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^{1}H-NMR-based metabolomics approach to understanding the drying effects on the phytochemicals in *Cosmos caudatus*

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ABSTRACT

Cosmos caudatus, locally known as *ulam raja*, is a local Malaysian herb traditionally used both as a food and medicinal herb to treat several maladies. Proton nuclear magnetic resonance (^{1}H-NMR) combined with principal component analysis (PCA) and partial least-squares analysis (PLS) was applied to distinguish variations among *C. caudatus* materials processed with various drying techniques. A discriminatory report on the metabolites responsible for the variation between air (AD), oven (OD) and freeze (FD) dryings of *C. caudatus* samples was successfully achieved using ^{1}H-NMR-based metabolomics. The correlation between antioxidant activity (AA) represented by radical DPPH scavenging activity and metabolite variation among processed samples dried in three different ways was also determined by partial least-squares analysis (PLS). The PCA and PLS scores showed a noticeable and clear separation into three clusters representing the three drying methods by PC1 and PC2, with an eigenvalue of 77.9%. Various assigned ^{1}H-NMR chemical shifts referring to the metabolites responsible for sample variation were ascribed. The identified compounds were β- and α-glucose, chlorogenic acid, catechin, rutin, quercetin, quercetin 3-*O*-rhamnoside, quercetin 3-*O*-β-arabinofuranoside and quercetin 3-*O*-β-glucoside. This research demonstrates that most of the identified compounds were present in all samples from the three processing methods. However, there was marked variance in the concentration of the constituents present.

1. Introduction

Plants are valuable to human life for numerous purposes including food, medicine and beauty. Thus, research on establishing the virtues related to their uses and the responsible constituents affecting it has been the attractive targets for many scientists. Because of their role in preventing various chronic maladies, plant consumption also plays a prominent nutritional role in the diet. There is a strong inverse correlation between the intake of plants and chronic maladies. Recently, our changing lifestyle obligates the search and characterization of the beneficial plant contents that offer defense against these diseases.

Cosmos caudatus (Asteraceae) is commonly known as *ulam raja* in Malaysia, and has been one of the most popular medicinal plant traditionally used to treat several diseases (Abas, Shaari, Lajis, Israf, & Kalsom, 2003; Rasdi, Samah, Sule, & Ahmed, 2010). This herb was discovered several decades ago in North America and over several decades, it has spread to numerous parts of the world, including tropical and subtropical countries (Abas et al., 2003; Huda-Faujan, Noriham, Norrakiah, & Babji, 2009; Rasdi et al., 2010; Shui, Leong, & Shih, 2005; Sukrasno, Fidriany, Anggadiredja, Handayani, & Anam, 2011). The leaves and stems of *C. caudatus* are consumed raw, whereas all its parts are extracted for medicinal purposes. These extracts have been claimed to be efficacious in treating decreased bone mineral density, lowering high blood pressure and improving blood circulation (Burkill, 1966). Several compounds have been reported from this herb which include quercetin 3-*O*-rhamnoside, quercetin 3-*O*-β-arabinofuranoside, quercetin 3-*O*-β-glucoside, quercetin, proanthocyanidin, crypto-chlorogenic acid, neo-chlorogenic acid, chlorogenic acid, catechin, epicatechin, myricetin, and naringenin (Abas et al., 2003; Mustafa, Hamid, Mohamed, & Bakar, 2010; Shui et al., 2005; Sukrasno et al., 2011). However, there is a lack of knowledge about other metabolic content. Although the medicinal efficacy of *C. caudatus* was previously reported (Shui et al., 2005), no report on its metabolomes has been published in the literature.

Metabolomics is a comprehensive tool for evaluating the metabolite variation of organisms under different conditions (Kim, Choi,

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& Verpoorte, 2010; Shuib et al., 2011; Verpoorte, Choi, & Kim, 2007). The metabolomics has also been used for monitoring plant metabolic changes (Abdel-Farid, Hye, Young, & Verpoorte, 2007; Shuib et al., 2011). In many physiological circumstances a wide range of metabolites were found to be changed including phenolic compounds, amino acids, fatty acids, organic acids, carbohydrates and sterol based compounds (Gershenzon, 1984). Changes in environmental pattern and processing methods can alter metabolic contents. This differentiation can be measured via metabolomics approach based on several techniques and the implementation of sophisticated statistical analysis. NMR spectrometry is one of the most utilized techniques for discrimination of these variations in numerous scientific fields (Defernez & Colquhoun, 2003; Georgiev, Ali, Alipieva, Verpoorte, & Choi, 2011; Kim, Choi, et al., 2010; Kim, Hyun, Yang, Choi, & Lee, 2010; Son et al., 2008, 2009; Verpoorte et al., 2007). The multivariate statistical methods used in this work help to reduce a huge data sets to a more manageable size (Cozzolino, Cynkar, Shah, & Smith, 2011; Lee et al., 2011).

Drying is generally applied to remove the excess of moisture from herbs to extend their shelf lives without any detection of spoilage during storage. It has also been used to control the dosage of some incorporated phytochemicals in food products. Air drying (AD) and oven drying (OD) are widely used for herbs processing due to their low cost and easy application even in a domestic setting. Various studies have indicated that the drying method influences the level of the phenolic and other compounds in *C. caudatus* (Sukrasno et al., 2011) as well as in other herbs (Arslan & Özcan, 2010; Chan et al., 2009; Hossain, Barry-Ryan, Martin-Diana, & Brunton, 2010; Larrauri, Ruperez, & Saura-Calixto, 1997; Lim & Murtijaya, 2007), which may possess AA and other medicinal values. However, there has not been any published data currently available on the drying effects on the majority of nutritional metabolites of this plant. Therefore, the aim of the present study is to distinguish the metabolic variation between the air, freeze and oven-dried *C. caudatus* samples using ^{1}H-NMR based metabolomics approach in combination with multivariate data analysis (MVDA). This information acquired will be useful as the basis for recommendation regarding the suitability of the processing method in order to preserve the metabolic features and beneficial values of the herbal material.

2. Materials and methods

2.1. Chemicals

Deuterated methanol-*d4* (CH_3OH-d_4), non deuterated KH_2PO_4, sodium deuterium oxide (NaOD), trimethylsilyl propionic acid-*d4* sodium salt (TSP) and deuterium oxide (D_2O) were supplied by Merck (Darmstadt, Germany). Liquid nitrogen was purchased from MOX Company (Petaling Jaya, Malaysia). Standard compounds used in this experiment such as quercetin 3-*O*-arabinofuranoside, quercetin 3-*O*-rhamnoside, quercetin 3-*O*-glucoside, rutin and quercetin were previously isolated by our group (Abas et al., 2003) while chlorogenic acid, sucrose and glucose were purchased from Sigma (St. Louis, USA).

2.2. Plant material

The plant seeds were provided by the Institute of Bioscience, Universiti Putra Malaysia (UPM) and were planted in the UPM Agricultural Park. A plot was established in an open field with exposure to a temperature of 28 °C during the day and 21 °C during the night; the relative humidity ranged from 80 to 90%. The plants were exposed to direct day sunlight. The soil was treated, fertilized, turned and covered with a black plastic. The seeds were planted in equidistant holes made in 2 rows in the plastic sheath, with every row contained 20 plants. During sowing, 5 to 7 seeds were placed in the center of each hole, and were later thinned to one plant per hole. The irrigation was carried out automatically with a hydraulic system as well as manually. Organic fertilization was performed every two weeks in addition to the first treatment at the beginning of planting. Pesticide treatment was avoided during growing period.

2.3. Sampling

The planted plot was divided into 10 sections, each of which contained 4 plants. Triplicate leave samples of the eight-week-old plants were randomly cut from each section with laboratory scissors for the three drying methods discarding any damaged leaves. To ensure the consistency of metabolite contents; similar leaves were harvested in the early morning. To avoid inconsistency in the resulting data due to technical variance the sampling and sample preparation were performed in group and in concurrent manner. A total of 30 samples for each of the three drying methods were prepared resulting in a grand total of 90 samples for analysis. The samples were weighed to 5 g, labeled in separate plastic bags and immediately subjected to further analysis.

2.4. Sample preparation

Upon harvesting, the samples were washed with distilled water and dried with tissue paper. The FD samples were immediately ground into a fine powder in a mortar with liquid nitrogen and kept in plastic tubes. These samples were then subjected to overnight freezing at −80 °C. The frozen samples were then lyophilized until a constant weight is reached. The oven dried (OD) samples were prepared depending on the optimized drying time and oven temperature conditions, which were previously determined. A Mammert laboratory oven was used for oven drying, which operated under forced-air ventilation at 44.5 °C for 4 h. The air-dried (AD) samples were exposed to a room temperature of 25 °C for 6 days and then ground in a laboratory blender to the similar particle size with FD and OD samples. The powders of all the three dried materials were kept and protected from light and humidity in aluminum pouch by vacuum packaging and labeled for further analysis.

2.5. Extraction method and NMR measurement

The protocol designed by Kim, Choi, et al. (2010) and Kim, Hyun, et al. (2010) was implemented for the extraction procedures, with a few modifications. A 25 mg sample of each dried materials was transferred to a 2 ml-micro tube. The samples were extracted with 0.375 ml CH_3OH-d_4 solvent and 0.375 ml KH_2PO_4 buffer in D_2O (pH 6.0) containing 0.1% TSP. The tubes were vortexed for 1 min at room temperature and then, ultrasonicated for 15 min without heating. The solutions were centrifuged for 10 min at 13,000 rpm to separate the supernatant from precipitate. The supernatant (0.6 ml) was transferred to an NMR tube and subjected to ^{1}H-NMR analysis. The ^{1}H-NMR measurements were performed using a 500 MHz Varian INOVA NMR spectrometer (Varian Inc., California, USA) functioning at frequency of 499.887 MHz and maintained at 26 °C. NMR analysis was performed on all the 90 samples. The acquisition time of each ^{1}H-NMR spectrum was 3.53 min, which consisted of 64 scans with a width of 20 ppm. The phasing and baseline corrections were conducted using the Chenomix software (v. 5.1, Alberta, Canada) with a consistent setting for all sample spectra. For some constituents, additional support for their identification was obtained using two dimensional ^{1}H–^{1}H *J*-resolved and heteronuclear multiple bond correlation (HMBC). The ^{1}H-NMR was run for expected metabolites and previously identified *C. caudatus* metabolites following the above described method.

2.6. Bucketing of ^{1}H-NMR spectra and multivariate data analysis

The bucketing of ^{1}H-NMR spectra was performed using Chenomix software (v. 5.1, Alberta, Canada). All spectra were automatically binned to ASCII files with identical parameters as follows; a spectral width (δ 0.04) forming a region of 0.5–10.0 to give a total of 216 integrated regions per NMR spectrum. The chemical shift ranges of δ 4.70–4.90, representing water and δ 3.23–3.36, representing residual methanol were excluded.

After binning the NMR spectra, sample triplicates were averaged to give a total of 30 samples that were discriminated with statistical analysis. Multivariate data analysis (MVDA) by principal component analysis (PCA) and partial least-squares analysis (PLS) were performed with SIMCA-P software (v. 12.0, Umetrics, Umeå, Sweden) using Parreto scaling method (Granato, Branco, Faria, & Cruz, 2011). In the resulting data matrix, the sample names were considered to be the observation and NMR chemical shift was the variables. Dimension of these resultant data matrices was 232×41. Thirty samples were used to develop the PLS model.

2.7. Extraction of samples for antioxidant DPPH radical scavenging assay

A portion of the dried sample (4 g) from each of the three drying methods was ground into a fine powder and immersed in 100 ml of 80% methanol in a 250 ml amber bottle. The bottles were shaken to mix the powder with the solvent, and the mixtures were subjected to a sonicator bath for 1 h at 25 °C. The sonication time was divided into two equal intervals, with a 15 min break to avoid a temperature increase in the bath. The mixtures were then filtered with cotton twice and filter paper to remove any debris. The residual solvents were then removed by rotary evaporation under a partial vacuum at 40 °C. The concentrated extracts were stored in amber bottles in a freezer for future utilization.

2.8. Free radical scavenging assay

A modified method was used for the DPPH assay (Chan et al., 2009). An aliquot (1 ml) of each diluted sample (6.25, 12.5, 25, 50, 100, and 200 μg/ml) and the solvent as a control were mixed with 2 ml of a DPPH solution (5.9 mg/100 ml of the solvent) in a test tube. The tubes were agitated to homogenize the mixtures and incubated in the dark for 30 min before recording the absorbance at a wavelength of 517 nm. The results are expressed as IC_{50} value in μg/ml of dry extract. To improve the precision of the results, ten replications were used. The radical scavenging activity was measured as a decrease in the absorbance of DPPH and thus, a lower absorbance indicated a higher free radical scavenging activity. For the purpose of correlating the activity with chemical composition, the results were converted to antiradical activity ($1/IC_{50}$) to invert the relation between absorbance and DPPH activity by the IC_{50}.

2.9. Statistical analysis

Data were analyzed using InStat V2.02 statistical package (GraphPad Software, San Diego, CA, USA). Differences in means were determined using ANOVA. Results are expressed as a mean of three determinations ± SD. Significance level was set as $p<0.05$.

3. Results and discussion

3.1. ^{1}H-NMR spectra of the three dried materials and metabolite identification

C. caudatus is a prominent medicinal herb that possesses potent biological activities (Abas et al., 2003). However, there is still a lack of research on its metabolites and their changes when this herb is processed with different drying techniques. Thus, in the present work, metabolomics analysis was applied to investigate the metabolite variations among the air, freeze and oven-dried *C. caudatus* samples. Metabolites from this plant including phenolic acid (chlorogenic acid), carbohydrates (sucrose and glucose), amino acids (alanine and valine), organic acid (formic acid) and flavonoids (rutin, quercetin, quercetin 3-*O*-α-rhamnoside, quercetin 3-*O*-β-arabinofuranoside, quercetin 3-*O*-β-glucoside and catechin), were well identified. ^{1}H-NMR spectra of the plant materials processed with the three drying methods displayed both quantitative and qualitative variations of some metabolites (Fig. 1A). The compounds were identified based on the two-dimensional (2D) NMR spectra and the comparison with the NMR signals of those isolated metabolites from this plant. Other compounds were assigned by comparison with the NMR spectra of reference compounds measured under the same conditions. The metabolites characterized using these methods are presented in Table 1. In aliphatic region (δ 0.5–3.0), some amino acids were recognizable such as alanine and valine. α-Glucose, β-glucose and sucrose were ascribed based on the signals displayed in the carbohydrate region (δ 3.0–5.5). In the aromatic region (δ 5.5–9.0), there were signals assigned for chlorogenic acid, rutin, quercetin, quercetin 3-*O*-α-rhamnoside, quercetin 3-*O*-β-arabinofuranoside, quercetin 3-*O*-β-glucoside and catechin. However, the three different drying treatment sample spectra showed a marked variation in the intensity of the signals, especially those in the aromatic region (Fig. 1B).

Despite the lower sensitivity of NMR as compared to mass spectrometry (MS) and the potential problem of signal overlapping in NMR, the 2D-NMR experiments have been greatly beneficial in

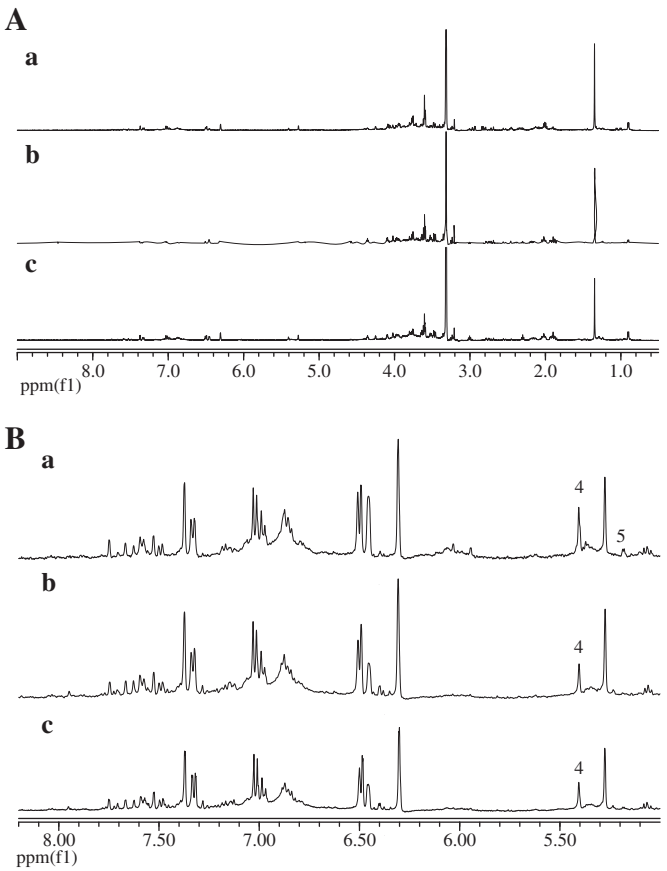

Fig. 1. (A) The representative ^{1}H-NMR spectra of the three *C. caudatus* extracts dried using different methods (a) FD, (b) AD and (c) OD. (B) The representative expanded ^{1}H NMR spectra of the three *C. caudatus* samples in the δ 5.00 to 8.2 range (a) FD, (b) AD and (c) OD: 1, quercetin derivatives; 2, chlorogenic acid; 3, catechin; 4, arabinosyl; 5, α-glucose.

Table 1
^{1}H-NMR characteristic signals of identified metabolites in *C. caudatus* extracts.

Metabolite	^{1}H-NMR characteristic signals	FD	AD	OD
Quercetin (1)	δ 6.20 (d, *J*=2.0 Hz), δ 6.40 (d, *J*=2.0 Hz), δ 7.73 (d, *J*=2.0 Hz), δ 6.83 (d, *J*=8.5 Hz), δ 7.60 (dd, *J*=8.5 Hz, 2.0 Hz)	+	+	+
Quercetin 3-*O*-β-arabinofuranoside (2)	δ 6.18 (d, *J*=2.0 Hz), δ 6.37 (d, *J*=2.0 Hz), δ 7.73 (d, *J*=2.0 Hz), δ 6.88 (d, *J*=8.5 Hz), δ 7.47 (dd, *J*=8.5 Hz, 2.0 Hz), δ 5.26 (d, *J*=7.6 Hz)	+	+	+
Quercetin 3-*O*-α-rhamnoside (3)	δ 6.18 (d, *J*=2.0 Hz), δ 6.35 (d, *J*=2.0 Hz), δ 7.73 (d, *J*=2.0 Hz), δ 6.88 (d, *J*=8.5 Hz), δ 7.30 (dd, *J*=8.5 Hz, 2.0 Hz), δ 5.49 (d, *J*=1.5 Hz). Methyl signal: δ 0.91 (d, *J*=6.0 Hz)	+	+	+
Quercetin 3-*O*-β-glucoside (4)	δ 6.20 (d, *J*=2.0 Hz), δ 6.39 (d, *J*=2.0 Hz), δ 7.73 (d, *J*=2.0 Hz), δ 6.86 (d, *J*=8.5 Hz), δ 7.60 (dd, *J*=8.5 Hz, 2.0 Hz), δ 5.16 (d, *J*=8.0 Hz)	+	+	+
Rutin (5)	δ 6.20 (d, *J*=2.0 Hz), δ 6.39 (d, *J*=2.0 Hz), δ 7.57 (d, *J*=2.0 Hz), δ 6.90 (d, *J*=8.5 Hz), δ 7.56 (dd, *J*=8.5 Hz, 2.0 Hz), anomeric proton glucosyl δ 4.97 (d, *J*=8.0 Hz), rhamnosyl δ 4.54 (d, *J*=1.0 Hz)	+	+	+
Chlorogenic acid (6) (5-*O*-caffeoyl quinic acid)	Signal for caffeoyl δ 7.62 (d, *J*=16.0 Hz), δ 6.37(d, *J*=16.0 Hz) and quinic δ 4.09 (m, *J*=3.0 Hz), δ 2.63 (dd, *J*=1.3, 5.0 Hz), δ 1.9 (d, *J*=10.0 Hz)	+	+	+
β glucose	δ 4.59 (d, *J*=8.0 Hz)	+	+	−
α glucose	δ 5.19 (d, *J*=3.5 Hz)	+	+	−
Sucrose	δ 5.40 (d, *J*=3.5 Hz)	+	+	+
Fatty acid	δ 1.35 (m)	+	+	−
Catechin (7)	δ 6.46 (d, *J*=2.0 Hz), δ 6.49 (d, *J*=2.0 Hz), δ 4.58 (d, *J*=7.2 Hz), δ 3.93 (m), δ 2.56 (dd, *J*=7.5 Hz, 16 Hz), δ 2.84 (m)	+	+	+
Formic acid	δ 8.47 (s)	+	+	−
Choline	δ 3.24 (s)	+	−	+
Alanine	δ 1.49 (d, *J*=7.5 Hz), 3.72 (q)	+	+	−
Valine	δ 1.01 (d, *J*=6.8 Hz), 1.04 (d, *J*=6.8 Hz)	+	+	+

addressing the faced problems in this work. The comprehensive information provided by the *J*-resolved regarding signal splitting and coupling constant helped in assigning and confirming the assigned metabolites indicated by 1D-NMR (Fig. 2). Most of the signals with marked variance between the spectra of the three dried samples were found within the aromatic and carbohydrate regions. There were missing carbohydrate peaks in the spectra of the oven-dried samples compared to the spectra of other drying treatments. On account of these the middle- and high-frequency regions of the spectra were given more emphasis due to their likely contribution to the variance between the chemical compositions of the three dried *C. caudatus* materials.

The metabolite signals assigned to the various carbohydrates were marked as overlapped peaks (Abdel-Farid et al., 2007; Georgiev et al., 2011; Shuib et al., 2011). Despite this, the anomeric proton signals of β-glucose, α-glucose and sucrose were detected at δ 4.59 (d, *J*=8.0 Hz), δ 5.19 (d, *J*=3.5 Hz) and δ 5.40 (d, *J*=3.5 Hz), respectively. These signals were visualized in the freeze and air-dried *C. caudatus* within the range of 3–5.5 ppm (Table 1). However, the intensity of the anomeric carbon signals in freeze-dried was markedly higher than those in the air-dried samples. In the aromatic region, the major compounds assigned in the three extracts were quercetin rhamnoside, quercetin arabinoside, quercetin glucoside and rutin. The characteristic signals for quercetin were those at δ 6.18–6.20 (d, *J*=2.0 Hz, H-6), δ 6.35–6.42 (d, *J*=2.0 Hz, H-8), and the ABX spin system at δ 7.30–7.60 (dd, *J*=8.5 Hz, 2.0 Hz, H-6′), δ 7.73 (d, *J*=2.0 Hz, H-2′) and δ 6.88–6.90 (d, *J*=8.5 Hz). The signals for the anomeric protons of the rhamnosyl, arabinosyl and glucosyl moieties were found at δ 5.49 (d, *J*=1.5 Hz), δ 5.26 (d, *J*=7.6 Hz) and δ 5.16 (d, *J*=8.0 Hz), respectively. Moreover, the methyl signal of the rhamnosyl moiety was attributed to a chemical shift at δ 0.91 (d, *J*=6.0 Hz). The characteristic signals for chlorogenic acid of the caffeoyl moiety at δ 7.62 (d, *J*=16 Hz), δ 6.37(d, *J*=16 Hz) and the quinic moiety at δ 4.09 (m, *J*=3.0 Hz), δ 2.63 (dd, *J*=1.3 Hz, 5.0 Hz), δ 1.9 (d, *J*=10 Hz) were observed in the spectra of the three dried materials. Catechin were also attributed due to the resonance signals at δ 6.46 (d, *J*=2.0 Hz), δ 6.49 (d, *J*=2.0 Hz), δ 4.58 (d, *J*=7.2 Hz), δ 3.93 (m), δ 2.56 (dd, *J*=7.5 Hz, 16 Hz) and δ 2.84 (m). All identified metabolites as shown in Fig. 3 were

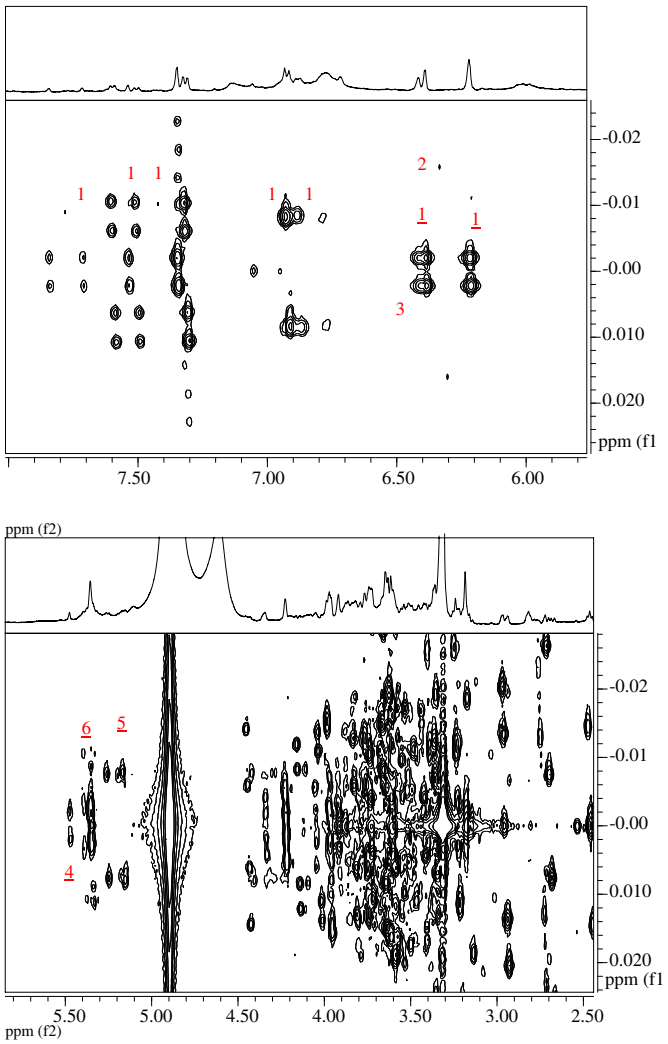

Fig. 2. ^{1}H–^{1}H *J*-resolved spectra of the *C. caudatus* extract in the region δ 2.5 to 8.0. The observed signal as follows: 1, quercetin derivatives; 2, chlorogenic acid; 3, catechin; 4, arabinosyl; 5, α-glucose; and 6, sucrose.

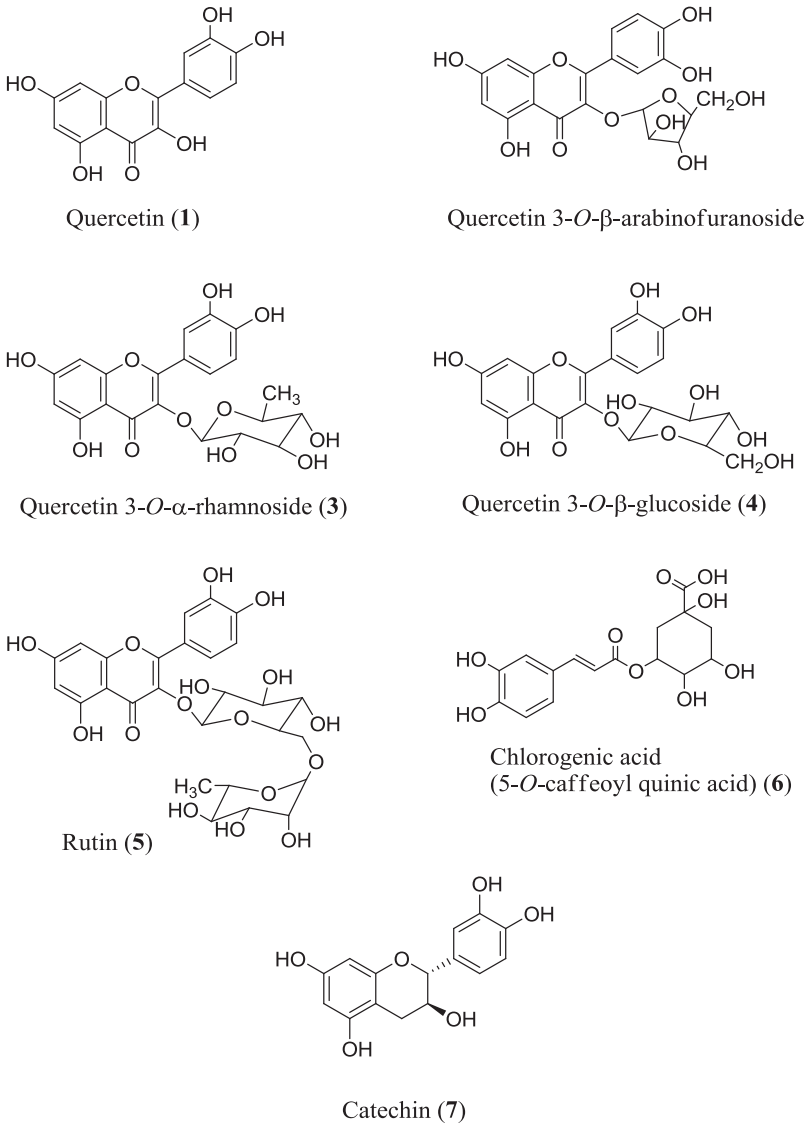

Fig. 3. Chemical structures of some identified metabolites in *Cosmos caudatus* extract. The numbering is according to the identified metabolites in Table 1.

confirmed by scrutinizing the spectral pattern and their characteristic signals as well as by comparison with those of the authentic compounds and an elucidated LCMS chromatogram (results not shown). Most of the signals in aromatic region were more intense in AD and FD as compared to those in the OD samples.

3.2. Classification of dried C. caudatus by PCA

The research on natural antioxidants from plant materials continues to attract interest due to their potential as replacement for synthetic antioxidants. Several processing methods, especially in the drying, can markedly affect the structures and bioactivity of some metabolites. Thermal processing and OD were reported to have an extensive negative impact on bioactivity of plant extracts because of their effect on the phytochemical content. Thus, it would be desirable to establish the variability of the product derived from different methods of drying. The information generated will be useful as the guide to select the best methods to be applied for the sake of efficiency, conservation of metabolite constituents and its antioxidant capacity.

The changes of metabolite content in air-, freeze- and oven-dried *C. caudatus* samples were further evaluated using MVDA. PCA was applied to understand the clustering features of the three dried samples and the metabolites contributing to the variability. The utilization of PCA in MVDA is to recognize the pattern and cluster of the samples depending on their variance by exposing the samples to different principal components (PCs). The PC is used by PCA to project original data depending on a certain feature that allows sample variability evaluation in a simple manner. The PCA score plot shows the available cluster between samples, and loading plot indicates the rate of variable contributions and their correlation to the sample differences (Son et al., 2008). In this study, PCA was performed on the NMR data of the *C. caudatus* materials dried using three different methods to evaluate the variance in their metabolite content and concentration. The score plot was performed to evaluate the variation within the processed samples with regard to the three drying methods, whereas the loading plot indicated the metabolite signals that may contribute to cluster differentiation.

From the score plot, three clear separated clusters were identified by PCA without any notable outliers (Fig. 4A). PC1 showed the most sample variation, followed by PC2. An eigenvalue of about 77.9% was described by the first two PCs, accounting for a variance of 49.5% by PC1 and 28.4% by PC2. The results of loading column plot indicated some metabolic variation, which caused separation (Fig. 4B and C). Flavonoids and flavonoid glycoside contents were higher in FD and

AD than in OD materials. However, their intensities were markedly higher in FD. Rutin and catechin, the representative examples of flavonoids, were also present in a large quantity in the FD samples. A high amount of both α- and β-glucose and chlorogenic acid in FD samples distinguished it from other drying methods. The low concentration of sugars in the AD samples is due to degradation possibly by thermal effect or air oxidation. These could also be affected by the residual oxidative enzyme during the drying process. The absence of sugar signals in the NMR data of the OD samples as opposed to those of the FD and AD samples (Table 1), might be due to the possible degradation of these components or the Maillard reaction induced by heat treatment (Puupponen-Pimiä et al., 2003). The concentration of the identified and unidentified amino and fatty acids in the FD samples was a marked variation from the AD and OD materials. This is not surprising since the low temperature applied in the FD methods can stopped most of the potential degradation events. An interesting and surprising finding was that OD materials were distinguished by their high content of sucrose and choline.

3.3. Comparison of DPPH scavenging activity of the three dried *C. caudatus* samples

Drying has a profound effect on metabolites responsible for antioxidant efficiency (Asami, Hong, Barrett, & Mitchell, 2003; Chang, Lin, Chang, & Liu, 2006; Lim & Murtijaya, 2007). However, this effect is different from one drying method to another. Numerous methods are used to evaluate the AA of plant extracts, which cannot be assessed by any single assay due to the multiple ways that AA can be affected. The DPPH assay has been widely applied to evaluate AA (Abas et al., 2003; Ali et al., 2008; Maisuthisakul, Pasuk, &

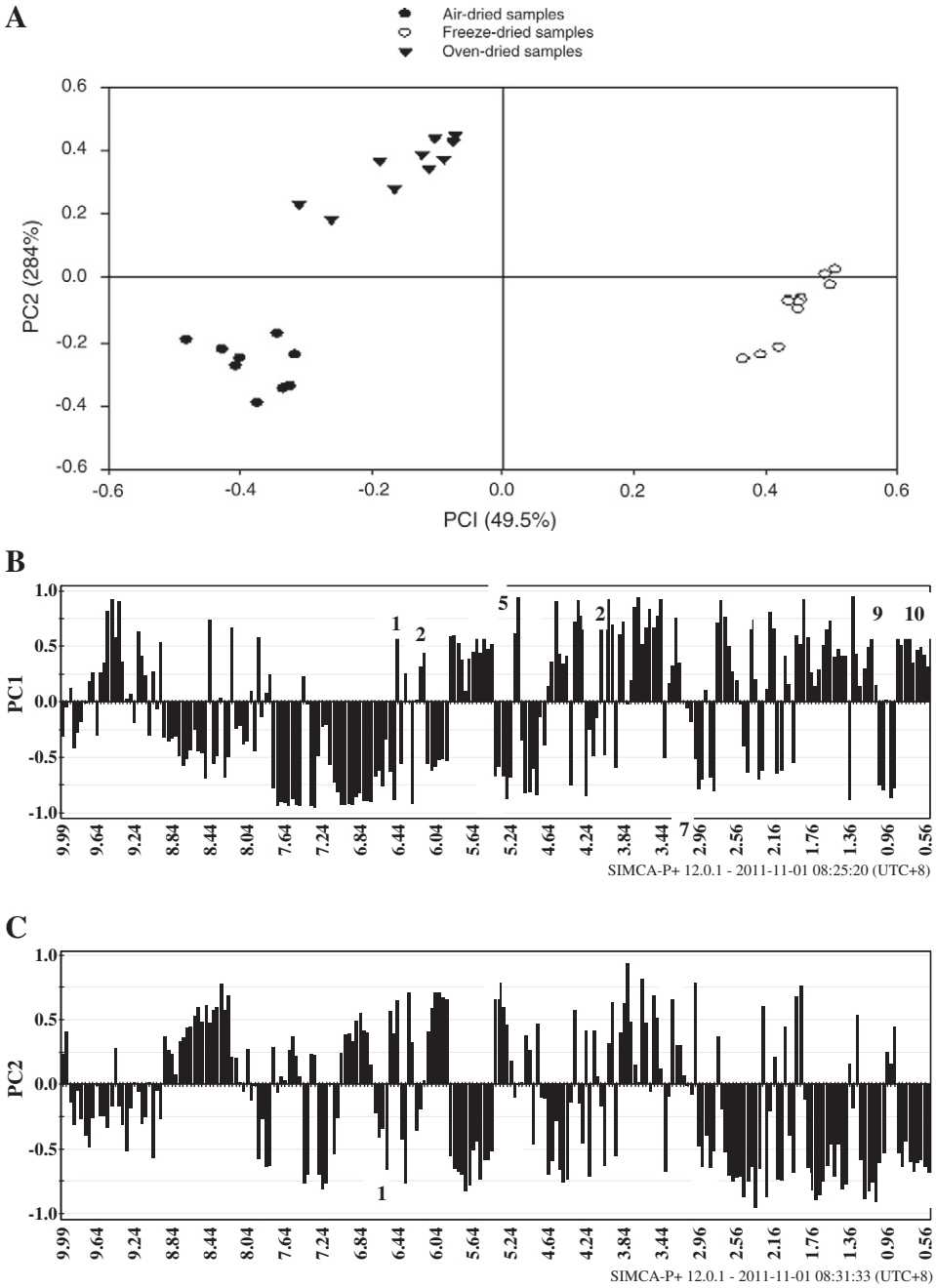

Fig. 4. The PCA score plot (PC1 vs. PC2, A) and the loading column plots of PC1 (B) and PC2 (C) of the ^{1}H NMR data representing all the dried *C. caudatus* materials. Assignments in (B and C): 1, quercetin derivatives; 2, chlorogenic acid; 3, catechin; 4, arabinosyl; 5, α-glucose; 6, sucrose; 7, β-glucose; 8, choline; 9, amino acids; 10, fatty acids.

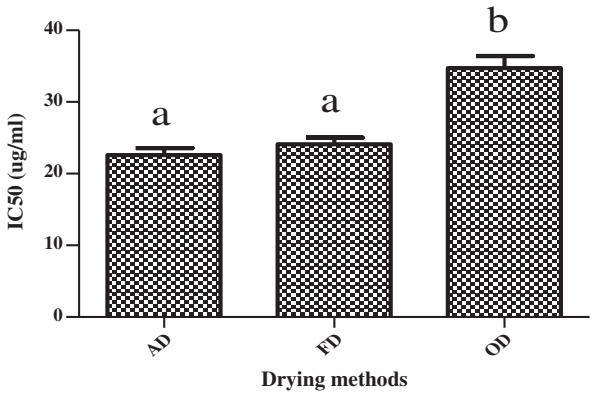

Fig. 5. IC_{50} value of dried *C. caudatus* samples by different drying methods. [a,b] Each different small letter above the column means a statistically significant difference ($p<0.05$; $n=10$).

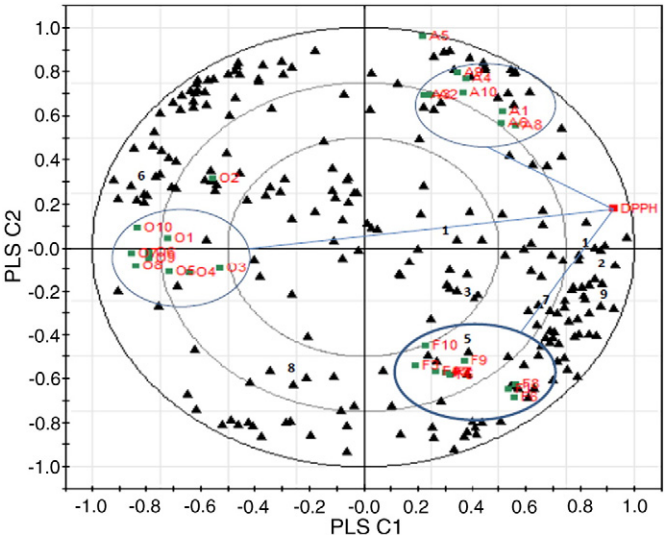

Fig. 6. The biplot obtained from PLS describing the variation between AD, FD and OD *C. caudatus* samples. 1, quercetin derivatives; 2, chlorogenic acid; 3, catechin; 5, α-glucose; 6, sucrose; 7, β-glucose; 8, choline; 9, alanine.

Ritthiruangdej, 2008). This method has many advantages, including its simplicity, rapidity, suitability of the solvent used for most extract and reproducibility. In addition, this assay shows a strong correlation with the metabolites previously known to be responsible for AA, such as phenolic compounds (Hossain et al., 2010; Lim & Murtijaya, 2007; Maisuthisakul et al., 2008; Mustafa et al., 2010; Sukrasno et al., 2011). The evaluation of significant difference between dried samples was conducted using one-way ANOVA test with Tukey comparison. The DPPH assay results showed that the extract from AD and FD samples exhibited non-significant ($p>0.05$) antioxidant capacities which were higher than the OD treated sample (Fig. 5). The DPPH values of the AD, FD and OD treated samples were 22.63 ± 0.97, 23.6 ± 0.94 and 34.73 ± 1.70 μg/ml, respectively. These results could be explained by taking into account the impact of heat treatment in the OD method, which may cause enzymatic denaturation by the Maillard reaction (Arslan & Özcan, 2010).

3.4. Correlation between antioxidant activity and metabolite variation among the samples treated with three drying methods

Several metabolites are associated with the AA of plant extracts including the phenolic compounds. These phenols are responsible for quenching free radicals that cause oxidative stress to plants. To further investigate the correlation between AA and the impact of the three drying methods on *C. caudatus*, PLS was applied using a validation model with a degree of overfit between the variables and the responses (Maisuthisakul et al., 2008). Furthermore, PLS was performed to obtain a more precise view on the correlation between the DPPH scavenging activity, the phytochemical present and the drying methods utilized. This approach was adopted because PLS has greater success in linking DPPH with phytochemicals and in providing models for prediction (Kim et al., 2011). The PLS model could indicate correlation between chemical and DPPH data, which is used in predicting variations among dried samples (Maisuthisakul et al., 2008). The same chances were given to the variables influencing the assessment of the DPPH variables by weighting data with the standard deviation. The PLS evaluation could assist in the determination of a correlation between the anti radical activity ($1/IC_{50}$) and phytochemical composition of extracts from AD, FD and OD samples.

From the PLS biplot that combines score and loading plots, three clusters were clearly noted (Fig. 6), as previously shown from the PCA score plot. A differentiation analysis of the chemical composition between the dried samples from the three drying methods and their contribution and correlation with radical scavenging activity were also performed (Fig. 6). The FD and AD samples were strongly correlated with DPPH scavenging activity. The OD samples were projected in the negative side of the plot far away from DPPH. The compounds contributed to this separation by PC1 were quercetin derivatives, chlorogenic acid, both the α- and β-glucose, catechin and alanine as previously marked from PCA results. All these compounds were located closer to both FD and AD samples and DPPH rather than OD samples. The OD samples were separated from the others by their high content of sucrose and choline. A high content of both the α- and β-glucose, catechin and chlorogenic acid were markedly higher in FD compared to other samples. This finding confirmed the DPPH bioassay results, which showed that the OD had the highest negative impact on the antioxidant capacity of the *C. caudatus* materials. This might result from the thermal processing effect during this drying method, which can alter compounds that may negatively contribute in AA. Chlorogenic acid, quercetin derivatives, rutin, amino acids and some unknown compounds were strongly correlated with DPPH activity. Other identified metabolites did not contribute in the variation and could have a negative correlation with AA. The high antioxidant capacity observed in FD samples could be due to the low temperature applied and the ability to preserve the natural compounds especially those known to be active as antioxidant compounds during the freeze-drying process.

PLS model validation was performed; by checking the Q^2 and R^2 accumulative means after cross validation and permutation test with 100 permutations. External validation was also performed. In the first validation, R^2 determines the model fitness significant and explains the quality of Y variables by the model, while Q^2 provides the predictive quality of the model. R^2 also defines the feature of mathematically reproducibility of the training set data. The closeness of both Q^2 and R^2 values to 1 reflects the higher performance of the model. The model is considered to have a good fit and perfectibility when the Q^2 and R^2 accumulative values ranged between 0.8 and 1. In addition, the more PC elements are included in the model shows the higher Q^2 and R^2 accumulative values.

In this study, all Q^2 and R^2 values fell between 0.8 and 0.93 thus, all model had great fitness and predictive qualities. The highest contributing component which shows the perfect performance was PC3. Permutation test and external validation met the criteria of a good performance PLS model. In external validation, the root-mean-square of both errors of the estimation and prediction was close to each other. These errors were markedly decreased as the number of PC included in the model is increased.

4. Conclusion

A discriminatory rapport on the metabolites responsible for the variation between AD, OD and FD samples was achieved using ^{1}H-NMR-based metabolomics approach coupled with MVDA. The PCA score plot allowed the separation of the products from three drying methods on *C. caudatus* samples to three clusters. Both freeze- and air-dried samples provided potentially beneficial and valuable products in term of their antioxidant capacity. They contained higher level of flavonoids and sugars than oven-dried samples. FD sample also exhibited a marked accumulation of amino and fatty acids compared to the other methods. The changes in some metabolites such as flavonoids caused the variation in AA of the three dried *C. caudatus* materials. PLS analysis showed a strong correlation between both the FD and AD samples with DPPH radical scavenging activity as compared to OD. The work presented herein demonstrates that FD and AD were the best drying methods to yield potentially valuable *C. caudatus* products in term of its radical scavenging activity and therefore may be towards producing high-value medicinal and nutritional *C. caudatus* product.

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