NEPHROPROTECTIVE EFFECT OF *Clinacanthus nutans* (BURM.F.) LINDAU ON CISPLATIN-INDUCED RAT AND HUMAN KIDNEY CELL LINES VIA NMR AND LCMS METABOLOMICS APPROACH

By

ILYA IRYANI BINTI MAHMOD

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

May 2019

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DEDICATIONS

This thesis is dedicated to my beloved parents, husband and children.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

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Renal toxicity or nephrotoxicity is frequently induced by either therapeutic drugs or environmental pollutants. Many plants have attracted attention due to their traditional medicinal claims as potential agents in treating and/or protecting against renal toxicity. Despite medicinal plants have been demonstrated to act as nephroprotective agents, there is still lack of scientific evidence to support these claims. An innovative, simple to perform, reproducible, reliable, and predictive assay such as *in vitro* cell line screening is crucial in taking up the challenge to establish a preliminary scientific evidence for the medicinal plant to be further studied.

Hence, the present study was aimed to evaluate the protective effect of *Clinacanthus nutans* (*C. nutans*) leaves on rat (NRK-52E) and primary human kidney cells (PCS-400-010) in cisplatin-induced nephrotoxicity through NMR and LCMS metabolomics approach. *C. nutans* Lind. locally known as Belalai Gajah or Sabah snake grass is a medicinal plant of Acanthaceae family. In Malaysia, this plant is traditionally used for treating skin rashes, insects and snake bites, diabetes mellitus, fever and for diuretic effect.

In order to evaluate the nephroprotective effect of *C. nutans* in cisplatin nephrotoxicity, six *C. nutans* leaves extracts in different ethanol-aqueous ratios (0, 20, 40, 60, 80 and 100 %) were tested on both cell lines by MTT assay. The *C. nutans* aqueous extract exhibited the most potential nephroprotective effect against cisplatin toxicity. To characterize the metabolic variations of intracellular metabolites and the compositional changes of the corresponding culture media in NRK-52E, ^{1}H NMR and LCMS coupled with multivariate data analysis were used. Analyses on both NRK-52E cell extract and media showed significant decrease in amino acid group (alanine, valine, serine, phenylalanine and glutamine). The increase levels of choline, glycerophosphocholine and phosphocholine also exhibited when the cells were induced by cisplatin. The

reduction of glucose uptake from media into the cells was observed in the cisplatin induced group as the level of the glucose increased compared to the control group. These changes showed that the cells have been successfully induced. The pre-treatment with *C. nutans* leaves aqueous extract has reduced choline level in 2 folds compared to the cisplatin-induced group, suggesting that the membrane degradation occurred due to cisplatin induction. Increased levels of alanine and valine were also observed in the *C. nutans* pre-treated group which suggesting the disturbance of amino acid metabolism.

Due to a very slow growth rate of PCS-400-010 which resulted in a small amount of cell extracts obtained, LCMS analysis was chosen to analyse the different treatment groups of this cell line. Major discriminant metabolites in cells which contributed to the separation between control and cisplatin-induced group were 3-indoxyl sulfate, phosphocholine (PC (14:0/14:0)), phosphatidylethanolamine (PE (15:0/16:0)), phenylalanine, lactic acid, uridine, leucine, proline, acetic acid, adenosine, guanosine and deoxyinosine. Glutamine, leucine, proline, valine, phenylalanine and lactic acid were identified from the culture media. The LCMS analysis revealed the altered metabolites, due to the cisplatin induction and then treatment of *C. nutans* aqueous extract on the cells, were correlated to the amino acids, glycerophospholipid, purine and glycolysis pathways. As a result, this study suggests that aqueous *C. nutans* leaves extract possessed nephroprotective effect on cisplatin-induced rat and human kidney cell lines via alterations in amino acid, lipid and purine metabolism.

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Abstrak yang telah dikemukakan kepada senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah.

KESAN NEFROPROTEKTIF *Clinacanthus nutans* (BURM.F.) LINDAU TERHADAP ARUHAN CISPLATIN KE ATAS SEL TIKUS DAN SEL MANUSIA MELALUI PENDEKATAN NMR DAN LCMS METABOLOMIK

Oleh

ILYA IRYANI BINTI MAHMOD

Mei 2019

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Toksisiti buah pinggang atau nefrotoksisiti sering kali disebabkan oleh ubat terapeutik atau pencemaran alam sekitar. Banyak tumbuhan menjadi perhatian kerana tuntutan perubatan tradisional mereka sebagai agen yang berpotensi dalam merawat dan / atau melindungi ketoksikan buah pinggang. Walaupun tumbuhan ubatan telah ditunjukkan untuk bertindak sebagai agen pelindung, namun masih terdapat kekurangan bukti saintifik untuk menyokong tuntutan ini. Satu inovatif, mudah untuk dilaksanakan, boleh direproduksi, boleh dipercayai, dan ramalan esei seperti kajian awal sel *in vitro* adalah penting dalam mengambil cabaran bagi menghasilkan bukti saintifik awal untuk tanaman perubatan agar dikaji lebih lanjut.

Oleh itu, kajian ini bertujuan untuk menilai kesan perlindungan dari daun pokok *Clinacanthus nutans* (C. nutans) pada sel buah pinggang tikus (NRK-52E) dan sel buah pinggang manusia (PCS-400-010) dalam nefrotoksiti yang disebabkan oleh cisplatin melalui pendekatan metabolomik NMR dan LCMS. *C. nutans* Lind. dikenali sebagai Belalai Gajah atau rumput ular Sabah adalah tumbuhan perubatan dari keluarga Acanthaceae. Di Malaysia, tumbuhan ini secara tradisinya digunakan untuk merawat ruam kulit, gigitan serangga dan ular, kencing manis, demam dan untuk kesan diuretik.

Untuk menilai kesan nefroprotektif *C. nutans* dalam nefrotoksisiti cisplatin, enam ekstrak daun *C. nutans* dalam nisbah etanol-akues yang berbeza (0, 20, 40, 60, 80 dan 100%) telah diuji pada kedua-dua jenis sel oleh ujian MTT . Ekstrak akueus *C. nutans* menunjukkan kesan nefroprotektif paling berpotensi terhadap ketoksikan cisplatin. Untuk mencirikan kepelbagaian metabolik daripada intraselular dan perubahan komposisi kultur media dalam NRK-52E, ^{1}H NMR dan LCMS serta analisis data multivariat digunakan. Analisis pada kedua-dua ekstrak sel NRK-52E dan media menunjukkan penurunan ketara dalam kumpulan asid amino (alanina, valina, serina, fenilalanina dan glutamina). Kadar peningkatan kolina, gliserofosfokolina dan

fosfokolina juga ditunjukkan apabila sel-sel telah diinduksi oleh cisplatin. Pengurangan pengambilan glukosa dari media ke dalam sel-sel telah diperhatikan di dalam kumpulan cisplatin induksi sebagai tahap peningkatan glukosa dibandingkan dengan kumpulan kawalan. Perubahan ini menunjukkan bahawa sel telah berjaya diinduksi. Pra-rawatan dengan daun ekstrak akues *C. nutans* telah mengurangkan kadar kolina dalam 2 kali ganda berbanding dengan kumpulan yang disebabkan oleh induksi cisplatin, menunjukkan bahawa degradasi membran berlaku disebabkan oleh induksi cisplatin. Peningkatan tahap alanina dan valina juga diperhatikan dalam kumpulan C. nutans yang mencadangkan gangguan metabolisma asid amino.

Oleh kerana kadar pertumbuhan yang sangat perlahan, PCS-400-010 yang menghasilkan sejumlah kecil ekstrak sel yang diperolehi, analisis LCMS telah dipilih untuk menganalisis kumpulan rawatan yang berbeza dari sel ini. Metabolit diskriminasi utama dalam sel yang menyumbang kepada pemisahan antara kawalan dan kumpulan yang disebabkan oleh induksi cisplatin ialah 3-indoksil sulfat, fosfokolina (PC (14: 0/14: 0)), fosfatidiletanolamina (PE (15: 0/16: 0)) , fenilalanina, asid laktik, uridina, leusina, prolina, asid asetik, adenosina, guanosina dan deoxiinosina. Glutamina, leusina, prolina, valina, fenilalanina dan asid laktik telah dikenal pasti dari media kultur. Analisis LCMS mendedahkan metabolit yang diubah, disebabkan induksi cisplatin dan kemudian rawatan akues estrak *C. nutans* pada sel-sel, dikaitkan dengan asid amino, gliserofosfolipid, purina dan glikolisis. Hasilnya, kajian ini mencadangkan bahawa ekstrak daun *C. nutans* mempunyai kesan nefroprotektif pada sel buah pinggang tikus yang disebabkan oleh cisplatin dan sel-sel buah pinggang manusia melalui perubahan dalam asid amino, lipid dan metabolisma purin.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

δ	Chemical shift
µg	Microgram
^{o}C	Degree in Celsius
^{1}H	Proton
bp	Boiling point
br	Broad
CC	Column chromatography
$CDCl_3$	Deuteriochloroform
$CHCl_3$	Chloroform
CKD	Chronic Kidney Disease
COSY	Correlation spectroscopy
d	Doublet
dd	Doublet of doublet
dt	Doublet of triplet
DMSO	Dimethyl Sulfoxide
EIMS	Electron Impact Mass Spectrum
EtOH	Ethanol
eV	Electron volt
h	Hour
H_2O	Water
HPLC	High Performance Liquid Chromatography
HRMS	High Resolution Mass Spctroscopy
Hz	Hertz
IC_{50}	Inhibition Concentration at 50 %
J	Coupling constant

LCMS	Liquid Chromatography Mass Spectrometry
m	Multiplet
MeOD	Tetradeuteriomethanol
MeOH	Methanol
m/z	Mass per charge
MHz	MegaHertz
MS	Mass Spectroscopy
MVDA	Multivariate Data Analysis
nm	Nanometer
NMR	Nuclear Magnetic Resonance
NOESY	Nuclear Overhauser Effect Spectroscopy
ppm	Parts per million
s	Singlet
SIMCA	Soft Independent Modelling of Class Analog
t	Triplet

CHAPTER 1

INTRODUCTION

1.1 Research background

Renal toxicity or nephrotoxicity is an adverse and side effect of a number of pharmaceutical agents and a common observation in preclinical safety assessment, clinical trial and clinical practice (Boudonck et al., 2009). Nephrotoxicity is frequently induced by a wide spectrum of therapeutic drugs (antibiotic, immunosuppressant, antineoplastic agent, nonsteroidal anti-inflammatory drugs, heroin, cocaine, and natural medicines) and environmental pollutants (heavy metals, organic solvents, insecticides, and glycols). Drugs are known to damage the glomerulus (puromycin and Adriamycin), papilla (NSAIDs), and various segments of the proximal tubules (aminoglycosides, cyclosporine and cisplatin) in kidney (Werner et al., 1995).

Plant extracts as natural products that contain several phytochemicals attract attention for their potential usage in treating and protecting against renal toxicity. Many medicinal plants have been demonstrated to act as nephroprotective agents. However, there is a lack of scientific evidence to support these claims. Developing a satisfactory, plant-based therapy to treat severe renal disorder, such as acute renal failure, nephritic syndrome, and chronic interstitial nephritis, requires systematic investigation on the potential medicinal plants (Mohana *et al*., 2012). Plants possess therapeutic properties due to the presence of the secondary metabolic constituents. Recent *in vitro* and animal studies have confirmed the biological activity and therapeutic effect of several plants on chronic kidney disease (CKD) (Zhong et al., 2013). However, the level of evidence supporting the efficacy of those medicinal plants is limited due to the non-proven claims and the various prescription patterns of the plant.

An innovative, simple to perform, reproducible, reliable, and predictive assay is crucial in taking up the challenge to establish a preliminary scientific evidence for the medicinal plant to be studied further, and thereafter possibly developed as a potential nephrotoxic alternative treatment. The extrapolation of observations in animal models, such as the rat and the rabbit to humans has been applied in contemporary research for toxicant identification and risk assessment. However, this method is inherently very costly, inaccurate, and ethically undesirable (Ellis *et al*., 2011). Hence, an *in vitro* model, which is cheaper and far less time-consuming for examining the nephroprotective effect of plants was developed utilizing two cell lines, the rat proximal tubular kidney (NRK-52E) and human proximal tubular kidney (PCS-400-010) cells. Based on the *in vitro* cell assays, a correlation, either through the similarities or the differences between these two cell lines of rat and human, could be established easily and rapidly. The rat cell line was chosen to correlate with the reported investigation on the anti-nephrotoxic assessment in an *in vivo* rat animal

model (Raghunath *et al*., 2017) done under the same supervision of Dr. Intan Safinar Ismail.

Cisplatin (*cis*-diamminedichloroplatinum (II)) was the choice to induce the nephrotoxicity in the cells. This compound is an important chemotherapeutic agent which is useful in the treatment of several cancers (Chirino *et al*., 2008). Unfortunately, there are major side effects in using this chemotherapeutic agent, as clinical nephrotoxicity or acute kidney injury reportedly to occur in about 30% of the patients (Kolfschoten *et al*., 2002). Clinically, cisplatin nephrotoxicity is often seen after 10 days of cisplatin administration, and results in a lower glomerular filtration rate, and higher serum creatinine and reduced serum magnesium and potassium levels (Brillet *et al*.,1994). On the other hand, exposure of tubular cells to cisplatin leads to cell injury and cell death. A high concentration of cisplatin induces necrotic cell death in confluent monolayers of proximal tubule cells, whereas lower concentrations lead to apoptosis (Daniyelle *et al*.,2002).

A specific, plant-based system for kidney treatment could be established when the active constituents which are responsible for the anti-nephrotoxic effects are identified. Metabolic profiling (metabolomics/metabonomics) is one strategy for evaluating cellular pathway analysis. It is based on the untargeted analysis of the small molecule composition of a chemical or biological sample, and it is a powerful and flexible tool in the discovery of biomedical markers in a complex plant matrix (Ellis *et al*., 2011). Proton Nuclear Magnetic Resonance (^{1}H NMR) spectroscopy has been used to study human kidney tissue in both a cancerous (carcinoma) (Moka *et al*., 1988) and normal (Tate *et al*., 2000) state. A recent study has evaluated the profiling of intracellular and extracellular metabolites derived from human renal proximal epithelial cells using an NMR approach (Ellis *et al*., 2011).

Thus, through a combination of *in vitro* assessment on nephrotoxic-induced rat and human cell lines, ^{1}H NMR data and LCMS data with metabolomics approach, the biomarkers and the mechanism of action exerted by the selected medicinal plants could be determined. This multi-faceted, integrated approach holds great promise for the identification of plant-based therapeutic regimen that could prevent and repair damage to the renal system.

1.2 Problem statement

The kidney disease has become a crucial issue in the developing and newly industrialized countries including Malaysia, China, and the Philippines. In Malaysia alone, the dialysis acceptance rate for 2015 was 249 patients per million population, with a prevalence of 1,220 patients per million population. The vast majority (92%) of the patients were placed on haemodialysis (HD), while 8% were on peritoneal dialysis (PD (24th Report Malaysian Dialysis and Renal Transplant Registry 2016). Synthetic corticosteroids (e.g. prednisolone) and non-corticosteroids (e.g. chlorambucil) have

been used in kidney disease therapy. However, these drugs give adverse and side effects such as decreased resistance to infections, bone marrow suppression with leucopoenia and rarely malignant transformation.

In response to this matter, other alternative therapies are urgently needed to replace the current drug treatments. Many medicinal plants have been demonstrated to act as nephroprotective agents, while many more are claimed to be nephroprotective. However, there is lack of scientific evidence to support these claims. Therefore, our proposed study is to establish a preliminary *in vitro* scientific investigation for the nephroprotective potential medicinal plants to be studied further, which then might open opportunities to be developed as nephrotoxic alternative treatments.

1.3 Scope and objectives

The current study was initiated to solve several issues and serve as platform in obtaining valuable extracts for the treatment of renal disease and for the improvement of its metabolic disturbances. The main aim of this study was to evaluate the nephroprotective effect of *Clinacanthus nutans* extracts by ^{1}H NMR and LCMS based metabolomics. To accomplish this aim, a few specific objectives were proposed as below:

1) To optimize the method that could be used to assess the nephroprotective effect of a selected medicinal plants on cisplatin induced-nephrotoxic in NRK-52E.

2) To screen the selected plants, *Clinacanthus nutans*, *Orthosiphon stamineus*, *Androghaphis paniculata*, *Phyllanthus niruri* and *Mitragyna speciosa* for nephroprotective effect on NRK-52E. (Note: *Clinacanthus nutans* extract was identified as having the highest nephroprotective effect on cisplatin-induced NRK-52E cell.

3) To determine the active extract fraction and concentration of *C. nutans* extract having anti-nephrotoxic properties. Selection of extract fraction for evaluation include 100 % EtOH, a series of EtOH: water fraction (i.e. 80:20, 60:40, 40:60, 20:80) and 100 % water.

4) To identify the biomarkers related to the nephroprotective properties of *C. nutans* on the cisplatin-induced nephrotoxic NRK-52E and PCS-400-010 cells.

5) To evaluate and correlate the modulatory effect of the *C. nutans* leaves extract in both cell lines.

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