NEPHROPROTECTIVE EFFECT OF *CLINACANTHUS NUTANS*, *ORTHOSIPHON STAMINEUS*, AND *SYZYGIUM CAMPANULATUM* LEAVES AGAINST CISPLATIN-INDUCED NEPHROTOXICITY; AND SAFETY ASSESSMENT OF *CLINACANTHUS NUTANS* LEAVES.

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NEPHROPROTECTIVE EFFECT OF *CLINACANTHUS NUTANS*, *ORTHOSIPHON STAMINEUS*, AND *SYZYGIUM CAMPANULATUM* LEAVES AGAINST CISPLATIN-INDUCED NEPHROTOXICITY; AND SAFETY ASSESSMENT OF *CLINACANTHUS NUTANS* LEAVES.

by

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LIST OF ABBREVIATIONS

A	Albumin
AECNL	Aqueous extract of *Clinacanthus nutans* leaves
A/G	Albumin/globulin ratio
ALT	Alanine aminotransferase
ALP	Alkaline phosphatase
APTT	Activated partial thromboplastin time
AST	Aspartate aminotransferase
ANOVA	Analysis of variance
CO_2	Carbon dioxide
Ctr1	High affinity copper uptake protein 1
dH_2O	Distilled Water
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
dNTP	Deoxyribonucleotide triphosphate
DPPH	2, 2-diphenyl-1-picrylhydrazyl
EC_{50}	Half maximal effective concentration
GFR	Glomerular filtration rate
GGT	Gamma glutamyl transferase
Hb	Hemoglobin concentration
HDL	High-density lipoprotein
HEOSL	Hydroethanolic extract of *Orthosiphon stamineus* leaves
HESCL	Hydroethanolic extract of *Syzygium campanulatum* leaves
IC_{50}	Half maximal inhibitory concentration

LDL	Low-density lipoprotein
LD_{50}	Median lethal dose
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
MCV	Mean corpuscular volume
NOAEL	No-observed-adverse-effect level
NRK-52E	*Rattus norvegicus*, proximal tubule cell line
OCT2	Organic Cation Transporter 2
OECD	Organisation for Economic Co-operation and Development
P	p-value
P53	Cellular tumor antigen p53
PBS	Phosphate-buffered saline
PCV	Packed cell volume
Plt	Platelet count
pH	Potential of hydrogen
RDW	Red cell distribution width
S_{Cr}	Serum creatinine
S_K	Serum potassium
S_{Na}	Serum sodium
SD	Sprague-Dawley
SD	Standard deviation
SE	Standard error
TNF-α	Tumor necrosis factor alpha
TP	Total protein
RBCs	Total red blood cells

WBCs	Total white blood cells
UA	Uric acid
U_{Cr}	Urine creatinine
U_{K}	Urine potassium
U_{Na}	Urine sodium

LIST OF UNITS

10^9/l	1.00×10^{12} m^{-3}
10^{12}/l	1.00×10^{15} m^{-3}
dl	Deciliter
g	Gram
g/l	Gram/liter
h	Hour
L	Liter
mg/ml	Milligram per millilitre
mg/kg	Milligram per Kilogram
ml	Millilitre
mM	Millmolar
mmol/l	Millimole per liter
nm	Nanometer
pg	Picogram
rpm	Rate per minute
U/l	Units per liter
Ug/ml	Unigram/ millilitre
µg/ml	Microgram per millilitre
µl	Micro litter
U/ml	Units per millilitre
µmol/l	Micromoles per liter

LIST OF SYMBOLS

α	Alpha
β	Beta
°C	Degree Celsius
=	Equal sign
%	Percentage
±	Plus or minus sign
<	Less-than sign

KESAN PERLINDUNGAN NEFRO DAUN-DAUN *CLINACANTHUS NUTANS*, *ORTHOSIPHON STAMINEUS*, DAN *SYZYGIUM CAMPANULATUM* TERHADAP KENEFROTOKSIKAN TERARUH-CISPLATIN DAN PENILAIAN KESELAMATAN DAUN *CLINACANTHUS NUTANS*

ABSTRAK

Cisplatin adalah ejen kemoterapi berkesan untuk tumor pepejal. Walau bagaimanapun, ia juga mendorong kenefrotoksikan yang tidak boleh diubah dan progresif bergantung kepada dos. Cisplatin mempunyai pelbagai kesan intrasel, termasuk menyebabkan kesitotoksikan langsung dengan spesies oksigen reaktif, mengaktifkan apoptosis, dan merangsang keradangan, yakni peristiwa-peristiwa yang menyebabkan disfungsi tiub dan vaskular. Model haiwan telah menunjukkan bahawa ketoksikan buah pinggang teraruh cisplatin dicirikan oleh kehilangan pesat penapisan glomerular, polyuria, hyperkalemia, hypernatremia dan azotemia. Baru-baru ini, banyak perhatian telah diberikan kepada peranan kemungkinan perubatan herba dalam melindungi ketoksikan buah pinggang teraruh cisplatin. Banyak produk herba yang kaya dengan fitokimia bersama dengan aktiviti anti-apoptotik, antioksidan, dan anti-radang. Faktor-faktor ini mungkin berguna dalam melindungi buah pinggang daripada kerosakan teraruh cisplatin dan, digabungkan dengan ciri-ciri proangiogenic mereka, membantu mempercepatkan proses penyembuhan. *Clinacanthus nutans, Orthosiphon stamineus* dan *Syzygium campanulatum* adalah herba tropika yang kaya dengan antioksidan yang telah digunakan secara tradisional untuk merawat penyakit buah pinggang. Kajian ini menyiasat kesan perlindungan ekstrak akueus daun *Clinacanthus nutans* (AECNL), ekstrak hydroethanolic daun *Orthosiphon stamineus* (HEOSL), dan ekstrak daun *Syzygium campanulatum* (HESCL) terhadap model ketoksikan in vitro

dan in vivo. Aktiviti perlindungan daun-daun tersebut pada sel tiub renal (NRK52-E) telah dinilai dari segi kebolehidupan selular (MTT assay) dan apoptosis (Hoechst dan Rhodamine 123 mengotorkan), dengan AECNL, HEOSL dan HESCL bersendirian atau dalam gabungan dengan cisplatin. Potensi membendung dinilai melalui gavaj oral pada haiwan dengan AECNL, HEOSL dan HESCL pada dos 100, 200 atau 400 mg / kg selama 90 hari, semasa menerima dos mingguan cisplatin (1 mg / kg). Aktiviti biologi AECNL terhadap angiogenesis dinilai menggunakan ex vivo cerakin gelang aorta tikus dengan mengukur tahap pertumbuhan vessel mikro daripada gelang tersebut. Aktiviti mutagen AECNL (500 μg /well) telah dinilai melalui ujian Ames, yang telah dilakukan ke atas Salmonella jenis TA98 dan TA100. Akut, dan kesan ketoksikan oral dos berulang 28- dan 90 hari AECNL juga dinilai. Untuk kajian ketoksikan akut, dos tunggal 5000 mg / kg AECNL diberikan bagi 28 dan 90 hari kajian, manakala 500, 1000, dan 2000 mg / kg / hari telah diberikan kepada kedua-dua jantina tikus Sprague-Dawley. Keputusan menunjukkan bahawa cisplatin menurunkan kebolehidupan sel, yang dikaitkan dengan peningkatan dalam kesitotoksikan dan apoptosis. Rawatan serentak dengan cisplatin dan AECNL, HEOSL atau HESCL, meningkatkan kebolehidupan dan kemandirian sel dan menurunkan kadar apoptosis dalam susunan menurun. Kemerosoton penting ketoksikan buah pinggang telah disahkan melalui penurunan tahap kreatinin serum dan protein, nitrogen urea darah, elektrolit air kencing, dan jumlah air kencing. Tambahan pula, daripada peningkatan kadar glomerular penapisan, elektrolit serum, dan air kencing kreatinin, kedua AECNL dan HEOSL menunjukkan potensi yang tinggi dan berkesan. Pengaruh AECNL pada angiogenesis adalah jauh (EC50: 47,37) positif, dan keputusan mendedahkan bahawa AECNL tidak mutagen dalam auxotrophs S. typhimurium. Dalam kajian ketoksikan akut, 28, dan 90 hari, tidak ada morbiditi atau kematian diperhatikan, dengan nilai-

nilai LD50 atas 5000 mg / kg. Walaupun perubahan ketara diperhatikan dalam hematologi dan parameter biokimia, pemeriksaan histopatologi dan berat relatif organ-organ utama tidak menunjukkan apa-apa tanda-tanda ketoksikan yang ketara. Berdasarkan penemuan ini, NOAEL daripada kajian dos oral berulang 28- dan 90-hari disimpulkan berada di bawah 2000 mg / kg untuk kedua-dua tikus jantan dan betina. Secara kolektif, data ini menyerlahkan potensi penggunaan AECNL dalam pengurusan dan rawatan kenefrotoksikan teraruh cisplatin melalui peningkatan kebolehidupan sel, penurunan apoptosis, angiogenesis, dan pengawalseliaan parameter klinikal serum dan air kencing. Keupayaan AECNL untuk mengurangkan kesan kemoterapi daripada cisplatin, bagaimanapun, mencegah pemberian serentak kepada pesakit-pesakit kanser. Tambahan pula, walaupun kajian ini menunjukkan tingkah laku yang bukan toksik AECNL pada tikus, langkah berhati-hati perlu dilaksanakan dalam aplikasi klinikal, terutama di kalangan pesakit yang mengalami masalah perubatan lain..

NEPHROPROTECTIVE EFFECT OF *CLINACANTHUS NUTANS*, *ORTHOSIPHON STAMINEUS*, AND *SYZYGIUM CAMPANULATUM* LEAVES AGAINST CISPLATIN-INDUCED NEPHROTOXICITY; AND SAFETY ASSESSMENT OF *CLINACANTHUS NUTANS* LEAVES.

ABSTRACT

Cisplatin is an effective chemotherapeutic agent for solid tumors. However, it also induces dose-dependent progressive and irreversible nephrotoxicity. Cisplatin has multiple intracellular effects, which includes causing direct cytotoxicity with reactive oxygen species, activating apoptosis, and stimulating inflammation, events of which cause vascular and tubular dysfunction. Animal models have demonstrated that cisplatin-induced renal toxicity is characterized by a rapid loss of glomerular filtration, polyuria, hyperkalemia, hypernatremia, and azotemia. Recently, much attention has been given to the possible role of herbal medicine in protecting against cisplatin-induced renal toxicity. Many herbal products are rich in phytochemicals with antiapoptotic, antioxidant, and anti-inflammatory activity. These factors may be useful in protecting kidneys from cisplatin-induced damage and, combined with their proangiogenic properties, help expedite the healing process. *Clinacanthus nutans*, *Orthosiphon stamineus*, and *Syzygium campanulatum* are antioxidant-rich tropical herbs that have been traditionally used to treat kidney diseases. This study investigates the protective effects of the aqueous extract of *Clinacanthus nutans* leaves (AECNL), the hydroethanolic extract of *Orthosiphon stamineus* leaves (HEOSL), and the extract of *Syzygium campanulatum* leaves (HESCL) on *in vitro* and *in vivo* toxicity models. Their protective activities on renal tubular cell (NRK52-E) were evaluated in terms of cellular viability (MTT assay) and apoptosis (Hoechst and Rhodamine 123 staining), with AECNL, HEOSL, and HESCL alone or in combination, with cisplatin. Their

ameliorating potential was evaluated via oral gavage on the animals with AECNL, HEOSL, and HESCL at doses of 100, 200 or 400 mg/kg for 90 days, while receiving weekly doses of cisplatin (1 mg/kg). The biological activity of AECNL on angiogenesis was evaluated using the *ex vivo* rat aortic ring assay by quantifying the degree of microvessel outgrowth from the rings. The mutagenic activity of AECNL (500 µg/well) was evaluated by Ames test, which was performed on *Salmonella* TA98 and TA100 strains. The acute, and the 28- and 90-day repeated dose oral toxicity effect of AECNL was also evaluated. For the acute toxicity study, a single dose of 5000 mg/kg of AECNL was administered for the 28 and 90-day studies, while 500, 1000, and 2000 mg/kg/day was administered to Sprague-Dawley rats of both sex. The results demonstrated that cisplatin decreased cell viability, which was associated with an increase in cytotoxicity and apoptosis. Simultaneous treatment with cisplatin and AECNL, HEOSL, or HESCL enhanced cell survival and viability, and decreased the apoptosis rate in a descending order. Significant attenuation of renal toxicity was confirmed by decreased levels of serum creatinine and proteins, blood urea nitrogen, urine electrolytes, and urine volume. Furthermore, from the increased glomerular filtration rate, serum electrolytes, and urine creatinine, both AECNL and HEOSL showed high potency and efficacy. The influence of AECNL on angiogenesis was significantly (EC50: 47.37) positive, and the results revealed that AECNL was not mutagenic in the auxotrophs of *S. Typhimurium*. In the acute, 28, and 90-day toxicity studies, neither morbidity nor mortality was observed, with LD_{50} values above 5000 mg/kg. While significant alteration was observed in hematological and biochemical parameters, the histopathological examination and the relative weight of the main organs did not show any significant toxicity symptoms. Based on these findings, the NOAEL of the 28- and 90-day repeated oral dose studies is concluded to be below

2000 mg/kg for both male and female rats. Collectively, these data highlight the potential use of AECNL in the management and treatment of cisplatin-induced nephrotoxicity through increased cell viability, decreased apoptosis, angiogenesis, and regulation of serum and urine clinical parameters. The ability of AECNL to decrease the chemotherapeutic effect of cisplatin, however, prohibits concurrent administration in cancer patients. Furthermore, even though these studies demonstrated the nontoxic behavior of AECNL in rats, caution should be exercised in clinical applications, especially in patients with other medical conditions.

CHAPTER ONE: INTRODUCTION AND LITERATURE REVIEW

1.1. Kidneys: Structure and function

Kidneys are paired, bean-shaped retroperitoneal organs located under the cover of the costal margin on either side of the vertebral column. A kidney is about 11 cm long, 6 cm wide, 3 cm thick, and weighs approximately 115–170 g. Macroscopically, kidneys are characterized by an inner fibrous medulla and outer granular cortex encompassed by a capsule of collagenous fibers. Microscopically, they consist of nephrons (functioning units of the kidney), blood vessels, lymphatics, and nerves. The cortex contains corpuscles (a glomerulus and a Bowman's capsule) and convoluted tubules. The medulla is composed of ascending and descending loops of Henle and collecting ducts (Figure 1.1) (Koeppen and Stanton, 2013).

Kidneys are the key organs of the urinary system and have numerous functions such as blood filtration, disposal of metabolic waste and exogenous chemicals, regulation of electrolytes, acid-base homeostasis, and regulation of blood pressure. Kidneys also have endocrine and metabolic functions. However, the pivotal function of the kidneys is excretion of exogenous or endogenous toxins. The kidneys use three general mechanisms to accomplish this goal: glomerular filtration, tubular reabsorption, and tubular secretion. Glomerular filtration is a process by which blood passes through the glomerulus and filtrates enter the Bowman's capsule. The filtrates move through the cortical and medullary loop of Henle where tubular reabsorption and secretion occurs. Finally, the filtrates and water enter collecting ducts to form urine (Daugirdas, 2012).

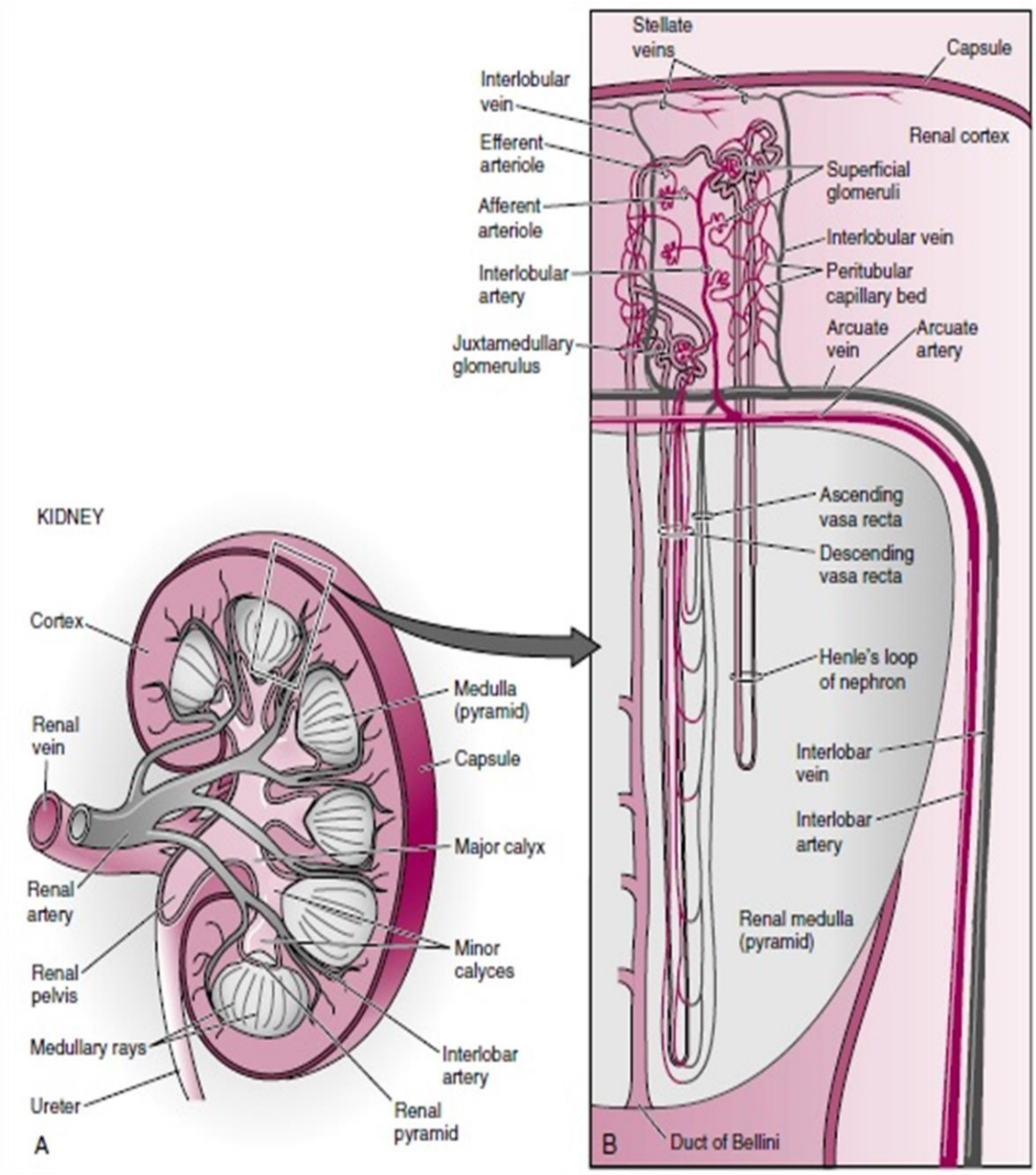

Figure 1.1. Gross anatomic features of the human kidney, the internal structures. A: The medial side of each kidney contains an indentation, through which pass the renal artery and vein, nerves, and pelvis. B Diagram of a nephron (Koeppen and Stanton, 2013).

1.2. Renal disease and diagnosis

Renal disease refers to any damage that disrupts the kidneys' normal glomerular filtration process. The renal disease, may be acute or chronic. Acute renal injury (AKI) is ascribed to an abrupt or a rapid decline in renal function and often causes severe damage to the kidney (Ozkok and Edelstein, 2014). The pathogenesis of AKI is very complicated and multifactorial (Table 1.1). However, the major causes of AKI among ambulatory or hospitalized patients are ischemia, toxins, or sepsis, alone or in combination (Chawla et al., 2014). Twenty percent of all AKI cases are triggered by drug-induced renal injury. AKI can lead to chronic kidney disease and end-stage renal failure (ESRF) (Naughton, 2008). Chronic kidney disease (CKD) is a category of progressive renal disorder that occurs complementary to other chronic medical conditions (Table 1.2). Kidney damage that lasts for three months or more, regardless of the etiology, is also considered CKD (NFK, 2016). CKDs are classified based on the causes of disease, glomerular filtration rate stages, and albuminuria stages. CKDs eventually lead to ESRF. The rate of developing ESRF, however, varies between patients and depends on the underlying cause. ESRF is a permanent kidney failure that necessitates the patient's dependence on life-long dialysis or renal transplant. It is estimated that two million of world's population suffer from ESRF (Levey and Coresh, 2012). Even though there have been significant improvements in medical treatment and patient management, the number ESRF cases increases annually by 6−8%. It is becoming a huge social and economic burden that needs to be addressed (NFK, 2016, Eknoyan et al., 2004).

Table 1.1. Common causes of acute kidney disease

Intrinsic renal disease	Interstitial nephritis	Hereditary renal disease
Acute tubular necrosis	Drug induced	Autosomal dominate polycystic kidney disease
Drug induced	Idiopathic	Autosomal recessive polycystic kidney disease
Toxin mediated	Glomerulonephritis	Alport's syndrome
Hypoxic/ischemic insults	Vascular lesions	Sickle cell nephropathy
Endogenous toxins - hemoglobin, myoglobin	Renal artery thrombosis	Juvenile nephronophthisis
Exogenous toxins - ethylene glycol, methanol	Renal vein thrombosis	Obstructive uropathy/lower tract lesions
Uric acid nephropathy and tumor lysis syndrome	Cortical necrosis	Obstruction in a solitary kidney

(Ozkok and Edelstein, 2014)

Table 1.2. Common causes of chronic kidney disease

Chronic Kidney Disease
Diabetes
Hypertension
Acute kidney injury

(Levey and Coresh, 2012)

1.3. Medication-induced nephrotoxicity

Kidneys are the recipients of 20 % of the resting cardiac output, which supports glomerular filtration and sustains renal metabolism (Guyton and Hall, 2006). High blood flow results in greater exposure to a wide range of drugs or their metabolites. Reabsorption of water along the nephron concentrates the drug in the lumen and tubular cells. The drug accumulates within the proximal tubule cells, which exposes the cells to higher concentrations of toxicants than normally occurs in circulation and leads to nephrotoxicity (Naughton, 2008). Drug-induced nephrotoxicity is a common complication. It involves many classes of prescribed or over-the-counter medicines such as anticancer, antibiotic and viral infection, antihypertensive, and antidiabetic (Table 1.3).

The mechanism of drug-induced nephrotoxicity may vary between different classes of medicines. However, most of the drug follows one or a combination of these pathogenic mechanisms such as crystal nephropathy, interstitial nephritis, vascular obliteration, glomerulonephritis, and tubular cell toxicity. Drug-induced nephrotoxicity is manifested by a decline in the glomerular filtration rate, azotemia, electrolyte imbalances, acid-base abnormalities, proteinuria, and polyuria (Uchino et al., 2005, Bonventre and Weinberg, 2003).

Table 1.3. Categories and examples of nephrotoxic drug

Chemotherapeutic	**Antiinfectives**	**Antihypertensives**	**Analgesics**	**Anti HIV**	**Hypoglycemic**
Cisplatin	Amikacin	Acetazolamide	Paracetamol	Didanosine	Glibenclamide
Carboplatin	Amoxicillin	Captopril	Phenacetin	Stavudine	Thiazolidinediones
Oxaliplatin	Amphotericin B	Furosemide	Acetaminophen	Zalcitabine	
Mithramycin	Benzylpenicillin	Hydralazine	Aspiri		
Zoledronate	Gentamicin	Hydrochlorothiazide	Diclofenac		
Ifosfamide	Metronidazole	Losartan	Ketorolac		
Nitrosoureas	Gentamicin	Mannitol	Aceclofenac		
Imatinib	Ciprofloxacin	Ramipril	Piroxicam		
Cyclophosphamide	Erythromycin	Quinapril	Tenoxicam		

(Naughton, 2008, Hoitsma et al., 1991)

1.4. Treatment for kidney disease

Dealing with kidney disease is challenging due to lack of a concise definition or etiology. Generally, AKI is defined as sudden reduction in the glomerular filtration rate (GFR) in response to an ischemia and nephrotoxins, or as a deteriorated GFR for a period of less than 90 days. There is no specific medication or cure for AKI, as it is normally secondary to other medical conditions (Ishani et al., 2009). However, several strategies based on the underlying causes and extent of illness have been explored in AKI management (NFK, 2016).

The first step is to take preventive actions to reduce the chance of AKI in susceptible individuals. The second step is identification and elimination of the exact causation. This approach, however, is not practical in conditions such as a life-threatening disease that urges an immediate medication. In the third step, which is after an episode of AKI, the patient should be closely monitored for any alteration in the GFR to prevent reoccurrence or progression of AKI (NFK, 2016).

Despite the efficacy of these strategies in delaying AKI or decreasing its side effects, there has been no success in treating this condition. Acute renal diseases are still catastrophic conditions with an increasing rate of morbidity and mortality. Therefore, finding new and effective treatments for patients affected by the renal disease is highly in demand (Chertow et al., 2005). Understanding the mechanisms of kidney dysfunction is of fundamental importance in finding new and effective treatments for patients suffering from renal disease. For this goal, animal models have proven extremely valuable to investigate the pathogenesis of kidney diseases and to evaluate the efficacy of novel therapeutic strategies (Dai et al., 2008, Hewitson et al., 2009).

1.5. Experimental models for kidney disease

To understand the pathology and mechanism of renopathy and to test the efficacy of drug candidates, many rodent and non-rodent models have been developed. Considering the ease of genetic manipulation and accessibility, the non-rodent model is favorable. However, the rodent model is still dominant as it mostly covers all the aspects of the nephropathy and mimics human physiology. Further, it provides an excellent platform to assess nephritis using urinary and hematological parameters and the glomerular filtration rate (Dai et al., 2008).

The best perspective on renal dysfunction has been achievable through medication-induced kidney failure. A number of popular medications such as cisplatin, gentamicin, and fluoxetine have been used to induce nephrotoxicity in animal models (Dai et al., 2008). Among them, the cisplatin-induced injury model has received special attention because of an increasing number of cancer cases and a high incidence of nephrotoxicity in cisplatin therapy. One-fifth of drug-induced acute renal failure is due to cisplatin-therapy, and 30% of the damage caused by cisplatin is irreversible (Arunkumar et al., 2012).

Cisplatin is a highly successful antineoplastic drug that has been increasingly administered recently due to the rising number of cancer cases and its remarkable efficacy. However, the clinical use of cisplatin often limited by life-threatening nephrotoxicity that may result in end-stage kidney disease (Leblanc et al., 2016). Cisplatin-induced nephrotoxicity is characterized by increased renal vascular damage and decreased GFR. The level of damage is measurable by changes in serum/urine clinical chemistry and urine output. In the cellular levels, cisplatin mostly targets proximal tubular cells, increases reactive oxygen species, and stimulates cell death mechanisms. These make the cisplatin-induced

nephrotoxicity an ideal model to study the pathophysiological features of nephrotoxicity (Arany and Safirstein, 2003).

1.6. Chemotherapy

In the turn of 20th century German scientist Paul Ehrlich, known as the father of chemotherapy, described the use of chemical substances in treating diseases as chemotherapy (chemo + therapy). In 1907 the first chemotherapy drug "Arsphenamine" was derived from arsenic to treat syphilis. The use of mustard gas as chemical warfare in World War I, and research on a similar compound known as "nitrogen mustard" during World War II, led to the discovery of the first cancer chemotherapy drug (Mustine). Since then, the use of cytotoxic, low molecular weight compounds in treating or limiting the growth of malignant cells was known as chemotherapy (Miller et al., 2011). Most of the chemotherapeutic drugs exert their effect mainly on proliferating cell cycles, as a rapid proliferation is the characteristic feature of cancer cells. Alteration of cell cycle phases includes interfering with nucleic acid (DNA and RNA) and protein synthesis, transcription, and hormonal balance. Chemotherapeutic agents are classified into two major groups based on their structure and their effects on different phases of the cell cycle. The classes were established as cell cycle-specific agents and cell cycle non-specific agents (Barton-Burke et al., 2001) (Table 1.4). The cycle-specific drugs act only during a particular cycle of division, whereas non-cycle-specific agents act both during the proliferating and resting status. This approach ultimately induces the irreversible death to neoplastic cells, and it has been a triumph to halt or diminish the progression of tumors (Barton-Burke et al., 2001).

Table 1.4. Chemotherapeutic agents

Cell cycle phase specific agents			
G_1[a] phase	**S[b] phase**	**G_2[c] phase**	**M[d] phase**
L-asparaginase	Cytarabine	Bleomycin	Docetaxel
Prednisone	Gemcitabine	Etoposide	Paclitaxel
	Hydroxyurea	Irinotecan	Vincristine
Cell cycle phase non-specific			
Alkylating agents	**Antibiotics**	**Nitrosoureas**	**Miscellaneous**
Cyclophosphamide	Dactinomycin	Carmustine	Dacarbazine
Cisplatin	Doxorubicin	Lomustine	Procarbazine
Carboplatin	Epirubicin	Semustine	

a: First gap or first growth phase; cell prepares for DNA synthesis during this phase.
b: Synthesis phase; Synthesis of DNA is the major event in this phase.
c: Second gap or second growth period; synthesis of RNA and proteins continues; production of the mitotic spindle apparatus occurs during this phase.
d: Mitosis phase, a cell divides into two daughter cells in this phase.
(Barton-Burke et al., 2001)

1.7. Chemotherapy side effects

Chemotherapy is the use of chemical or natural agents to stop the proliferation and metastasis of cancer cells. Most of the drugs in this class exert their antitumor activity by suppression of cancer cells propagation with no specific drug targets. The non-targeted chemotherapeutic agent indiscriminately interacts with cancer and healthy cells with rapid division rates. In this circumstance, the drug induces immediate or late sides effect (Table 1.5). The immediate or common side effects of all chemotherapeutic agents are nausea, vomiting, fatigue, alopecia, anemia, and leukopenia. However, the most explicit drawbacks are often late or organ-specific side effects. These include neurotoxicity, cardiotoxicity, nephrotoxicity, hepatotoxicity, pulmonary toxicity, and ototoxicity (Sayed-Ahmed, 2010). Organ-specific toxicity is a limiting step in the treatment of cancer, as the administration of an adequate dose is hampered by increased side effects (Chabner and Longo, 2011).

Table 1.5. Side effects of chemotherapy

Immediate (common)	Late (Organ-specific)		
Alopecia Anemia Gastrointestinal Immunosuppression Infertility Leucopenia Myelosuppression Nausea and vomiting Neurotoxicity Ototoxicity (hearing loss) Thrombocytopenia	Cardiotoxicity	:	Cyclophosphamide Doxorubicin
	Hepatotoxicity	:	Vincristine
	Nephrotoxicity	:	Ifosfamide Cisplatin
	Neurotoxicity	:	Axonal Vinca
	Pulmonary toxicity	:	Bleomycin
	Cardiotoxicity	:	Cyclophosphamide Doxorubicin

(Skeel and Khleif, 2011)

1.8. Cisplatin

Cisplatin or cis-diamminedichloroplatinum (II) is a structural isomer of Pt (IV) complex, which in 1845 synthesized by Michele Peyrone and named as Peyrone's salt. In 1965, it was found to inhibit binary fission in *Escherichia coli* (E. coli) via blocking cell division but not growth (Rosenberg et al., 1965).

Cisplatin was approved by the Food and Drug Administration (FDA) in 1978 as an alkylating antineoplastic agent and cytotoxic drug based on platinum for solid tumors. Since then, cisplatin became one of the most successful chemotherapy agents ever. It is clinically proven to combat different types of cancer including soft tissue, bone, muscle, cervical, head and neck, esophageal, and colon cancer (Schrauzer, 2013). Despite the remarkable victory, significant challenges remain about the use of cisplatin. The drug resistance and its considerable side effects such as ototoxicity, neurotoxicity, and severe dose-dependent nephrotoxicity are the major stumbling blocks (Yao et al., 2007).

1.9. Mechanism of action of cisplatin

The neutral and inorganic cisplatin initially enters the cell via passive diffusion then by active transport to react with biomolecules and exerts its therapeutic effect. For the reaction to occur, cisplatin undergoes series of hydrolytic process and form monohydrated complexes by sequential replacement of cis-chloro ligands with water molecules (Figure 1.2). The highly reactive monoaquated binds to DNA, forms DNA-DNA and inter- and intrastrand DNA-protein cross links. The formed adducts interfere with normal DNA replication and transcription in rapidly dividing cells and arrest the cells' proliferation. Intact DNA lesion leads to irreversible injury and ultimately cell death. This is the most probable mechanism of action that has been elucidated for cisplatin-induced cell death.

Besides adduct formation, cisplatin binds to phospholipids and phosphatidylserine at the cell membrane level. In the cytoplasm, it interacts with cellular constituents and nucleophilic sites such as cytoskeletal microfilaments, thiol-containing proteins, peptides, and RNA (Figure 1.3) (Jordan and Carmo-Fonseca, 2000). Some studies reported that less than 1% of the cisplatin molecules that enter the cell binds to nuclear DNA so that most of them end up binding to proteins and other biomolecules. Binding to non-DNA targets may contribute to the mechanism of cytotoxicity of cisplatin in cancer cells as well (Yao et al., 2007). Even though the mechanism of action of cisplatin is still not clear after 30 years of clinical use, it is still an effective chemotherapeutic drug that changed the course of treatment in cancer therapy to the extent that some of deadliest malignant tumors are now curable (Yao et al., 2007). Unfortunately, the profound activity of cisplatin is undermined by irreversible toxicity in kidney tissues. This necessitates the development of protective agents against cisplatin-induced toxicity (Arany and Safirstein, 2003).

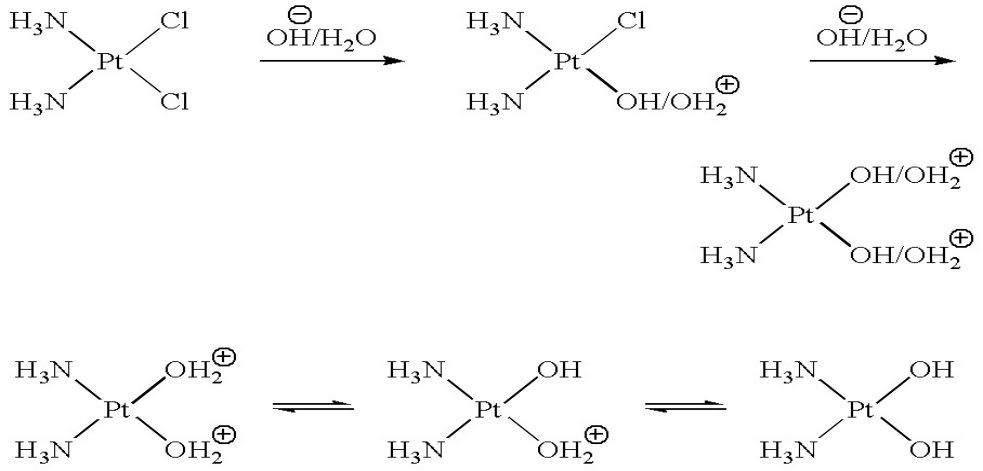

Figure 1.2. Mechanism of cisplatin activation. After entering cells, cisplatin loses chloride ligands to form aquated species.

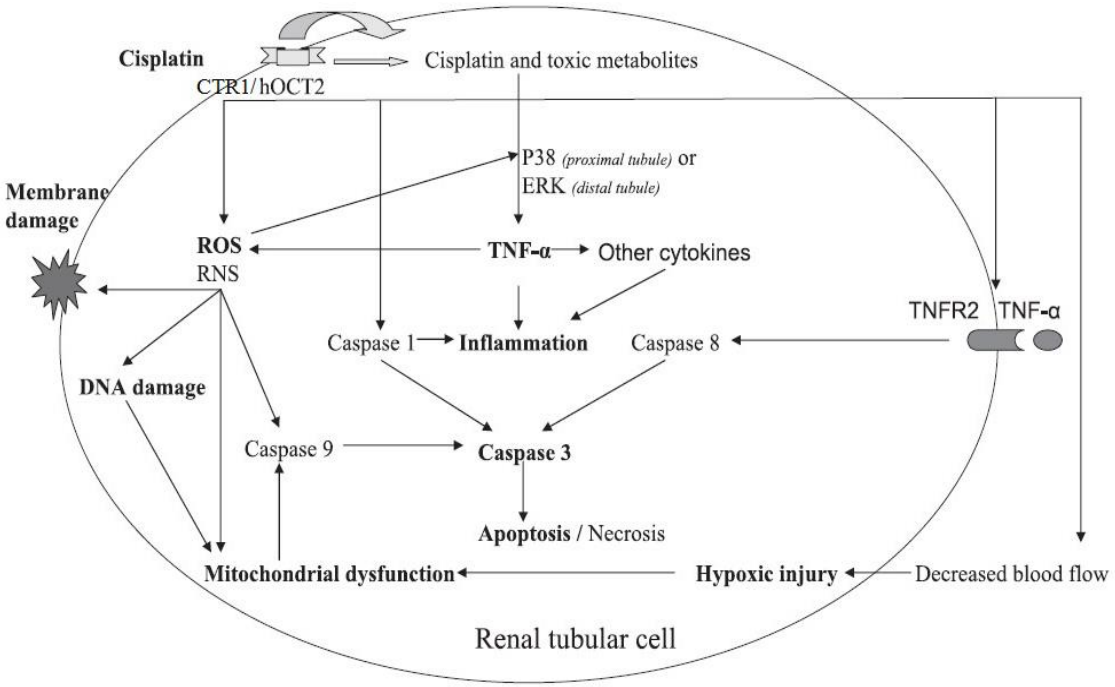

Figure 1.3. Schematic diagram of major pathways in cisplatin-induced acute tubular cell injury. CTR1-membrane copper transporter-1; ERK-extracellular regulated kinase; hOCT2- human organic cation transporter 2; RNS- reactive nitrogen species; ROS- reactive oxygen species; TNF- α tumor necrosis factor-alpha; TNFR2- tumor necrosis factor receptor 2.

1.10. Cisplatin-induced nephrotoxicity

Kidneys are the major excretory organs for cisplatin, and are highly vulnerable to its cytotoxic effect. Due to high blood flow in the kidneys, low molecular weight, and uncharged character of cisplatin, up to 80% of non-active cisplatin passes through glomerulus in 24 hours. Renal tissue uptakes and accumulates the cisplatin in the proximal tubular epithelial cells up to five times of its serum concentration. The excessive accumulation of cisplatin in the renal cortex causes nephrotoxicity (Oh et al., 2014). The cisplatin-induced nephropathy is characterized by renal tubular cell injury. The deposited cisplatin is metabolized in the proximal tubule epithelial cells. Once the reactive cisplatin is formed, it renders the toxic effect through multiple mechanisms. The mechanisms are

described as oxidative stress, inflammation, apoptosis, and vascular injury (Figure 1.4) (Pabla and Dong, 2008).

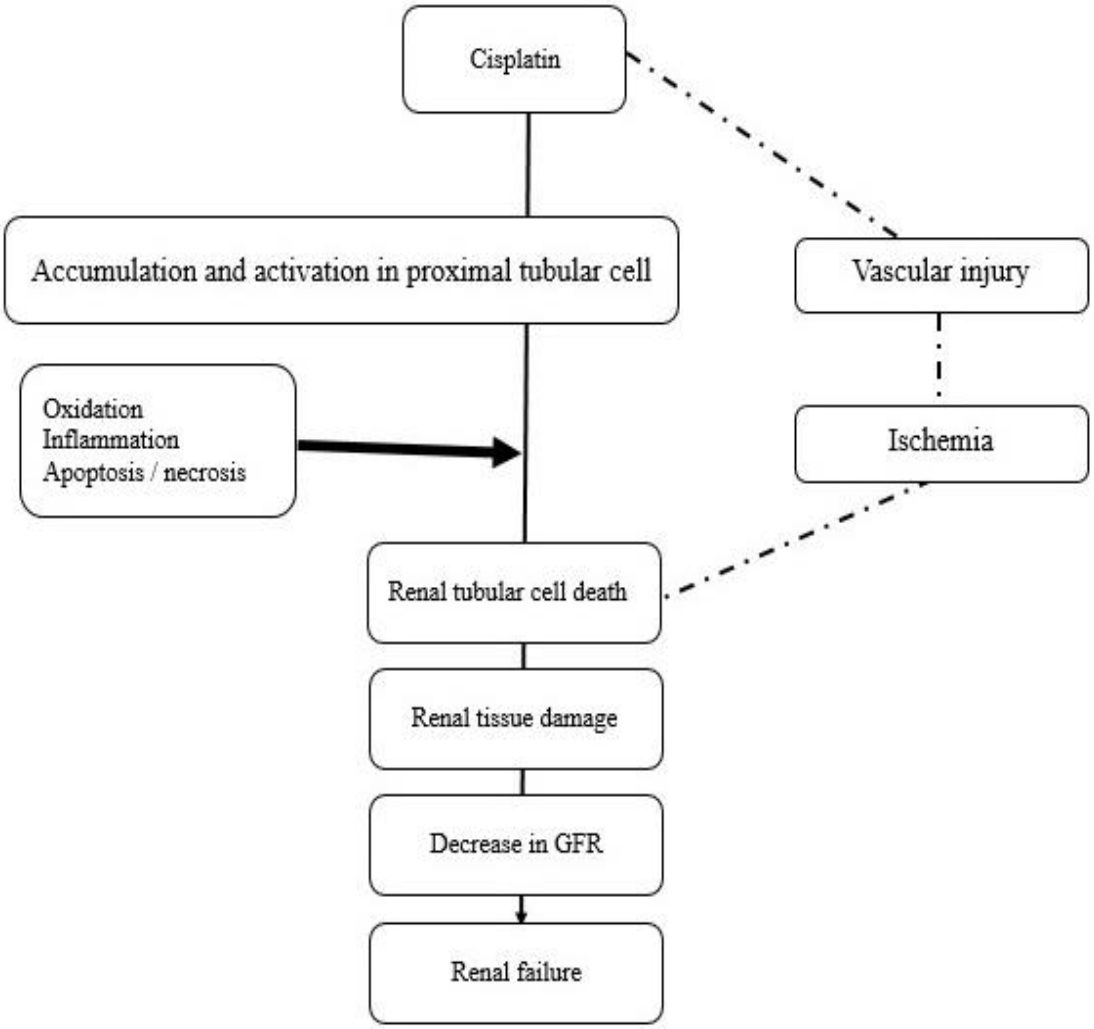

Figure 1.4. Overview of the pathophysiological events in cisplatin nephrotoxicity (Pabla and Dong, 2008).

1.10.1. Cisplatin-induced nephrotoxicity: Oxidative stress

Cisplatin induces oxidative stress by the production of reactive oxygen species (ROS) and attenuation of antioxidant defense capacity. Monoaquated cisplatin inactivates the antioxidant enzyme, which leads to depletion of the antioxidant defense system and increased oxidative stress. Cisplatin also produces ROS by disruption of mitochondrial function. The excess of ROS directly act on cell constituents such as lipids, proteins, RNA and DNA, destroy their structure and modulate cell survival (Pabla and Dong, 2008).

1.10.2. Cisplatin-induced nephrotoxicity: Inflammation

Cisplatin renal injury is related to robust inflammatory responses that contribute to renal tissue and vascular damage. The cascade of inflammatory responses starts with the stimulation of pro-inflammatory cytokines and chemokines in the presence of cisplatin. Tumor necrosis factor alpha (TNF-α), as a major cytokine, has a central role in mediating the renal injury. TNF-α interferes with the cell death process through the production of ROS, and activation of a myriad of apoptotic responses in the kidney. Moreover, it increases the expression of inducible nitric oxide synthesis and enhances the production of nitric oxide, which leads to cell damage. The level of contribution of each pathway to renal injury, however, is not yet defined (Pabla and Dong, 2008).

1.10.3. Cisplatin-induced nephrotoxicity: Apoptosis

Apoptosis is the programmed mode of cell death that is an integral part of normal a cell's death and is characterized by morphological and energetic changes. Sporadic apoptosis, however, underlies many medical conditions such as degenerative diseases and ischemic damage (Elmore, 2007). Cisplatin initiates apoptosis via intrinsic as well as extrinsic pathways. Intrinsic processes start by DNA damage, compromised mitochondrial and

endoplasmic reticulum function, and finally cell death. In the extrinsic pathway, engagement of death receptors such as TNF-α results in cisplatin-induced renal cell death (Arany and Safirstein, 2003). Morphologically, apoptosis starts with renal cell shrinkage, chromatin condensation, and membrane blebbing and eventually cells turn into dark oval bodies (Elmore, 2007).

1.10.4. Cisplatin-induced nephrotoxicity: Vascular injuries

Kidneys are highly vascular organs with the unique spatial arrangements. The proper assembly of kidney vessels with their nephrons is a crucial event leading to the formation of a functioning and healthy kidney. The integrity of the vascular endothelium is critical for the health of kidney and is determined by the balance of physiological endothelial cell turnover. After an inflammatory injury, damaged cells are replaced through induced proliferation of surrounding endothelial cells. In disease conditions, constant injury and inefficient delivery of angiogenesis factors accelerate vascular injury. A decrease in proangiogenic factors are directly related to endothelial cell proliferation and reverse the degree of glomerular capillary loss. Loss of functioning vascular systems and disruption of blood supply and limited oxygen availability in kidney induces acute hypoxia, and eventually ischemic renal failure (Sequeira Lopez and Gomez, 2011).

Cisplatin is directly toxic to endothelial cells through inflammation and necrosis, as well as dysfunction and impairment of vascular autoregulation. Cisplatin induces vasoconstriction and develops a hypoxic injury, decreases effective renal plasma flow, and finally reduces GFR. In this circumstance, it has been demonstrated that delivery of angiogenesis factors and the stimulation of the angiogenesis response can be therapeutic to promote glomerular endothelial repair (Goligorsky, 2015).

1.11. Pathophysiologic hallmarks of cisplatin-induced nephrotoxicity

Cisplatin-induced nephrotoxicity is dose-related and cumulative in both animals and humans. A dosage of more than 50 mg/m^2 can cause renal injury initiated by an acute, mainly proximal tubular impairment and preceded by alterations in renal hemodynamics. At 48 to 72 hours after cisplatin administration, a depressed renal function is observed due to impairment of proximal and distal tubular reabsorptive capacities. The damage is symptomized by polyurea, azotemia, and fall in serum sodium, potassium, magnesium, phosphorous, and occasionally calcium (Arany and Safirstein, 2003).

1.12. Current strategies to treat cisplatin-induced nephrotoxicity

The broad clinical applications of cisplatin not only rely on the therapeutic effect, but also on controlled and minimized nephrotoxicity. There are numbers of pathological conditions that lead to cisplatin-induced nephropathy. Therefore, the protective strategies are classified accordingly to ameliorate or eliminate cisplatin-induced nephrotoxicity. The approaches are summarized in Table 1.6 (Pabla and Dong, 2008).

Clinical efficacy of these nephroprotective strategies is under debate, as most of them have been tested only in cultured cell and animal models. Further, many of the same pathological conditions are responsible for the cytotoxic activity of cisplatin in malignant cells. Regardless of which strategies have been the method of choice, whether it is antiinflammation, antioxidant, or antiapoptosis, it reduces cisplatin cytotoxicity. Attenuation of cisplatin's chemotherapeutic activity, restricted the clinical applications of current strategies. Therefore, any proposed strategy must be carefully studied to ensure that the chemotherapeutic efficacy of cisplatin is not vitiated (Pabla and Dong, 2008).

Table 1.6. Experimental strategies to prevent cisplatin-induced nephrotoxicity

Strategies
Reduction of renal cisplatin accumulation
OCT2[a] inhibitors, e.g., cimetidine or metformin
Ctr1[b] inhibitors, e.g., copper Micellar/liposomal cisplatin
Antioxidant
Selenium
Vitamin E catalase
Superoxide dismutase
N-acetyl cysteine
Antiapoptosis
Caspase inhibitors
p53[c] inhibitors
Antiinflammatory
TNF-alpha[d] antagonists
Salicylates

a: Organic cation transporter 2, b: High affinity copper uptake protein 1
c: Cellular tumor antigen p53, d: Tumor necrosis factor alpha
(Miller et al., 2010).

1.13. Herbal medicine in treating cisplatin-induced nephrotoxicity

Despite the lack of consistent information to conclude the efficacy of the herbal products in treating chemotherapy side effects, some data show adjunctive administration of plants is receiving notable attention recently due to the increase in the life span and quality of life of patients (Metri et al., 2013). The data further showed that over 80% of patients have been using complementary and alternative approaches adjuvant to chemotherapy. There is an agreement within a number of published work that shows that complementary formulas from different traditional systems of medicine with various levels of efficacy is gaining popularity in reducing side effects of chemotherapy (Cassileth and Deng, 2004).

Ayurveda is a system of traditional medicine in the Vedic culture of India, and dates back to 5000 B.C. Some studies have shown that concurrent administration of Ayurvedic herbal formulas with chemotherapy increases the lifetime of patients, compared with those who received chemotherapy alone (Metri et al., 2013). Chinese herbal formulas have been used widely to neutralize the side effects of cancer treatment. Clinical research has proven that Fu Zhen, a combination of Chinese herbal medicines, increased the life span of certain cancer patients up to five years (Efferth et al., 2007). PHY906 is a traditional Chinese medicine formulation consists of *Scutellaria baicalensis Georgi, Glycyrrhiza uralensis Fisch., Paeonia lactiflora Pall., and the fruit of Ziziphus jujubeMill*. Co-administration of PHY906 with current anticancer drug provides a survival benefit. Clinical data showed, patients had stable condition after two treatment cycles. Furthermore, Asian patients had a higher median overall survival (16.5 months) than non-Asian patients (6.2 months) (Yen et al., 2009).

Herbal intervention is not limited to traditional practice. Many herbal preparations have been tested for their efficacy to ameliorate cisplatin-induced nephrotoxicity in preclinical

setting (Table 1.7). Previous research suggested that methanolic extract of *Angelica sinensis* protected human kidney cells from the cytotoxic effect of cisplatin (Bunel et al., 2015b). Another study revealed that silymarin, a natural flavonoid extracted from *Silybum marianum* (milk thistle) seeds, demonstrated a protective effect against cisplatin-induced alteration in glomerular and proximal tubular function as well as proximal tubular morphology change (Bokemeyer et al., 1996). The effect was further confirmed by one study that shows pretreatment with methanolic extract of milk thistle seeds helped to reduce cisplatin-induced kidney damage by reducing blood urea nitrogen and serum creatinine (Karimi et al., 2005) and produced a significant antioxidant effect (Abouzeinab, 2015). Post treatment with thymoquinone, a phytochemical from *Nigella sativa* significantly ameliorate elevation in biochemical and physiological parameters as well as histopathological changes induced by cisplatin administration (Ali et al., 2015). It also protects kidney against doxorubicin (Elsherbiny and El-Sherbiny, 2014), acetaminophen-induced damage (Aycan et al., 2015). The concurrent administration of thymoquinone with omega-3 increased kidney tissue content and antioxidant enzymes in ischemia/reperfusion model (Fayez et al., 2014). Water preparation of *Nigella sativa* protects kidney against paracetamol induced oxidative stress (Hamza and Al-Harbi, 2015). *Chrysanthemum indicum* is traditional herbal remedies for inflammatory conditions, ethanolic preparation of leaves protect kidney via increasing antioxidant activity and porcine kidney cell (LLC-PK1) viability both in *in- vivo /-vitro* (Kim et al., 2015).

The use of herbal remedies to ameliorate immediate and late side effects of cisplatin therapy has some basis in scientific research. However, the concomitant administration of some plants with chemotherapy may cause potential herb-drug interaction. Simultaneous

administration of herbs with chemotherapy should be closely monitored as it may render the chemotherapeutic agent to be ineffective (Cassileth and Lucarelli, 2003).

Table 1.7. List of herbal preparation with nephroprotective activity against cisplatin-induced toxicity.

Test sample	**Activity**	**Reference**
Azadirachta indica	Antioxidant	(Abdel Moneim et al., 2014)
Angelica sinensis /Ferulic acid	Increasing renal cell proliferation and motility Inhibition of β-catenin pathway	(Bunel et al., 2015a)
Curcuma longa	Increasing renal cell proliferation	(Kuhad et al., 2007)
Glycine max	antioxidant and antiinflammatory	(Ekor et al., 2010)
Momordica cochinchinensis	Antiapoptotic	(Jung et al., 2016)
Phyllanthus maderaspatensis	Antioxidant Genotoxicity	(Chandrasekar et al., 2006)
Paeonia suffruticosa	Increasing renal cell proliferation	(Sohn et al., 2009)
Scutellaria barbata	Increasing renal cell proliferation	(Sohn et al., 2009)
Schisandra sphenanthera	Antioxidant	(Jin et al., 2015)
Scutellaria barbata	Antiinflammatory	(Lee et al., 2010)
Sinapis alba	Increase cell proliferation	(Sohn et al., 2009)
Silymarin	Antiapoptotic	(Kabel et al., 2013)
Silibinin	Proximal tubular morphology protection	(Behling et al., 2006)
Tinospora cordifolia	Antioxidant	(Uppuluri et al., 2013)
Trichosanthes kirilowii	Antioxidant, Antiinfalmmatory Increasing renal cell proliferation Increased cell viability	(Seo et al., 2015)
Ribes diacanthum	Antioxidant	(Tilyek et al., 2016)

1.14. Description of *Clinacanthus nutans*

Clinacanthus nutans is an annual plant indigenous mainly to South China, Indonesia, Malaysia, Myanmar, the Philippines, Singapore, Thailand, and Vietnam. *Clinacanthus nutans* grows as an erect herb or rambling shrub. The stem is green and cylindrical, and it turns yellow after the plant is dried. Sulcate, bifarious, and pubescent petioles of 0.3–2.0 cm in length connect leaves to the stem. The leaf blade is green, lancelot, and linear, and can grow up to 12 mm long and 4–6 mm wide (Figure 1.5) (GlobinMed, 2015).

1.14.1. Taxonomy

Kingdom	:	Plantae
Order	:	Lamiales Bromhead
Family	:	Acanthaceae
Genus	:	*Clinacanthus*
Species	:	*Clinacanthus nutans*
Scientific name		*Clinacanthus nutans*
English name	:	Sabah snake grass
Common name		
Indonesia	:	Kitajan (Sunda); Dandang Gendis (Java); Gendis (Central Java)
Malaysia	:	Belalai Gajah
South China	:	E zui hua , and niu xu hua

Figure 1.5. *Clinacanthus nutans*; whole plant (https://florafaunaweb.nparks.gov.sg).

1.14.2. Traditional uses

Despite the accessibility of modern medicine, *Clinacanthus nutans* has been and still is being used as a healing herb in South China and South East Asia (GlobinMed, 2015). In some cases, it is the first step in health care; in others, it provides a real alternative to primary health care approaches in treating conditions such as kidney dysfunction, gastrointestinal diseases, inflammation, viral infection lesions, and snake or scorpion venom (Siew et al., 2014, Gao et al., 2014). *Clinacanthus nutans* was also used to promote urination, discharge kidney stones, and promote blood circulation that invigorates the kidneys.