UNIVERSITI PUTRA MALAYSIA

NUCLEAR MAGNETIC RESONANCE METABOLOMICS APPROACH IN CHEMICAL AND PROTECTIVE EVALUATIONS OF Orthosiphon stamineus BENTH. LEAF EXTRACTS ON CISPLATIN-INDUCED NEPHROTOXICITY

RAGHUNATH PARIYANI

IB 2016 23

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By

RAGHUNATH PARIYANI

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Doctor of Philosophy

December 2016

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DEDICATION

This thesis is dedicated to my beloved parents

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the Degree of Doctor of Philosophy

NUCLEAR MAGNETIC RESONANCE METABOLOMICS APPROACH IN CHEMICAL AND PROTECTIVE EVALUATIONS OF *Orthosiphon stamineus* BENTH. LEAF EXTRACTS ON CISPLATIN-INDUCED NEPHROTOXICITY

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December 2016

Chairman : Associate Professor Intan Safinar Ismail, PhD
Institute : Bioscience

Orthosiphon stamineus (OS), locally known in Malaysia as 'Misai Kucing', is a herbaceous shrub belonging to the family Lamiaceae. Dried leaves of OS is gaining wide acceptance and marketed in the form of herbal tea, known as Java tea, owing to its traditional and scientific claims on various health benefits. OS has been a well-known renoprotective agent primarily due to its diuretic potential. This research investigated the effects of commonly employed drying methods of OS leaves on their chemical constituent profile, and *in vivo* biological properties of the protective role in cisplatin induced nephrotoxicity using rats, through Nuclear Magnetic Resonance (NMR) metabolomics approach. The NMR spectra of rat urine and the OS leaf extracts were analysed and correlated using multivariate data analysis techniques employing metabolomics platform.

The ^{1}H NMR metabolite profiling of aqueous extract of OS leaves resulted in the identification of 31 metabolites. The presence of biologically active secondary metabolites including phenylpropanoids such as caffeic acid, protocatechuic acid, chlorogenic acid, flavonoids such as luteolin and apigenin, gallic acid and orthosiphol derivatives were confirmed by J resolved NMR technique. The HPLC - MS/MS analysis further confirmed the presence of these secondary metabolites. Metabolite fingerprinting in combination with multivariate analysis has successfully differentiated the three differently dried (Freeze, microwave and shade) OS leaves and established that the levels of 15 metabolites were varied significantly between the samples. The shade drying method retained maximum secondary metabolites followed by the microwave, while freeze drying retained the least. Assessment of the main beneficial properties, such as antioxidant, total phenolic and flavonoid contents of any tea preparation, confirmed that all the differently dried Java tea leaves gave good antioxidant activity, with the shade dried leaves recorded the highest level with an IC50 of 48.09 μg/mL. The chemical constituents correlated to the high antioxidant activity of the shade dried leaves were extracted from a Partial Least Square regression

(PLS) model. In addition, the toxicity profile of the microwave dried OS leaves was investigated through acute oral toxicity test in Sprague Dawley (SD) rats of both sexes, whereby, the no-observed-adverse-effect level (NOAEL) of aqueous, 50% ethanolic and ethanolic extracts of the microwave dried OS was determined as 5000 mg/kg body weight/day. Thus, it is presumed that the microwave dried leaves are safe to be used as an oral health supplement.

Cisplatin is an anticancer drug, which induces nephrotoxicity in a long term use. Metabolomic analysis of the rats' urine revealed the involvement of a total of 17 biochemical markers from TCA cycle, carbohydrate, amino acid, and polyamine metabolic pathways in cisplatin nephrotoxicity. To the best of knowledge, 6 of the 17 involved metabolites are newly established in this study. In order to evaluate the protective efficacy of OS in cisplatin nephrotoxicity, shade and microwave dried OS extracts were administered at doses of 100, 200 and 400 mg/kg body weight to rats. The results suggested the dose independency of the extracts. Treatment with 50% aqueous ethanolic extract of shade dried OS leaves (OSFS) exhibited moderate ameliorative effect observed through a statistically significant reduction in the levels of 8 biomarkers. It was also revealed that the aqueous extract of the shade dried leaves (OSAS) exhibited slightly deteriorative activity via disturbance in the energy metabolism and gut microflora. The higher concentration of the secondary metabolites such as caffeic acid, chlorogenic acid, protocatechuic acid and orthosiphol in OSFS could be correlated to the ameliorative activity as revealed from a Principal Component Analysis (PCA) between OSAS and OSFS. A prediction model on nephroprotective effect of OS was constructed through PLS regression analysis.

Thus, the impact of different drying techniques on chemical constituents of OS leaves was established. The metabolomics approach has proved to be successful in shedding light to the even minute variations in the biological profiles of the low intensity metabolites involved in the renal toxicity caused by cisplatin. A global comprehensive view of the OS effect in cisplatin toxicity was successfully profiled and correlated.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Doktor Falsafah

PENILAIAN KIMIA DAN KESAN PERLINDUNGAN MELALUI PENDEKATAN NUKLEAR MAGNETIK RESONAN METABOLOMIK EKSTRAK DAUN *Orthosiphon stamineus* BENTH. TERHADAP NEFROTOKSIK CISPLATIN

Oleh

RAGHUNATH PARIYANI

Disember 2016

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Orthosiphon stamineus (OS), dikenali di Malaysia sebagai 'Misai Kucing'. Ia merupakan sejenis pokok herba renek dalam keluarga Lamiaceae. Daun OS yang telah dikeringkan semakin diterima ramai lalu dipasarkan dalam bentuk teh herba, yang dikenali sebagai teh jawa. Ini terjadi atas kepelbagaian manfaat kesihatan yang diuar-uarkan melalui pendekatan tradisional dan saintifik. OS telah dikenali sebagai ejen perlindungan buah pinggang yang terkenal kerana potensi diuretiknya. Kajian mengenai kesan kaedah pengeringan yang telah biasa diamalkan turut dilakukan pada daun OS bagi menentukan profil konstituen kimianya serta kajian biologi turut dilakukan secara *in vivo* bagi memastikan peranan perlindungannya pada tikus aruhan cisplatin yang mengakibatkan kesan nefrotoksik. Ia dilakukan melalui pendekatan metabolomik Nuklear Magnetik Resonan (NMR). Spektrum NMR daripada sampel air kencing tikus dan estrak daun OS dianalisis dengan mengaitkan platform metabolomik menggunakan teknik analisis data multivariat.

Metabolit profil pada ^{1}H NMR ekstrak air daun OS telah mengenalpasti 31 jenis metabolit. Kehadiran metabolit biologi sekunder yang aktif termasuk phenyl-propanoids seperti asid caffeic, asid protocatechuic, asid chlorogenic, flavonoid seperti luteolin dan apigenin, asid Gallic dan derivatif orthosiphol telah disahkan melalui teknik “J resolve” NMR. Analisis lanjut HPLC - MS / MS mengesahkan kehadiran metabolit sekunder ini. Teknik pengkelasan metabolit ditambah dengan analisa multivariat telah berjaya membezakan tiga kaedah pengeringan (sejuk beku, ketuhar gelombang mikro dan bawah teduhan) daun OS dan telah membuktikan bahawa 15 metabolit telah mengalami perbezaan ketara antara sampel. Kaedah pengeringan dibawah teduhan berjaya mengekalkan kehadiran metabolit sekunder yang paling tinggi diikuti oleh pengeringan gelombang mikro, serta penyejuk bekuan menunjukkan nilai yang paling rendah. Penilaian terhadap manfaat utama seperti antioksida mendapati, jumlah fenolik dan kandungan flavonoid sesuatu penyediaan teh mengesahkan bahawa kesemua daun teh jawa yang berbeza kaedah

pengeringannya memberikan aktiviti antioksidan yang baik di mana daun yang dikeringkan dibawah teduhan telah merakamkan nilai IC50 yang paling tinggi pada 48.09 μg / mL. Selanjutnya analisa separa persegi (PLS) telah digunakan untuk mengenalpasti konstituen kimia yang bertanggungjawab terhadap aktiviti antioksida yang tinggi dalam daun OS yang dikeringkan di bawah teduhan ini. Di samping itu, profil toksik daun OS yang dikeringkan dengan menggunakan gelombang mikro telah dikaji dengan menjalankan ujian oral toksiti pada kedua-dua jantina tikus Sprague Dawley (SD). Keputusan menunjukkan tiada pemerhatian-taraf-kesan-buruk (NOAEL) bagi ekstrak air, 50% etanol dan etanol pada dos 5000 mg/kg berat badan/hari. Oleh itu, daun OS yang telah dikeringkan menggunakan gelombang mikro dianggap selamat untuk digunakan sebagai makanan tambahan kesihatan melalui oral.

Cisplatin adalah ubat anti-kanser yang boleh menyebabkan implikasi nefrotoksik jika digunakan dalam jangka masa panjang. Analisa metabolomik ke atas air kencing tikus mendedahkan sebanyak 17 penanda biokimia daripada kitaran TCA, karbohidrat, asid amino, dan laluan metabolik poliamina dalam aktiviti nefrotoksik yang disebabkan oleh cisplatin. Enam, daripada 17 metabolit merupakan metabolit terbaru yang telah dibuktikan dalam kajian ini. Untuk menilai keberkesanan perlindungan OS dalam menangani kesan nefrotoksik ini, daun OS yang diekstrak melalui pengeringan di bawah teduhan dan ketuhar gelombang mikro telah digunakan dan diberi pada beberapa dos rawatan iaitu 100, 200 dan 400 mg / kg berat badan tikus. Hasil kajian mendapati keberkesanan ekstak tidak bergantung kepada dos rawatan yang diberikan. Sementara itu, awatan dengan 50% ekstrak ethanol daripada daun OS yang dikeringkan di bawah teduhan (OSFS) menunjukkan kesan rawatan yang sederhana. Ini disebabkan oleh pemerhatian terhadap kadar penurunan yang signifikan pada 8 penandabio. Ia juga mendedahkan bahawa ekstrak air daripada daun OS yang dikeringkan di bawah teduhan (OSAS) hanya mempunyai sedikit penurunan nilai aktiviti melalui gangguan dalam metabolisme tenaga dan usus mikroflora. Metabolit sekunder yang lebih tinggi kepekatannya seperti asid caffeic, asid chlorogenic, asid protocatechuic dan orthosiphol dalam OSFS boleh dikaitkan dengan aktiviti membaik pulih seperti yang dinyatakan daripada Analisia Komponen Utama (PCA) antara OSAS dan OSFS. Satu model ramalan mengenai kesan nefroprotektif OS telah dibina melalui analisis regresi PLS.

Oleh itu, kajian ini telah membuktikan bahawa teknik pengeringan yang berbeza akan memberi kesan kepada konstituen kimia dalam daun OS. Pendekatan metabolomik juga telah berjaya memberi penjelasan terhadap perubahan kecil dalam profil biologi metabolit yang terlibat dalam masalah toksik buah pinggang yang disebabkan oleh cisplatin. Rumusannya, liputan yang menyeluruh terhadap kesan toksik cisplatin telah berjaya diprofilkan dan dihubungkaitkan.

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I certify that a Thesis Examination Committee has met on 28 December 2016 to conduct the final examination of Raghunath Pariyani on his thesis entitled "Nuclear Magnetic Resonance Metabolomics Approach in Chemical and Protective Evaluations of *Orthosiphon stamineus* Benth. Leaf Extracts on Cisplatin-Induced Nephrotoxicity" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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LIST OF ABBREVATIONS

°C	Degree centigrade
%	Percentage
α	Alpha
β	Beta
γ	Gamma
δ	Delta
µm	Micro meter
µg	Microgram
µL	Micro litre
ANOVA	Analysis of variance
BCAA	Branched chain amino acids
COSY	Homonuclear correlation spectroscopy
DOSY	Diffusion ordered-NMR spectroscopy
DPPH	1,1-diphenyl-2-picrylhydrazyl radical
GAE	Gallic acid equivalent
GFR	Glomerular filtration rate
HCA	Hierarchical cluster analysis
HMBC	Heteronuclear multiple bond coherence
HPLC	High performance liquid chromatography
JRES NMR	J resolved NMR spectroscopy
MS	Mass spectrometry
NKEA	National Key Economic Area
NMR	Nuclear magnetic resonance spectroscopy

NP	Natural product
OPLS	Orthogonal Partial Least Squares
OPLS-DA	Orthogonal Partial Least Squares - Discriminant Analysis
OS	*Orthosiphon stamineus*
OSAM	Aqueous extract of microwave dried OS
OSFM	50% aqueous ethanolic extract of microwave dried OS
OSAS	Aqueous extract of shade dried OS
OSFS	50% aqueous ethanolic extract of shade dried OS
PC	Principal components
PCA	Principal Component Analysis
PLS	Partial Least Squares
PLS-DA	Partial Least Squares - Discriminant Analysis
QE	Quercetin equivalent
ROS	Reactive oxygen species
SD	Sprague Dawley
SD	Standard deviation
SEA	South - East Asia
TFC	Total flavonoid content
TPC	Total phenolic content
TSP	Trimethylsilylpropionic acid-d4 sodium salt

CHAPTER 1

INTRODUCTION

1.1 Research background

Historically, plants have been the forerunners in the prevention and cure against a wide spectrum of diseases. Apart from their use in traditional system treatment, the abundant store of unique and diverse chemical compounds present in plants has served as a prominent source of lead molecules in the modern drug discovery process. This is evident from the fact that natural products (NP) and their derived compounds constitute a nearly 50% share of total new chemical entities approved in the span of the past 33 years, until December 2014 (Newman and Cragg, 2012; Newman and Cragg, 2016).

However, the progress made in drug discovery in terms of the number of novel drugs based on NP is not in proportion with the magnitude of ethno-pharmacological claims on various plants. The commonly adopted approaches in NP research such as bioassay guided isolation of active principles and high throughput screening often failed to elicit the optimum activity. This is primarily due to the fact that the bioactivity of a plant often resulted from a cumulative interaction of a large number of phytoconstituents, which deprives the isolated compound from exhibiting the activity as how a complex matrix of crude extract does (Cordell and Colvard, 2012; Yuliana et al., 2013) .

Recent developments in the field of systems biology such as metabolomics allow the evaluation of the biological activity of unfractionated complex extracts using proper bioassays (Robinette et al., 2011). Here, a broad range of analytical techniques such as nuclear magnetic resonance (NMR) spectroscopy, mass spectrometry (MS) and/or chromatography characterises the complex diverse metabolite classes present in the crude extract and an overall picture of metabolites correlating to the bioactivity could be derived using proper data mining methods. Thus, this holistic approach facilitates the identification of multiple active compounds from a single extract and their interaction either synergistically or antagonistically in *in vivo* systems.

Metabolomic fingerprinting has been applied to novel research areas such as pharmacological properties of medicinal plants, drug discovery via bioprospecting and quality control of herbal drugs. High-resolution NMR spectroscopy is a simple, powerful and fastest approach and has been used in the NP research to identify and correlate both primary and secondary plant metabolites with the elucidated bioactivity. The applications of NMR in metabolic profiling of plant extracts have been well reviewed in several recent articles (Kim et al., 2011; Schripsema, 2010).

Orthosiphon stamineus (OS) is a herbal remedy used traditionally in the cure of various system disorders, primarily that of kidney. It is locally known as *Misai Kucing*

in Malaysia. An aqueous infusion of dried leaves of OS, known as Java tea, is widely consumed by the people in South - East Asia (SEA) and Europe as a health adjuvant and general tonic (Ameer et al., 2012; Yuliana et al., 2009). The standardization of herbal products is of paramount importance in order to ensure consistent biological effects. So far the standardisation of OS was based on the quantification of certain marker compounds such as rosmarinic acid or sinensetin, and the practice was to generalize the observed biological activity to the variations of these selected markers. However, the metabolomic approach offers a platform to realistically correlate the responsible metabolites to the activity, and enables simultaneous standardization of several compounds present in the extract with minimal time and effort.

Variations in the metabolic profiles of NP might be due to multitude of factors including differences in species, pre- and post-harvesting methods, adulteration, and extraction among many others (Van der Kooy et al., 2009; Wang et al., 2009). The subsequent effects on bioactivity profile warrant proper monitoring of these metabolite changes in order to ensure their safe and effective usage. Drying technique deserves special attention as one of the most important variables in the preparation of Java tea (OS) leaves. A metabolomic analysis on the differently dried (shade, microwave and freeze) Java tea leaves thus would be helpful in fingerprinting the metabolites, which serves as the basis for standardisation and quality control tools.

The metabolomics platform has proved its usefulness in the field of toxicology as it derives a comprehensive picture of the effect of toxin in the body by the determination of global metabolome levels and their interrelations (Ramirez et al., 2013). OS has been known particularly for the beneficial effects on renal system owing to its diuretic and free radical scavenging activities (Arafat et al., 2008; Olah et al., 2003). However, to the best of our knowledge, the potential of OS in the protection of kidney from toxins, which is one of the most important sites of toxicity, has not yet been studied. A systematic exploration using metabolomics allows the simultaneous understanding of the complex mode of action of the toxin as well as the potential intervention of the OS. The toxico-metabolomics approach thus helps in understanding the mechanisms of toxicity, identify the biomarkers, and predict the bioactivity of the extract, thus, results in improvement of safety, to the shortening of the lead identification and a cost reduction (Robertson et al., 2011; Ulrich-Merzenich et al., 2009).

A holistic evaluation of long term perspective rational evidence-based herbal treatment could lead to the discovery and development of effective phytomedical intervention, taking into account the interaction of multiconstituents in synergism or antagonism, thus ensuring better safety and efficacy of the usage of herbs used in traditional system treatment.

In this research, it is hypothesized that different drying methods employed in the production of OS leaves affect the chemical profile and biological properties of OS. Identification of a proper drying method, which retains maximum beneficial chemical constituents, and safe, as well as efficient pharmacological activity is important to be ensured before its usage.

1.2 Aims and objectives

The work presented in this thesis aimed to investigate the effects of the drying methods exerted on the chemical and biological properties of OS leaves using NMR metabolomics approach. The metabolomics tool was employed to detect and discriminate the modulatory effects of OS on cisplatin nephrotoxicity. The general objective of this research was to evaluate the quality, safety, efficacy and consistency (QSEC) parameters of OS leaves with regard to different drying techniques employed.

These were achieved through the following set of specific objectives:

- To establish the metabolic fingerprint of shade, microwave and freeze dried OS leaf extracts, and to correlate their antioxidant activity with the overall bioactive compounds.
- To determine primary toxicity profile of microwave dried OS leaves.
- To identify the biomarkers and underlying metabolic pathways involved in cisplatin nephrotoxicity.
- To evaluate the modulatory effect of various OS extracts in cisplatin-induced nephrotoxic biomarkers and to develop a validated regression model, correlating the phytoconstituents to nephroprotective activity.

1.3 Outline of thesis

This thesis is presented in seven chapters. The general introduction is described in Chapter 1. Chapter 2 focusses on the comprehensive review of the literatures related to this research. Relevant literatures on pharmacological and phytochemical studies on OS, metabolomics and cisplatin nephrotoxicity are reviewed. Chapter 3 discusses the application of nuclear magnetic resonance (NMR) spectroscopy and chemometric methods in achieving the metabolic fingerprint of shade, microwave and freeze dried leaves. The correlation of antioxidant activity of the OS leaf to its phytoconstituents was established using Partial Least Square (PLS) regression analysis. Chapter 4 emphasizes on the preliminary phytochemical and toxicological studies on microwave dried OS leaves. A comparative evaluation of the microwave and shade dried OS leaves chemical constituent profile using liquid chromatography-mass spectrometry (LC-MS/MS) analysis and an acute oral toxicity test to assess the safety of microwave dried OS leaves are described here. Chapter 5 deals with the NMR spectroscopic profiling of the metabolites in cisplatin nephrotoxic and normal rats. The chemometric data analysis tools were used to identify the biomarkers and the underlying metabolic pathways involved in cisplatin nephrotoxicity. Chapter 6 discusses the NMR metabolomic analysis of rat urine in order to understand the metabolic perturbations induced by the OS intervention in cisplatin nephrotoxic biomarkers. A correlation model comprising of the nephroprotective activity with the phytoconstituents of OS was established using PLS. Finally, the overall conclusions are summarised in chapter 7, along with the future perspectives of the results obtained in this thesis.

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BIODATA OF STUDENT

Raghunath Pariyani was born on 15th May, 1983 in Palakkad, Kerala, India. He did his early schooling in Palakkad. In the year 2000, after passing pre-degree examination, he joined University of Calicut for Bachelor degree in Pharmacy (B. Pharm). In 2005, the degree was awarded with gold medal for securing the highest marks in University of Calicut B. Pharm examinations, and then he registered as a Pharmacist in Kerala State Pharmacy Council. After securing a state merit scholarship from the Government of Kerala, he joined in University of Kerala in 2006 to further Master in Pharmacy (M. Pharm), specialized in Pharmaceutical Chemistry, and completed in 2008. During M. Pharm, he gained experience as a toxicological analyst and pursued the research on evaluation of antimycobacterial activity of selected compounds. After which, he started career as Lecturer in Pharmacy in Masterskill University College of Nursing & Health (renamed as Asia Metropolitan University), Malaysia, until 2011. His passion towards the research in natural product sources as potential drug and health supplements led to join for PhD, through which he aimed to develop and enhance the systematic research aptitude. He started the PhD research on September 2011 at Laboratory of Natural Products, Institute of Bioscience, Universiti Putra Malaysia, in *Orthosiphon stamineus,* which is a traditional herb as well as health supplement. He applied NMR-based metabolomics approach in studying the chemical and biological properties of *Orthosiphon stamineus,* the results of which are presented in this thesis.

LIST OF PUBLICATIONS

Pariyani, R., Safinar Ismail, I., Azam, A.A., Abas, F., Shaari, K., Sulaiman, M.R., 2015. Phytochemical Screening and Acute Oral Toxicity Study of Java Tea Leaf Extracts. BioMed research international 2015.

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P Raghunath, IS Ismail., Discriminating the effect of different drying methods on the biological and chemical properties of Java tea through metabolomics approach, The TriSys Asian Regional Conference on Systems Biology 2015 (ARCSB), Bangi, Malaysia, 08 - 09 September 2015, (*Best Young researcher presentation award)*

P Raghunath, IS Ismail, Amalina Ahmad Azam, Alfi Khatib, Faridah Abas and Khozirah Shaari, Metabolomic study on the effect of *Orthosiphon stamineus* leaf extracts in cisplatin-induced nephrotoxicity, Inaugural Symposium of the Phytochemical Society of Asia (ISPSA), Tokushima, Japan, 30 August - 2 September 2015

P Raghunath, IS Ismail., ^{1}H NMR based metabolomics approach in identifying urinary biomarkers associated with cisplatin nephrotoxicity in rat model, 3rd International Postgraduate Conference in Pharmaceutical Sciences, UiTM, Malaysia, 13-14 August 2014

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