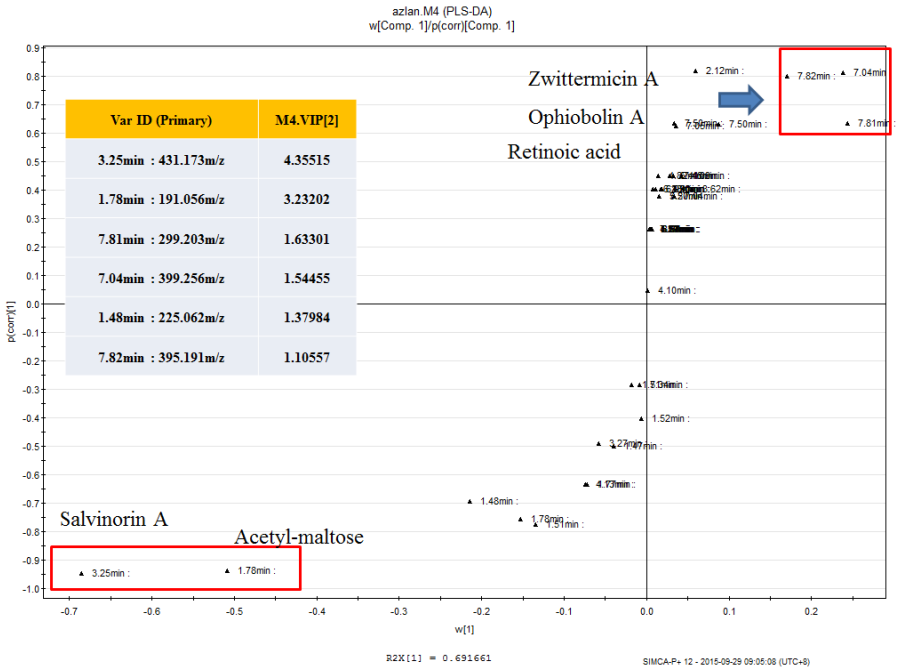

Figure 2. VIP and S-plot obtained from PLSDA. VIP values are used to select the most relevant variables to the separation.

As shown in Figure 2, these candidate marker compounds are located at the lower-left and upper-right quadrants of the S–plot. These marker compounds displayed the most discriminative Rt-*m/z* pairs obtained from ethanol and water extracts and thus can be considered as candidate marker compounds.

Following PLSDA, we used random forest (RF) variable important measures and a two-way hierarchical clustering heat map to confirm and visualize the levels of candidate marker compounds. Figure 3 displays the top 15 Rt-*m/z* pairs that are ranked based on the mean decrease in accuracy value. A total of six Rt-*m/z* pairs with the highest mean decrease in accuracy were identified as candidate marker compounds. Further observation using a two-way hierarchical clustering heat map led to two grouping between the six Rt-*m/z* pairs. Specifically, the levels of 1.78 min: *m/z* 383.1212 (Acetyl-maltose), 1.77 min: *m/z* 195.0515 (1,3 - Dimethyluric acid / 1,7- Dimethyluric acid), 1.51 min: *m/z* 179.0568 (D-fructose) and 1.48 min: *m/z* 225.0610 (AFMU) were higher in water extract while Rt-*m/z* pairs of 7.04 min: *m/z* 399.2574 (Ophiobolin A) and 7.82 min: *m/z* 395.1920 (Zwittermicin A /

1-Hexanol arabinosylglucoside) were higher in ethanol extract. The combination of PLSDA and RF analysis led to eight candidate marker compounds.

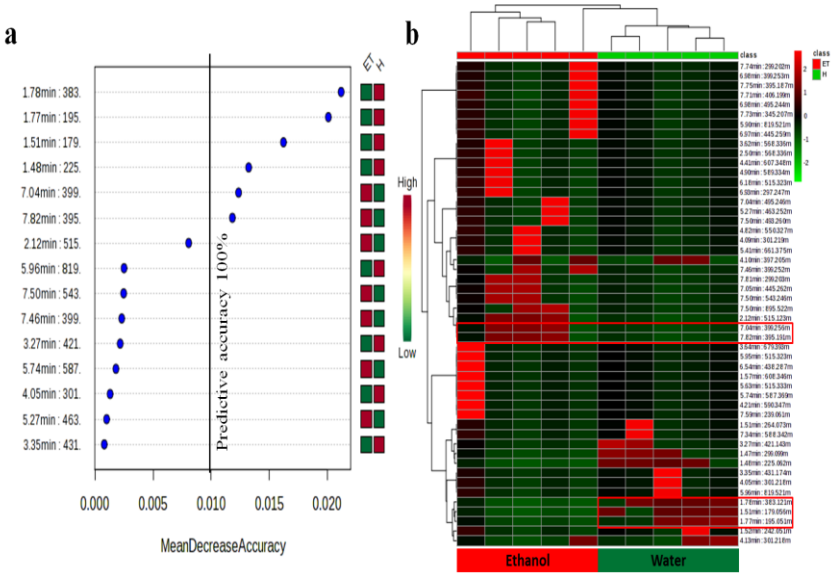

Figure 3. Top 15 Rt-*m/z* pairs and its levels according to solvent extractions.

3.2. The identification of candidate marker compounds from ethanol and water extracts

The candidate marker compounds and the relative identification of the marker compounds are reported in Table S1. The water extract of *W. trilobata* generally contained alkaloids, diterpenoids, and monosaccharides. The major Rt-*m/z* pairs that stood out in the water extract were 3.25min: *m/z* 431.1735 and 1.78min: *m/z* 383.1217 (Figure 1b). The putative identification of 3.25min: *m/z* 431.1744 with fragmentation ions of 341, 373 and 387 and the predicted molecular formula of $C_{23}H_{28}O_8$ led to salvinorin A (SA). The pattern was similar to those described by Medana et al. 2006 [38] which mentioned *m/z* 373 and *m/z* 387 as the fragmentation ion to detect SA. SA is a trans-neoclerodane diterpenoid, an active compound in *Salvia divinorum* (Lamiaceae) [13]. Meanwhile, the identification of 1.78min: *m/z* 383.1212 with a fragmentation ion of *m/z* 191 led to acetyl-maltose ($C_{14}H_{24}O_{12}$). Interestingly, a further observation of the fragmentation ion of this Rt-*m/z* showed loss of $[C_7H_{12}O_6\text{-}H]^-$, which corresponded to quinic acid [14]. In particular, several Rt-*m/z* pairs with a fragmentation ion of 191 have been identified. 1.48min: *m/z* 225.0610 with fragmentation ions of 179 and 191 proposed that the molecular formula of $C_8H_{10}N_4O_4$ was putatively identified as 5-acetylamino-6-formylamino-3-methyluracil (AFMU). 1.77min: *m/z* 195.0515 with a prominent fragmentation ion of *m/z* 191 was identified as purine alkaloids of 1,3-dimethyluric acid and 1,7-dimethyluric acid ($C_7H_8N_4O_3$). Specifically, quinic acid and its ester conjugates have roles in plant defense against environmental stress and plant pathogens, which can be potentially applied as herbicides [15].

The ethanol extract of *W. trilobata* was characterized by the presence of diterpenoid and sesterterpenoid compounds. The major Rt-*m/z* pairs that stood out in the ethanol extract corresponded to 7.04min: *m/z* 399.2574 and 7.81min: *m/z* 299.2026. The putative identification of 7.04min: *m/z* 399.2574 with fragmentation ion of *m/z* 144 and 182 led to Ophiobolin A (OPA), a sesterpene and 16-alpha, and 17-Isopropylidenedioxy-6α-methylprogesterone, a methylprogesterone acetate (MPA). Ophiobolins are phytotoxic sesterpene (C_{25}) with potential natural herbicides against grass weeds [16]. The putative identification of 7.81min: *m/z* 299.2026 with fragmentation ions of 182 and 299 corresponded to retinoic acid (RA) and hydroxyl steroid compounds. The allelopathic effects of retinoic acids however, have not been reported yet. Additionally, we have also putatively identified diterpene of kauren and resin acids (DRAs) of the abietane and pimarane type (abietic, neobietic,

dehydroabietic, palustric, pimaric and isopimaric acid), that corresponded to Rt-*m/z* 4.13min: *m/z* 301.218 and proposed a molecular formula of $C_{20}H_{30}O_2$.

4. Conclusions

LC-QTOF-MS based metabolomics of ethanol and water extracts of *W. trilobata* detected a number of potential secondary metabolites with allelopathic effects. The PLSDA and RF analysis identified eight candidate marker compounds that can be used for monitoring allelopathic interactions of *W. trilobata*. The ethanol and water extracts showed the presence of diterpenoid and sesterterpenoid compounds, while compounds of alkaloid including caffeine metabolism and monosaccharide were found in the water extract only. This study successfully reported the candidate marker compounds of *W. trilobata* extracted using ethanol and water extracts.

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