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Phytochemical and biological features of *Phyllanthus niruri* and *Phyllanthus urinaria* harvested at different growth stages revealed by ^{1}H NMR-based metabolomics

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ABSTRACT

Several studies have suggested that plants are potential sources of bioactive compounds. *Phyllanthus* is a plant genus that has been used in traditional medicine due to its phytomedicinal metabolites content. The variation between two *Phyllanthus* species (*P. niruri* and *P. urinaria*) was studied using proton nuclear magnetic resonance (^{1}H NMR) combined with multivariate data analysis (MVDA). The total phenolic content (TPC), DPPH radical scavenging activity and α-glucosidase inhibitory activity of the *Phyllanthus* species were also evaluated and correlated with their phytochemical constituents at different growth stages (8, 10 and 12 weeks) using partial least square regression (PLS). Principal component analysis (PCA) and PLS indicated separation between the two species based on the identified metabolites and the screened bioactivities. A comparison of the two species indicated that *P. urinaria* was separated from *P. niruri* due to its larger quantity of fatty and amino acids, choline, phyllanthin and sucrose. However, *P. niruri* contained higher quantities of hypophyllanthin and phenolic compounds. The loading column plot, which was used to compare the *P. niruri* at different growth stages, indicated that the eight-week-old plant contained a higher amount of fatty acids, amino acids (leucine and alanine), phyllanthin and choline. The dominant substances in the *P. niruri* at 10 weeks of growth by PC1 were identified as hypophyllanthin, malic acid, sucrose, and identified phenolics. The 12 week sample was differentiated by its higher sugars contents as well as malic acid and leucine. The harvested samples of both *Phyllanthus* species at ten weeks of age exhibited significant bioactivities with the highest content and number of metabolites.

1. Introduction

Plant extracts and their derived components have become important as various herbal therapies and phytopharmaceuticals. The connection between diseases and oxidative stress indicates that antioxidants possess the ability to protect against similar diseases, which was confirmed by the strong inverse link between the intake of the plant and the reduction of various diseases (Ali et al., 2014; Maisuthisakul et al., 2008). Recent studies indicated a positive link between the plants and the reduction of chronic diseases, such as diabetes (Bansal et al., 2011; Lee et al., 2014). Polyphenols are the most important phytochemicals found in plants with potent antioxidant properties, which partially contribute to the positive influence on the prevention of chronic diseases (Ali et al., 2014; Fischer et al., 2011; Verpoorte et al., 2007).

The *Phyllanthus* species (Euphorbiaceae) are important and popular medicinal ingredient that is commonly used to treat different ailments in several countries around the world (Markom et al., 2007; Moreira et al., 2013). *Phyllanthus* originated from India, but is also grown in the rainforests of the Amazon and other tropical region worldwide, such as the Bahamas, China and Malaysia (Sabir and Rocha, 2008). In Brazil, 'Quebra Pedra' is the traditional name of *Phyllanthus*, which means 'stone breaker'. This species is also used to cure pathological conditions, such as jaundice and other liver

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related diseases. In Malaysia, *Phyllanthus* is known as 'dukung anak', which means 'to carry a child', referring to the fruits at the bottom of each leaf petiole. This genus includes many species and the most popular ones are *Phyllanthus niruri* and *Phyllanthus urinaria*, which exhibit antioxidant and α-glucosidase inhibitory activities (Khatoon et al., 2006; Kumaran and Karunakaran, 2007). Therefore, the two species were selected for this study. *Phyllanthus niruri* has been utilized as a traditional medicine to cure kidney problems, diarrhea, fever, diabetes and colic (Kumaran and Karunakaran, 2007). *Phyllanthus niruri* also has the ability to reduce bad lipids, as well as exhibit an analgesic effect (Amin et al., 2012; Kumaran and Karunakaran, 2007). In China, this plant is used as a traditional folk medicine and is known as 'pearls under the leaves' (Murugaiyah and Chan, 2009). *Phyllanthus urinaria* has several uses in traditional medicine to treat many diseases including liver protection, reduce fever, laxative, cure inflammation, suppress hyperactivity of the liver, improve eyesight diminish, increase problems of urine flow and detoxify poison from the body (Amin et al., 2012; Kumaran and Karunakaran, 2007). Currently, *P. urinaria* is also widely used as an anti-inflammatory and antioxidant agents as well as a smooth muscle relaxer (Catapan et al., 2000; Kumaran and Karunakaran, 2007). Various bioactive and phytochemical compounds are found in *Phyllanthus*, which include the *fl*avonoids, tannins, lignans and other benzenoid compounds (Chang et al., 2003; Khatoon et al., 2006; Kumaran and Karunakaran, 2007; Sprenger and Cass, 2013). In addition, corilagen, gallic acid, caffeolquinic acid, geraniin, and rutin have been identified in this plant (Khatoon et al., 2006; Kumar et al., 2015). This plant is a rich source of many bioactive compounds that have attracted much attention from researchers to study its health benefits and to cure or prevent certain chronic diseases.

The biological activity of plants can be affected by several factors, such the growth condition which might reduce their quality and beneficial effects (Maulidiani et al., 2012). Metabolites, which are responsible for antioxidant activity, could dramatically differ throughout the plant growing stages. The variations in growth might influence the germination of the plant and its quality (Mediani et al., 2012a; Ribeiro et al., 2015). The differences in the levels of secondary metabolites of some plants, harvested at different growth stages, have been recognized. Nevertheless, the information regarding the chemical composition, nutritional properties and their variations in the *Phyllanthus* species are very limited. Therefore, additional studies of *Phyllanthus* are needed in order to gain insight into their uses as medicines or food supplements. Determination of appropriate species and growth stage for harvesting can provide the valuable information on its nutritional and bioactivity effects.

Metabolomics is defined as the quantitative and qualitative analyses of all of the metabolites in an organism subjected to different factors (Maulidiani et al., 2013, 2012; Mediani et al., 2012b; Shuib et al., 2011; Torras-Claveria et al., 2014). Numerous advanced tools have been used in metabolomics for the high throughput analysis of targeted and untargeted metabolites (Choi et al., 2004). Nuclear magnetic resonance is a sufficient and suitable technique for these analyses due to its ability to allow simultaneous identification of the different groups of secondary metabolites as well as the abundant primary metabolites. In addition, this method is fast and reproducible and involves simple sample preparation. Recently, the NMR techniques, which were combined with multivariate data analysis have been employed for metabolic profiling and characterization of different types of plants (Choi et al., 2004; Maulidiani et al., 2013). However, there is no data on the variation in the bioactivity and metabolic discrimination of *P. niruri* and *P. urinaria* harvested at different growth stages using advanced tools. Therefore, this study is performed to fill the information gap that exist for these two *Phyllanthus* species and to determine the correlation between the metabolite profiles with their biological activities using ^{1}H NMR spectroscopy coupled with multivariate data analyses.

2. Materials and methods

2.1. Chemicals and reagents

The chemicals and reagents used included KH_2PO_4, methanol-d_4 (CD_3OD, 99.8%), sodium deuterium oxide (NaOD), deuterium oxide (D_2O, 99.9%), trimethyllsilyl propionic acid-d_4 sodium salt (TSP) and α-glucosidase enzyme, which were supplied by Merck (Darmstadt, Germany). The Folin–Ciocalteu reagent, sodium carbonate (Na_2CO_3), 1,1-diphenyl-2-picrylhydrazyl (DPPH), potassium dihydrogen phosphate, glycine, and *p*-nitrophenyl α-D-glucopyranoside were supplied by Sigma–Aldrich (St. Louis, USA). Acetonitrile (LCMS grade) was purchased from Merck (Darmstadt, Germany). The reference standards were purchased from Sigma–Aldrich (St. Louis, MO, USA) which included gallic acid, ellagic acid, chlorogenic acid; naringin, quercetin, quercetin rhamnoside, kaempferol, rutin, catechin, epicatechin, phyllanthin, hypophyllanthin and corilagin. Liquid nitrogen was supplied by the MOX Company (Petaling Jaya, Malaysia).

2.2. Plant materials

Both *Phyllanthus* species were identified by Dr. Shamsul Khamis, an in-house botanist of the Institute of Bioscience and the voucher specimen (SK2462/14 for *P. urinaria* and SK2463/14 for *P. niruri*) were deposited at the Herbarium of the Institute. These two species were planted in the Sendayan Commodities Development Center in Seremban, Negeri Sembilan, Malaysia. Three plots (40 plants each) of each species were divided into 10 parts, and 4 whole plants of each species one biological replicates from each part were randomly collected. A total of 10 replicates were used from each species at every growth stage. The plants were harvested in the early morning, cleaned and dried with tissue paper to remove all of the residues. The samples (whole plant) were immediately placed at −80 °C prior to freeze drying.

2.3. Extraction of samples

The freeze dried samples were ground into a fine powder and 4 g of each sample was immersed in 100 ml of 80% ethanol in an amber bottle. The mixtures were shaken to mix the samples with the solvent followed by sonication for 1 h under a controlled temperature at 25 °C. Then, the mixture was filtered through filter paper twice to completely remove the debris. The residual solvents were removed using a rotary evaporator under a partial vacuum at 40 °C. The samples were frozen at −80 °C and then lyophilized in a freeze dryer to ensure removal of the residual water. The dried extracts were stored in an amber bottle at 4 °C for prior to use.

2.4. NMR analysis

The ^{1}H NMR and 2D (*J*-resolved) NMR determinations were implemented using a 500 MHz Varian INOVA NMR spectrometer (Varian Inc., California, USA), functioning at a frequency of 499.887 MHz at room temperature (25 °C). The CD_3OD was used as an internal lock. The extraction procedures were carried out according to the protocol designed by Maulidiani et al. (2013) and Verpoorte et al. (2007) with slight modifications. Ten milligrams of the crude extracts were placed in 2 ml Eppendorf tubes and 0.375 ml of both CD_3OD solvent and KH_2PO_4 buffer in D_2O (pH 6.0) containing 0.1% TSP were added. The mixture was vortexed for 1 min followed by ultrasonication for 15 min at room