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Phytochemical diversity of *Clinacanthus nutans* extracts and their bioactivity correlations elucidated by NMR based metabolomics

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ABSTRACT

Clinacanthus nutans is a medicinal herb and a traditional remedy for herpes viral infection, cancer, and diabetes mellitus. Despite its popular use, there is limited information on the chemical constituents and their relationship with the bioactivities of the herb. The choice of drying and extraction methods will greatly influence the metabolite profile and the bioactivities of an herbal extract. In order to maximize retention of the original chemical profile of the herb and quality assurance, optimization of processing methods is needed. Using nuclear magnetic resonance (NMR) based metabolomics approach, we have carried out a discriminative analysis of the metabolite profiles of the leaves and stems of the herb when different drying (air, oven, and freeze) and extraction (soaking and sonication) methods were used and correlated the metabolite profiles with the total phenolic content (TPC), antioxidant and α-glucosidase inhibitory activities. Identification of primary and secondary metabolites was performed using 1D- and 2D-NMR techniques as well as ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS). Results showed that the leaf extracts, which were richer in phenolic compounds and terpenoids, showed significantly higher bioactivities compared to the stem extracts. Several newly reported compounds in the herb, identified using tandem mass spectrometry, included gendarucin A, a gendarucin A isomer, 3,3-di-*O*-methylellagic acid, ascorbic acid, and two isomeric oxoprolinates. From the NMR metabolomics analysis, the PLS biplot model indicated that the presence of some phenolics compounds, terpenoids, and sulfur-containing glucosides in the oven and air dried leaf extracts are the main components responsible for the antioxidant and α-glucosidase inhibitory activities. This study has provided additional information on the chemistry and biology of the herb that may be useful in future development phytomedicinal preparations of *C. nutans*.

1. Introduction

Clinacanthus nutans (Burm f.) Lindau, is a small herb belonging to the Acanthaceae family. The plant is native to tropical Asia where it is regarded as an important herb in traditional medicine, notably in Thailand, Indonesia, and Malaysia where it is known as Sabah snake grass or 'belalai gajah' (Sakdarat et al., 2009). In Thailand, the leaf of *C. nutans* is used in primary healthcare for the treatment of herpes virus infections and as an anti-inflammatory agent (Wanikiat et al., 2008). In recent years, the leaves and the aerial part of this plant have also gained popularity in Malaysia, particularly as a traditional remedy for diabetes mellitus and cancer (Arullappan et al., 2014; Yong et al., 2013).

Diabetes mellitus is a metabolic disorder characterized by chronic postprandial hyperglycemia and is accompanied by disturbances in the carbohydrate metabolism (Zhou et al., 2012). This leads to an imbalanced production of excessive reactive

Abbreviations: GLM, general linear model; TPC, total phenolic content; MVA, multivariate data analysis.

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oxygen species (ROS), which overwhelms the organism's endogenous antioxidant defenses, resulting in degenerative diseases. The current clinical drugs for diabetes include α-glucosidase inhibitors which slow down the digestion of carbohydrates in the small intestine and therefore, reduce the after meal blood sugar levels. However, the use of synthetic α-glucosidase inhibitors, such as acarbose and miglitol, have been reported to cause side effects such as liver disorders, flatulence, renal tumors, and hepatic injury (Zhou et al., 2012). Using botanical extracts having α-glucosidase inhibition activity holds great promise as an alternative treatment for diabetes since they are expected to have lesser side effects. Furthermore, several studies have shown a positive correlation between the high phenolic contents of several medicinal herbs, used as a remedy for diabetes, to their strong antioxidant activities (Lee et al., 2014; Wan et al., 2012).

An intrinsic part of the concept and philosophy of herbal medicine is synergism or combined effects of its chemical constituents rather than the action of a single constituent (Williamson, 2001). Thus, it is of paramount interest to preserve the original chemical profile of the herb in order to ensure that the quality and efficacy of the therapeutic effect of the complex mixture is not lost. Suitable processing methods for a particular herb will have to be established and optimized so that alterations to the chemical profile of the herb are kept to a minimum. Currently, very little is known about the possible alterations in the chemical profiles of *C. nutans* resulting from different drying and extraction methods. Previous phytochemical studies on *C. nutans* reported the presence of flavonoids such as belutin, vitexin, isovitexin, shaftoside, isomollupentin-7-*O*-β-glucopyranoside, orientin, and isoorientin (Wanikiat et al., 2008). The presence of sulfur-containing glucosides, glycoglycerolipids, cerebrosides, pheophytins, and sterols were also reported (Sakdarat et al., 2009; Teshima et al., 1998).

Metabolomics is a holistic, non-biased approach in identification and quantitation of all metabolites in an organism. It strives to generate complete metabolic fingerprints, to detect chemical variations between the organisms and to provide a possible explanation for the differences (Georgiev et al., 2011). Detection and analysis of the metabolic changes can be carried out using various analytical platforms depending on the required sensitivity. ^{1}H NMR offers a versatile analytical tool that allows the simultaneous detection of as many metabolites as possible. Furthermore, since the NMR signals of a given chemical compound are proportional to their molar concentration, quantification can be carried out without the need for any calibration curves (Liu et al., 2010). Meanwhile, additional application of two dimensional NMR (2D-NMR) allows structural elucidation of a chemical constituent without a need for further purification since overlapping signals can be easily resolved (Kim et al., 2010b). Similarly, the highly robust and sensitive UPLC-DAD-ESI–MS/MS can help to fast-track targeted metabolite analysis, giving better peak separations in comparison with conventional HPLC systems (Farag et al., 2013).

In continuation of our studies towards bioprospection of the medicinal value of herbs, we investigated the potential of *C. nutans* as an alternative source of antioxidants for protection against chronic diseases, in particular, diabetes mellitus. Thus, extracts of the herb were evaluated for antioxidant and α-glucosidase inhibitory activities. The chemical profiles of the leaf and stem parts of *C. nutans* processed using different drying and extraction methods were determined via ^{1}H NMR and UPLC-DAD-ESI–MS/MS. Additionally, the correlation between the DPPH free radical scavenging and α-glucosidase inhibitory activities to the possible bioactive metabolites in the tissues extracts were investigated through the use of ^{1}H NMR metabolomics. The results of this study will throw some light into the potential use of *C. nutans* as a phytomedicinal preparation.

2. Results and discussion

2.1. Effects of drying and extraction methods on the total phenolic content (TPC), DPPH radical scavenging and α-glucosidase inhibitory activities

The influence of drying and extraction methods on the total phenolic content (TPC) of *C. nutans* can be discerned from the results tabulated in Table 1. The overall results indicated that the combined factors produced a significant effect on the TPCs of *C. nutans*, giving values ranging from 1.04 to 7.29 mg GAE/g dw. All of the considered factors had equal *P* values (Table S1) and based on the General Linear Model (GLM) statistical analysis, the factor that exhibited the most significant influence on TPC was the plant part (*F* value = 16637.91), whereas the drying method had the least influence (*F* value = 909.59). Overall, the leaf extracts were shown to contain higher TPCs (3.19–7.29 mg GAE/g dw) than the stem extracts (1.04–2.24 mg GAE/g dw). By considering the leaf extracts, it was observed that air drying resulted in higher TPC (7.29 mg GAE/g dw) compared to oven (6.37 mg GAE/g dw) and

Table 1
Effect of drying and extraction methods on total phenolic content (TPC), DPPH radical scavenging activity, and α-glucosidase inhibition activity of *C. nutans* leaf and stem.

Plant part	Extraction method	Drying method	TPC, (mg GAE/g dw sample) at 5000 ppm	DPPH radical scavenging activity, % at 5000 ppm	α-glucosidase inhibition activity, % at 5000 ppm
Leaf	Sonication	Freeze dry	$5.10 \pm 0.14_{a}^{ca}$	$25.65 \pm 0.59_{a}^{ba}$	$34.69 \pm 3.68_{a}^{aa}$
		Oven dry	$6.37 \pm 0.06_{a}^{ba}$	$44.31 \pm 3.16_{a}^{aa}$	$41.70 \pm 1.97_{a}^{aa}$
		Air dry	$7.29 \pm 0.11_{a}^{aa}$	$42.66 \pm 0.86_{a}^{aa}$	$37.34 \pm 0.49_{a}^{aa}$
	Soaking	Freeze dry	$3.19 \pm 0.06_{a}^{bb}$	$26.12 \pm 1.03_{a}^{ba}$	$5.31 \pm 0.93_{a}^{cb}$
		Oven dry	$5.23 \pm 0.02_{a}^{ab}$	$35.18 \pm 1.74_{a}^{ab}$	$30.35 \pm 0.35_{a}^{ab}$
		Air dry	$5.01 \pm 0.14_{a}^{ab}$	$33.38 \pm 0.97a^{ab}$	$26.91 \pm 1.80_{a}^{bb}$
Stem	Sonication	Freeze dry	$1.68 \pm 0.05_{b}^{ba}$	$23.40 \pm 1.92_{a}^{ba}$	$16.56 \pm 0.89_{a}^{ca}$
		Oven dry	$2.18 \pm 0.02_{b}^{aa}$	$25.40 \pm 3.03_{b}^{bb}$	$25.20 \pm 3.60_{b}^{ba}$
		Air dry	$2.24 \pm 0.14_{b}^{aa}$	$34.19 \pm 1.11_{b}^{aa}$	$35.70 \pm 2.71_{a}^{aa}$
	Soaking	Freeze dry	$1.04 \pm 0.02_{b}^{cb}$	$15.44 \pm 2.21_{b}^{bb}$	$12.57 \pm 1.91_{b}^{cb}$
		Oven dry	$1.40 \pm 0.04_{b}^{bb}$	$30.37 \pm 0.46_{b}^{aa}$	$17.31 \pm 0.83_{b}^{bb}$
		Air dry	$2.05 \pm 0.02_{b}^{aa}$	$31.38 \pm 3.26_{a}^{aa}$	$23.38 \pm 0.90_{b}^{ab}$

Values are the means ± standard deviation based on six replicates. The subscripts represent comparison between plant parts. The superscripts represent comparison between the drying methods (first) and between the extraction methods (second). Means with different subscript and superscript or subscript letters were significantly different ($P < 0.05$). The mean ± standard deviation for % of DPPH inhibition of standard, quercetin at 62.5 μg/ml was 67.00% ± 4.06. The mean ± standard deviation for α-glucosidase inhibition of standard, quercetin at 62.5 μg/ml was 73.15% ± 9.15. GAE: gallic acid equivalent; dw: dry weight.