Anti-diabetic properties of three common *Bidens pilosa* variants in Taiwan

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**A B S T R A C T**

*Bidens pilosa* L. var. *radiata* (BPR), *B. pilosa* L. var. *pilosa* (BPP), and *B. pilosa* L. var. *minor* (BPM) are common variants of a plant often used as a folk remedy for diabetes in Taiwan. However, the three variants are often misidentified and little is known about their relative anti-diabetic efficacy and chemical composition. In this paper, we have first developed a method based on GC–MS and cluster analysis with visualization to assist in rapidly determining the taxonomy of these three *Bidens* variants. GC–MS was used to determine the chemical compositions of supercritical extracts, and differences and similarities in the variants were determined by hierarchical cluster analysis. Next, the HPLC profiles of the methanol crude extracts in the *Bidens* plants and evaluated anti-diabetic effects of methanol crude extracts were compared, as well as three polyacetylenic compounds of the *Bidens* plants using db/db mice. Single-dose and long-term experiments showed that the BPR extract had higher glucose-lowering and insulin-releasing activities than extracts from the other two variants, and that cytopiloyne was the most effective pure compound among the three polyacetylenic compounds. BPR extract and cytopiloyne also significantly reduced the percentage of the glycosylated hemoglobin A1c in db/db mice. Besides, both animal studies and HPLC analysis demonstrated a good correlation between anti-diabetic efficacy of the *Bidens* extracts and the particular polyacetylenes present.

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1. Introduction

Diabetes mellitus is a serious metabolic disease resulting from defects in either insulin secretion, insulin action, or both. Over 90% of diabetic patients have Type 2 diabetes which results from insulin resistance or insulin secretion defects (Attele et al., 2002). Control over blood glucose levels is gradually lost and levels of glycosylated hemoglobin A1c (HbA1c) rise in patients with Type 2 diabetes. Currently, several anti-diabetic agents are available, but these are unsatisfactory in terms of efficacy and adverse side-effects (Howlett and Bailey, 1999; Lebovitz, 1998). Recently, the search for novel anti-diabetic medicines has focused on medicinal plants because of their efficacy in human clinical trials and the minimal side-effects of drugs derived from medicinal plants (Rates, 2001; Suba et al., 2004).

Plants are known to be an extraordinary source of anti-diabetic medicines (Marles and Farnsworth, 1995). The genus *Bidens* includes approximately 230 species worldwide (*Encyclopedia Britannica Inc.*, 2008), and a wide range of biological activities have been reported for many plants of this genus (Alarcon-Aguilar et al., 2002; Brandão et al., 1997; Chang et al., 2005; Chih et al., 2004; Cihl et al., 1995; Dimo et al., 2001, 2002; Geissberger and Séquin, 1991; Gilbert et al., 1999; Jäger et al., 1996; Mahonge et al., 2008; Rücker et al., 1992; Rabe and van Staden, 1997; Ssegawa and Kasenene, 2007; Ubillas et al., 2000; Yang et al., 2006; Yoshida et al., 2006), although most of the presumed activities have not been studied scientifically. Several *Bidens* species are currently used as herb diabetic treatments by people in American, African, and Asian regions (Brandão et al., 1997; Chih et al., 1995; Hernandez-Galicia et al., 2002; Jäger et al., 1996; Lans, 2006). In Taiwan, the species most often used is *Bidens pilosa* (Lin, 1992). There are three common variants of this species in Taiwan: *B. pilosa* L. var. *radiata* (BPR), *B. pilosa* L. var. *pilosa* (BPP) and *B. pilosa* L. var. *minor* (BPM). All three variants share similar morphological characteristics and habitat preferences (Chaw et al., 1998), so they are often misidentified. To help in the correct identification of these plants, we have developed a chemotaxonomic method, a
method to identify relationships among plant taxa using concentration of several pre-selected compounds (Ge et al., 2008), based on GC–MS analysis and cluster analysis with visualization of the chemical profiles.

We have previously shown that three of the polyacetylenic glucosides in *Bidens pilosa* can prevent Type 1 diabetes (Chang et al., 2007, 2004; Chiang et al., 2007). In addition, two of the polyacetylenic glucosides were shown elsewhere to be anti-hyperglycemic (Ubillas et al., 2000). These studies suggest that polyacetylenic glucosides present in *B. pilosa* are the major active phytochemicals against both types of diabetes. In this paper, we first developed a combinational method of GC–MS analysis and cluster analysis to help in the authentication of the three *Bidens pilosa* variants including BPR, BPP, and BPM. Next, we examined anti-diabetic efficacies of the crude extracts and polyacetylenic compounds of the *Bidens* variants in db/db mice, a model of Type 2 diabetes.

2. Results and discussion

2.1. GC–MS chromatograms and cluster analysis of the chemical profiles of *Bidens pilosa* variants

To aid the taxonomy of the three common *B. pilosa* variants, namely BPR, BPP, and BPM, in Taiwan, GC–MS analysis of supercritical CO₂ extracts was used to determine their chemical compositions (Fig. 1). Cluster analysis tools, such as hierarchical cluster tree (HCT), principal component analysis (PCA) and multidimensional scaling (MDS), have been used previously in the analysis of inter- and intra-species chemical variations (Medina-Holguin et al., 2008; Nyman and Julkunen-Tiitto, 2005). Although all of these cluster tools illustrated very well the grouping structure of the samples, they were not able to depict the sources of similarities and differences. In this study, we adopted HCT with matrix visualization (MV) in generalized association plots (GAP) (Chen, 2002; Tien et al., 2008; Wu et al., 2008) to compare similarities and differences in chemical profiles of BPR, BPP and BPM (Fig. 2). Fig. 2A has the matrix visualization of the sizes of the GC–MS peaks in percent relative areas [abbreviated as RA (%), the area of a GC–MS peak normalized against the area of the largest peak in the chromatogram, presented in unit of percentages] of 12 samples (four samples for BPR, four samples for BPP, and four samples for BPM) with 68 retention times between 15.38 and 34.95 min. A rainbow spectrum is adopted to color code the sizes of the chromatographic peaks with red denoting the largest peak [maximum RA(%)] and blue denoting the smallest peak [minimum RA(%)]. Since a few extremely large peaks with retention times near 23.82 min may suppress the whole visualization effect, peaks with percent relative areas larger than 18.49 are coded as if their percent relative areas were 18.49. Each row in Fig. 2A represents one sample while each column represents one particular retention time. Average linkage hierarchical clustering trees (HTCs) for retention time and for samples were then built on corresponding correlation matrices for retention time and for samples respectively. Fig. 2A is then two-way permuted into Fig. 2B according to the orders of terminal nodes in HCTs for retention time and for samples in order to obtain sample clustering structure and retention time grouping pattern (see Tien et al., 2008 for detailed description of permutation algorithm). A bi-directional blue-white-red spectrum is used to denote negative-to-positive correlation in Fig. 2C. The most profound observation to be noted in Fig. 2C is that BPR and BPP samples form a larger group with high positive within-group correlation (in dark red) while BPM samples form a separate cluster. The white to light blue colors in off-diagonal area indicate the relationship structure between groups which could vary from uncorrelated (BPR to BPM) to negatively correlated (BPM to BPP), although one of the BPM sample (BPM1) shows some positive correlation to BPR samples. The GC–MS profiles in Fig. 2A show that BPR and BPP have three peaks in common (i.e., peaks F, D, and G with retention times of 23.82 min, 21.81 min, and 30.30 min) while BPR and BPM share only one peak in common (peak B, retention time 18.06 min). The GC–MS profiles of BPR are more similar to those of BPP than to those of BPM. Some peaks appeared only in the GC–MS profiles of one plant: the peak at 16.44 min (peak A) is unique to the BPP variant, while the peak at 33.95 min (peak H) is unique to BPM. The peaks that are unique to a particular variant may be used to distinguish one variant from the others. One practical application of these species-specific peaks is the detection of adulterated plant materials in herbal medicines.

By converting Fig. 2B to Fig. 2D using individually ranged color spectrums for each retention time so that between-variant structure within each retention time (column) can be clearly depicted, we found the combination of matrix visualization for GC–MS chemical profiles with hierarchical cluster tree using GAP to be a fast and reliable chemotaxonomical method to assist in the identification of *Bidens* plants.

Clustering and visualization data shown in Fig. 2 show that GC–MS profiles can be used to determine the taxonomy of plant samples from the three common *B. pilosa* variants. For validation of the clustering and visualization methods use in Fig. 2, we adopted the classification tree method (Brieman et al., 1984) because of its ease.
Fig. 2. Matrix visualization with hierarchical clustering tree (HCT) for GC–MS profiles of the Bidens variants. (A) GC–MS profiles of four batches of BPR, BPP and BPM from Fig. 1 are displayed as a matrix map with 12 rows each representing one sample and 68 columns each representing one retention time. A rainbow spectrum is adopted to color code the size of the chromatographic peaks [expressed in percent relative area, RA(%)] in the whole matrix with red denoting the largest peaks [maximum RA(%)] and blue denoting the smallest peak [minimum RA(%)]. (B) Two-way permuted data matrix of (A). The tree structure above the data matrix represents average linkage hierarchical clustering tree (HCT) for 68 different retention times. (C) Map of correlation matrix for the 12 samples (four BPR samples, four BPP samples, and four BPM samples). A bi-directional blue–white–red spectrum is used to denote negative-to-positive correlation. The tree structure beside the correlation matrix represents average linkage HCT for 12 samples. (D). Identical data matrix map as in (B) except each column is colored using a rainbow color spectrum scaled individually for that particular column instead of a rainbow color spectrum scaled for all columns in the matrix.
of interpretation and the high-dimensional nature of our data. Blinded sample analyses using both leave-one-out (LOO) (Devijver and Kittler, 1982) and leave-one-per-class-out (LOPCO) validation procedures were carried out for all 68 univariate and 4556 (68 x 67) bi-variate classification tree models. For univariate classification tree model, the GC–MS peak with retention time of 31.66 min outperformed all other components with zero error rate for all LOO and LOPCO validations. For bi-variate classification tree models, combinations of one component from groups a or c in Fig. 2D and another from groups b or d always produce good results with minimum classification error rate, as validated by either LOO or LOPCO validations.

By choosing components with both high discriminant power from the aforementioned validation procedures (groups a, b, c, and d in Fig. 2D) and significant relative peak areas in Fig. 2B, we have identified peaks A, B, D, F, and H (Fig. 2B) as components for further analysis. Upon further analysis, we found the pair of components H and A turned out to be associated with the best validation error rate of zero for all LOO and LOPCO validation procedures, suggesting the feasibility of chemotaxonomic analysis.

2.2. HPLC profiles of the Bidens methanol extracts and polyacetylenic glucosides

The existence of polyacetylenic glucosides, shown as compounds 1, 2, and 3 in Fig. 3 in the crude extracts of BPR was confirmed in previous publications (Chang et al., 2007, 2004; Ubillas et al., 2000). These polyacetylenic glucosides can serve as index compounds for chemical analyses and for quality control of different batches of the Bidens crude extracts. As shown in Fig. 3, BPR, BPP and BPM have different levels of the polyacetylenic glucosides. Based on the HPLC analyses of triplicate samples for BPR, BPP, and BPM, the percentages of compounds 1, 2, and 3 in the methanol extract were determined to be 0.61 ± 0.02%, 0.44 ± 0.02%, and 0.32 ± 0.01% in BPR, 0.17 ± 0.01%, 0.19 ± 0.01%, and 0.27 ± 0.01% in BPP and 0.16 ± 0.02%, 0.15 ± 0.02%, and 0.27 ± 0.01% in BPM, respectively. The composition of BPR (percentages of compounds 1–3 in the crude extract) is statistically different from those of BPP and BPM. (The statistical analysis was carried out using ANOVA followed by post hoc using Bonferroni’s multiple comparison tests.) The difference between compositions of BPP and BPM, on the other hand, are not statistically significant. In addition, BPP

Table 1

<table>
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<th>Treatment (n)</th>
<th>Oral dose (mg/kg)</th>
<th>Blood glucose level (mg/dl)</th>
</tr>
</thead>
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<td>1</td>
</tr>
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<td>380.3 ± 10.5</td>
</tr>
<tr>
<td>GLM (6)</td>
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</tr>
<tr>
<td>BPR extract (5)</td>
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<td>364.2 ± 7.0</td>
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<td>BPR extract (5)</td>
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</tr>
<tr>
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<td>CMPD 3 (5)</td>
<td>2.5</td>
<td>391.2 ± 19.7</td>
</tr>
</tbody>
</table>

The number of mice used (n) is indicated in parentheses in the first column.

* Indicates P < 0.05, as determined by ANOVA followed by post hoc test using Bonferroni’s multiple comparison test vs. the control (PBS) group.

Fig. 3. Representative HPLC profiles of BPR, BPP and BPM methanol extracts. The most representative HPLC profiles for each variant were chosen from one out of three HPLC analyses. The UV detection wave length was set at 240 nm.
Table 2

<table>
<thead>
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<th>Treatment (n)</th>
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<th>Serum insulin level (ng/ml)</th>
</tr>
</thead>
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<td></td>
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</tr>
<tr>
<td>PBS (5)</td>
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<td>10.5 ± 2.4</td>
</tr>
<tr>
<td>GLM (5)</td>
<td>1.0</td>
<td>12.9 ± 2.9</td>
</tr>
<tr>
<td>BPR extract (5)</td>
<td>50</td>
<td>10.7 ± 0.7</td>
</tr>
<tr>
<td>BPP extract (5)</td>
<td>50</td>
<td>10.8 ± 2.2</td>
</tr>
<tr>
<td>BPM extract (5)</td>
<td>50</td>
<td>10.8 ± 2.3</td>
</tr>
<tr>
<td>CMPD 1 (5)</td>
<td>0.5</td>
<td>10.8 ± 0.3</td>
</tr>
<tr>
<td>CMPD 2 (5)</td>
<td>0.5</td>
<td>11.9 ± 2.1</td>
</tr>
<tr>
<td>CMPD 3 (5)</td>
<td>0.5</td>
<td>10.1 ± 1.3</td>
</tr>
</tbody>
</table>

The number of mice used (n) is indicated in parentheses in the first column. Indicates P < 0.05, as determined by ANOVA followed by post hoc test using Bonferroni’s multiple comparison test vs. the control (PBS) group.

and BPM may have additional polyacetylenic compounds based on examination of their HPLC chromatograms (Fig. 3).

2.3. Single-dose effects of the Bidens methanol extracts and polyacetylenic glucosides on blood glucose levels and serum insulin levels of db/db mice

Methanol crude extract and a mixture of two polyacetylenes of Bidens pilosa have been shown to reduce blood glucose levels in diabetic mouse models (Alarcon-Aguilar et al., 2002; Ubillas et al., 2000). Therefore, we evaluated single-dose effects of the Bidens methanol extracts and polyacetylenic glucosides, compounds 1–3, on the regulation of blood glucose in db/db mice. Administration of a single oral dose [10, 50 and 250 mg/kg body weight (BW)] of BPR, BPP and BPM crude extracts lowered postprandial blood glucose levels in db/db mice for up to four hours (Table 1). The reduction in blood glucose levels appeared to be dose dependent. Collectively speaking, BPR extract of the same dose had higher reduction of blood glucose levels than the other extracts. A single oral dose of compounds 1–3 also lowered postprandial blood glucose levels in db/db mice in a dose-dependent manner. Compound 2 had more anti-hyperglycemic activity than compounds 1 and 3.

Next, we examined one-dose effects of the above crude extracts and phyto compounds on serum insulin levels in db/db mice. In these experiments, we only used a dose of 50 mg/kg for crude extracts and a dose of 0.5 mg/kg for polyacetylenes because these dosage levels could differentiate the efficacy of the crude extracts and compounds. BPR crude extract and compounds 1–3 significantly increased serum insulin level in db/db mice (Table 2). In contrast, BPP or BPM extract had a slight increase in serum insulin levels. However, this increase by BPP or BPM is not statistically significant (Table 2). Serum insulin reached a plateau at 0.5 h after an oral dose of the crude extracts of BPR, BPP, BPM or of compounds 1–3, in contrast to glimepiride, where peak serum insulin occurred 1 h after an oral dose of 1.0 mg/kg. The insulin kinetics of the Bidens extracts and their compounds appeared to be slightly different from that of glimepiride. Coincidently, all extracts, polyacetylenes and glimepiride increased serum insulin levels.

Collectively, the glucose-lowering activities of the Bidens crude extracts (e.g., BPR extract) and the polyacetylenes in db/db mice correlate with their insulin-increasing activities, implying that the above crude extracts and compounds reduced blood glucose levels in db/db mice by increasing insulin release.

2.4. Long-term effects of the Bidens methanol extracts and polyacetylenic glucosides on the regulation of blood glucose, insulin and HbA1c

We next verified the long-term efficacy of the Bidens crude extracts and compounds 1–3 in controlling blood glucose levels, serum insulin levels and the HbA1c levels in db/db mice. Before treatment, diabetic mice had postprandial glucose levels ranging from 370 to 420 mg/dl. As shown in Fig. 4, the blood glucose level rose to over 600 mg/dl in db/db mice of control group which was treated for 28 days with phosphate buffer solution (PBS). In addition, 28-day treatment with glimepiride significantly reduced blood glucose levels in db/db mice. Furthermore, 28-day treatment with the crude extracts of BPR, BPP or BPM and compounds 1–3 lowered blood glucose levels in db/db mice. The range of the
The data presented in Tables 1 and 2 and Figs. 4 and 5 showed that the anti-diabetic effect of BPR (in terms of reduction of blood glucose and increase of serum insulin) is greater than those of BPP and BPM, and that there is no statistically significant difference between the anti-diabetic effects of BPP and BPM. In addition, the anti-diabetic effect of compound 2 is greater than compound 1 while the anti-diabetic effect of compound 1 is approximately equal to that of compound 3. The data also show that the anti-diabetic effects of crude extracts are related to the quantities of the three polyacetylenes present in the extracts. The body weight of the db/db mice were monitored during the 28-day treatment period and the data are presented in Table 3. The body weight of the db/db mice did not show statistically significant changes based on analysis using ANOVA followed by post hoc test using Bonferroni’s multiple comparison test.

As shown in Figs. 4–6 and Tables 1 and 2, Bidens crude extracts and their polyacetylenic active compounds lowered blood glucose levels and HbA1c and elevated insulin levels in db/db mice. This is likely due to the polyacetylenes, particularly compound 2, in the Bidens extracts acting as active compounds to treat diabetes (reduction of blood glucose and HbA1c) via up-regulation of insulin secretion. Alternatively, the compounds could have increased insulin secretion via their effect on either β cell hyperplasia or enhancement of incretin action. Further mechanistic study of the polyacetylenes needs to be done. In addition, blood dilution, variant hemoglobin translation, and decrease in erythrocyte number could account for the reduction of HbA1c by the active compounds. However, the data on complete blood counts, hemoglobin content and hematocrit are against this possibility (data not shown). Furthermore, insulin secretion can be controlled at the levels of transcription/translation or post-translation. The level at which dosages was between 10 mg/kg BW and 250 mg/kg BW. However, only treatments with the BPR crude extract and compounds 1–3 resulted in blood glucose reductions that were statistically different from control. 28-day treatment with glimepiride significantly increased the serum insulin levels in db/db mice (Fig. 5). Similarly, 28-day treatment with the BPR crude extract and compound 2 significantly increased insulin levels (Fig. 5). The percentage of glycosylated HbA1c in the blood of diabetic db/db mice aged 10–12 weeks was 7.9 ± 0.5% (Fig. 6). In contrast, glycosylation levels in the blood of age-matched mice following treatment with the BPR crude extract (50 mg/kg), glimepiride (1 mg/kg) and compound 2 (0.5 mg/kg), were 6.6 ± 0.2%, 6.1 ± 0.3% and 6.2 ± 0.3% respectively (Fig. 6). These data were in good agreement with the glucose-lowering and insulin-releasing effects of the BPR extract and compound 2 (Figs. 4 and 5). Overall, crude extract of BPR showed the largest anti-diabetic effects (reduction of blood glucose, increase in serum insulin and reduction of glycosylated HbA1c).

Fig. 5. Insulin-releasing effects of the methanol crude extracts and polyacetylenic glucosides of BPR, BPP and BPM in db/db mice. Postprandial serum insulin levels of diabetic db/db mice which have been orally administered with a daily dose of PBS at 0.2 ml, glimepiride (GLM) at 1.0 mg/kg BW, methanol crude extracts of the Bidens plants at 50 mg/kg BW, or pure bioactive compounds 1, 2, and 3 of the Bidens plants at 0.5 mg/kg BW for 33 days. The glycosylated HbA1c levels of the db/db mice were then determined. Glycosylation levels are presented here as a percentage of total HbA1c.

Fig. 6. Effects of the methanol crude extracts of the Bidens plants and polyacetylenic glucosides of BPR, BPP and BPM on the glycosylation of HbA1c in db/db mice. Diabetic db/db mice were orally administered with a daily dose of phosphate buffer solution (PBS) at 0.2 ml, glimepiride (GLM) at 1.0 mg/kg BW, methanol crude extracts of the Bidens plants at 50 mg/kg BW, or pure bioactive compounds 1, 2, and 3 of the Bidens plants at 0.5 mg/kg BW for 33 days. The glycosylated HbA1c levels of the db/db mice were then determined. Glycosylation levels are presented here as a percentage of total HbA1c.

Table 3
Effect of the Bidens crude extracts and active compounds on the body weight of the db/db mice during a 28-day treatment period with Bidens crude extracts and active compounds.

<table>
<thead>
<tr>
<th>Treatment (n)</th>
<th>Oral dose (mg/kg)</th>
<th>Mean body weight (g/mouse)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td>0</td>
</tr>
<tr>
<td>PBS (5)</td>
<td>–</td>
<td>27.3 ± 1.0</td>
</tr>
<tr>
<td>GLM (5)</td>
<td>1.0</td>
<td>28.0 ± 1.0</td>
</tr>
<tr>
<td>BPR extract (5)</td>
<td>50</td>
<td>27.8 ± 1.0</td>
</tr>
<tr>
<td>BPP extract (5)</td>
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<td>28.3 ± 0.7</td>
</tr>
<tr>
<td>BPM extract (5)</td>
<td>50</td>
<td>27.9 ± 0.3</td>
</tr>
<tr>
<td>CMPD 1 (5)</td>
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<td>27.7 ± 0.9</td>
</tr>
<tr>
<td>CMPD 2 (5)</td>
<td>0.5</td>
<td>29.8 ± 0.9</td>
</tr>
<tr>
<td>CMPD 3 (5)</td>
<td>0.5</td>
<td>29.0 ± 0.8</td>
</tr>
</tbody>
</table>

The number of mice used (n) is indicated in parentheses in the first column.

Statistical analysis was performed by ANOVA and no statistically significant difference between control (PBS) and experimental groups was observed.
the polyacetylenic and Bidens crude extracts regulate this secretion needs to be ascertained.

Gross pathological examination did not show any acute or chronic toxicity in db/db mice with the treatment of the crude extracts and Bidens active compounds, as evidenced by organ weight and death (data not shown).

3. Conclusions

Using the three Bidens pilosa variants BPR, BPP and BPM as examples, we have demonstrated that a combination of GC–MS analysis and cluster analysis with matrix visualization is a fast and reliable taxonomical method to chemically distinguish plants with similar habitats and morphologies. We also found that the methanol crude extract of BPR has higher anti-diabetic activities than that of BPP and BPM, as evidenced by the increase in serum insulin, the decrease in blood glucose and the increase in glycosylated HbA1c levels. This may be due to BPR crude extracts having a higher level of compound 2, the most effective compound for increasing serum insulin, reducing blood glucose levels, and for reducing glycosylated HbA1c levels, than the crude extracts of BPP and BPM. This hypothesis is supported by HPLC-based quantitative analysis of the polyacetylenes in the Bidens extracts and dose–effect study in db/db mice as these data showed a strong correlation between the anti-diabetic efficacy of Bidens crude extracts and the quantity of compound 2 as well as compounds 1 and 3 in the crude extracts.

4. Experimental

4.1. Plant materials

Bidens pilosa variants (BPR, BPP, and BPM), which belong to the plant family Asteraceae, were collected in Taiwan and authenticated by Dr. Ching-I Peng (Biodiversity Center, Academia Sinica, Taiwan), and voucher specimens were deposited as 120085 (BPR), 120086 (BPP), and 120087 (BPM) at Academia Sinica Herbarium, Taiwan. The plants were then grown from seeds in green houses at Academia Sinica.

4.2. Chemicals and animals

HPLC grade MeOH, CH3CN and biochemical grade CF3CO2H (TFA) were purchased from Mallinckrodt Baker (Phillipsburg, NJ). H2O was purified with a Milli-Q deionization unit (Millipore, Bedford, MA). Glimepiride was purchased from Sigma–Aldrich (St. Louis, MO), whereas Type 2 diabetes model, db/db mice, was from the Jackson Laboratory (Bar Harbor, ME). All animals were main-
4.6. Measurement of HbA1c, blood glucose, and serum insulin

Levels of glycosylated HbA1c in mouse blood samples were measured on a DCA 2000 analyzer according to the manufacturer’s instructions (Bayer, Pittsburgh, PA). The glucose levels from either mouse blood or cell supernatant were determined using an Elite glucometer (Bayer). After blood clotting, blood samples were centrifuged to obtain sera and the serum insulin levels were measured by ELISA using a commercial kit (Mercodia, Uppsala, Sweden). The percentage of HbA1c in blood samples was measured using a DCA 2000 analyzer (Bayer, Pittsburgh, PA, USA).

4.7. Cluster analysis of GC–MS chemical profiles

Cluster analysis with matrix visualization of the GC–MS profiles of *Bidens* supercritical CO2 extracts was performed using generalized association plots (GAP) (Chen, 2002; Tien et al., 2008; Wu et al., 2008). Four replicates of the GC–MS chromatograms of each *Bidens* plants were analyzed. The peaks in the GC–MS chromatograms were assigned standardized retention times in order to account for the variations in retention times during GC–MS analysis. The standardized retention times were obtained by averaging the retention times obtained from four separate analyses. Peaks with retention times between 15 min and 35 min were chosen for the hierarchical cluster tree analysis with matrix visualization. Relationships between samples of the *Bidens* plants were represented as a correlation matrix map and tree diagram with the length of the dendrogram denoting the level of distance between the GC chromatograms of the samples (Fig. 2C).

4.8. Validation of inter-variant discrimination power of the GC–MS profiles

It is necessary to validate the clustering and visualization results identified in Fig. 2 that GC–MS profiles can be used to determine the taxonomy of the three common *B. pilosa* variants. Many statistical classification methods coupled with validation procedures are available for discriminating the three *B. pilosa* variants and for assessing the discriminant accuracy. For our study, classification tree method (Brieman et al., 1984) was used for classifying twelve *B. pilosa* samples into three variants (BPR, BPP, and BPM) using the GC–MS profiles with 68 retention times. A classification tree recursively splits each intermediate node into purer (in terms of sample variations) daughter nodes using variables (the percent relative area of GC–MS peaks) with the best discriminant power till all terminal nodes contain sample from only one variant or when certain criteria are satisfied. In selecting an appropriate validation procedure for estimating the accuracy (classification error rate) of the classification tree method, a LOO validation procedure (Devijver and Kittler, 1982) and a modified LOO approach, LOPCO procedure, were conducted. In the LOO procedure, we used one single sample for validation (testing set) while using the other eleven samples as training set. The procedure was then repeated twelve times such that each sample was used once as validation (testing) set. In the LOPCO procedure, one sample from each class was taken as testing set while the other nine samples (three from each variant) were used as training set. The procedure was repeated 64 (4 × 4 × 4) times in such a way that all combinations of one sample in each group were used for validation.

4.9. Statistical analysis

Data from five or more independent measurements in each group are presented as mean ± SEM. Repeated measurement analysis of variance (ANOVA) was used to analyze changes in blood glucose levels and other parameters. Bonferroni’s multiple comparison test was used for post hoc comparisons when appropriate to determine the source of significant differences. *P* values less than 0.05 were considered to be statistically significant.

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