Lebanese American University

The Protective Effects of *Daucus carota* L. ssp. carota and *Cannabis sativa* L. Extracts against Cisplatin Induced Nephrotoxicity in Animal Models

By

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DEDICATION

I’d like to dedicate my thesis work to Al-Muntazar.

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The Protective Effects of *Daucus carota* L. ssp. carota and *Cannabis sativa* L. Extracts against Cisplatin Induced Nephrotoxicity in Animal Models

Sahar Shawki Al Toufaily

ABSTRACT

Cisplatin is a standard antineoplastic drug that has been incorporated in many first-line chemotherapy regimens for the treatment of solid tumors such as, advanced ovarian, lung, head and neck, testicular and bladder. However, its use is associated with dose dependent nephrotoxicity, and ongoing attempts have been made to reduce the cisplatin-induced nephrotoxicity. The current study investigates the nephroprotective effects of two native Lebanese plants, *Daucus carota* and *Cannabis sativa*, in animal models. Previous studies in our laboratory showed that *Daucus carota* oil extract (DCOE) possesses antioxidant, anti-inflammatory, and anticancer properties. The major component of DCOE, β-2-himachalen-6-ol (HC), has been isolated and found to be a potential safe and potent chemotherapeutic agent. Additional recent studies showed that *Cannabis sativa* oil extract (COE) demonstrated potent anti-inflammatory effects. DCOE (100 mg/kg body weight) and HC (25 and 50 mg/kg body weight) were administered daily to rats for 10 days, and a single dose of cisplatin (7.5 mg/kg body weight) was administered on day five. In another protocol, 2.5, 5, 10, and 20 mg/kg body weight of COE were given daily to mice for three days, and cisplatin (20 mg/kg body weight) was administered on day one. Serum creatinine and urea, proteinuria, and inflammatory markers were measured. All used doses of COE showed significant decrease in serum urea; however, a significant decrease in serum creatinine was observed only at 20 mg/kg COE. On the other hand, treatment with HC (50 mg/kg) or DCOE (100 mg/kg) demonstrated significant decrease in serum urea, but little or no effects was

observed on serum creatinine. In addition, COE treatment caused a significant decrease in urinary albumin excretion. Furthermore, Western blot analysis with the antibodies against COX-2 was significantly lower in cisplatin-intoxicated mice with COE in comparison to mice treated with cisplatin alone. In conclusion, the current findings demonstrates that doses of HC (50 mg/kg) and DCOE (100 mg/kg), and all used doses of COE could be a promising approach to protect against cisplatin-induced nephrotoxicity, partially by ameliorating inflammatory response.

Keywords: Medicinal plants, Daucus carota, Cannabis sativa, Cannabis, Cisplatin, Nephrotoxicity, Cisplatin-induced nephrotoxicity.

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List of Abbreviations

CB	Cannabinoid
CBC	Cannabichromene
CBD	Cannabinoid
CBG	Cannabigerol
CBL	Cannabicyclol
CBN	Cannabinol
COE	Cannabis oil extract
COX-2	Cyclooxygenase 2
Ctr1	Copper transporter
DAMPs	Damage-associated molecular pattern molecules
DCOE	*Daucus carota* oil extract
eGFR	Estimated glomerular filtration rate
ER	Endoplasmic reticulum
FasL	Fas ligand
FDA	Food and Drug Administration
HC	B-2-himachalen-6-ol
MAPK	Mitogen activated protein kinases
MW	Molecular weight
NSAIDs	Nonsteroidal anti-inflammatory drugs
OCT2	Organic cation transporter
PI3K	Phosphatidylinositol 3 kinase
ROS	Reactive oxygen species
SEM	Standard error of mean
THC	Δ^9-tetrahydrocannabinol
TLR4	Toll-like receptor 4
TNF alpha	Tumor Necrosis Factor alpha

Chapter One

Introduction

1.1. Natural Remedies: A Gift from Nature

1.1.1. Natural Remedies

Natural products have a great importance in human's life. Humans have used different natural compounds and remedies from plants and animals as medicine to treat different diseases. The use of plants by human as medicine is traced back to sixty thousand years ago according to some studied fossil records (Yuan et al., 2017).

1.1.2. Lebanese Natural Remedies

Lebanon is famous for its great biodiversity and is characterized by the existence of plant in its different geographical area (Medail & Quezel, 1997). There are more than 2500 flora species in Lebanon. Many of them are reported to have medicinal benefits for different diseases (Dagher-Kharrat et al., 2018. A recent study done by Abi-Rizk shows that four different Lebanese plants extracts, *P. asperula*, *S. ehrenbergii*, *S. multicaulis* and *H. lobelia*, have antitumor and antioxidant effects on human pulmonary adenocarcinoma cells (Abi-Rizk et al., 2020). Still, more studies need to be conducted to prove the medicinal benefits of Lebanese natural products. In our study, two well-known Lebanese plants are chosen to evaluate its nephroprotective effects against cisplatin-

induced nephrotoxicity. These two plants are *Daucus carota* ssp. carota and *Cannabis sativa* L. ssp. indica.

1.2. Nephrotoxicity

1.2.1. Drug-induced nephrotoxicity

Drugs can be a double-edged sword. While many drugs are used to treat and prevent certain illness, they cause 20% of acute kidney failure episodes (Kaufman et al., 1991; Bellomo, 2006). In older patients, the incidence of nephrotoxicity caused by drugs can reach 66% (Kohli et al., 2000). Compared to forty years ago, people today are expected to live longer, and this is associated with an increased incidence of chronic diseases like cardiovascular disease and diabetes. Such diseases indicate an increased exposure to medications and procedures which have a potential to induce kidney damage (Hoste & Kellum, 2006). Although drug-induced renal impairment is often reversible, it is still a life-threatening and costly condition (Gandhi et al., 2000).

1.2.2. Mechanisms for Drug-Induced Nephrotoxicity

Different drugs cause nephrotoxicity through different pathways. It can cause toxicity to the tubular cells, alter the intraglomerular hemodynamics, rhabdomyolysis and crystal nephropathy (Schetz et al., 2005; Schnellmann et al., 1999). It is important to know what drug and what pathway of nephrotoxicity is present in each patient as it helps in their treatment. For example, analgesics like acetaminophen and aspirin can cause chronic interstitial nephritis (Perneger et al., 1994). Antimicrobials like aminoglycosides can

cause tubular cell toxicity (Guo & Nzerue, 2002). Other medications like the proton pump inhibitors that are used for ulcers such as omeprazole can cause acute interstitial nephritis (Simpson et al., 2006). Finally, the most dangerous medications on the kidneys are chemotherapeutics like cisplatin, carboplatin, and oxaliplatin that can cause both chronic interstitial nephritis and tubular cell toxicity (Appel, 2002; Jagieła et al., 2021).

1.2.3. Detection of Acute and Chronic Renal Failure

When renal impairment is detected early and the offending drug is discontinued, renal damage will be mostly reversible (Choudhury & Ahmed, 2006). The main signal that indicates possible drug-induced nephrotoxicity is the increase in serum creatinine after the administration of the drug except in the case of cimetidine or trimethoprim administration as these two drugs compete with creatinine in tubular secretion and can cause elevated serum creatinine levels without kidney injury (Naughton, 2008). Renal damage can be viewed as acute or chronic kidney injury. Since their first descriptions in the nineteenth century by physicians such as Bright, Heberden, and Abercrombie, chronic kidney disease and acute kidney injury have been viewed as significant but distinct pathologies (Berry, 1992). Until recently, conventional thinking held that oliguric acute kidney injury was often fatal if left untreated, but with the emergence of dialysis, complete recovery was possible (Liano et al., 2007).

Acute kidney failure strikes suddenly and is frequently reversible. Accidents, wounds, disease, infection, shock, or the ingestion of a poison or a drug are examples of etiologies (James et al., 2015). When the kidneys become damaged, they cease to produce

urine. Poisons accumulate in the bloodstream, causing the patient to become confused or unconscious, as well as overloaded with fluids. Patients with acute kidney failure are put on a special diet, have fluid restrictions, and are put on dialysis until their kidneys heal. Kidney function may return to normal with treatment (Ferenbach & Bonventre, 2016).

Chronic kidney failure develops gradually and is generally irreversible (Naeem et al., 2020). Dialysis is usually required once the diagnosis has been made and kidney function is reduced to 10% - 15% (Cibulka & Racek , 2017). Dialysis mimics the functions of healthy kidneys but is not a cure for kidney disease. In most cases, the patient will need dialysis for the rest of his or her life or a kidney transplant (Ortiz et al., 2014).

1.2.4. Biochemical Markers for Testing Renal Function

Different biochemical markers can be used to assess kidney function. Such markers can be radioactive and non-radioactive chemicals. They can indicate the glomerular filtration rate (GFR) and tubular function of the kidneys. Any increase or decrease in the values of these markers may imply kidney dysfunction.

Creatinine, a breakdown product of creatine phosphate in muscle, is produced at a fairly constant rate in the body depending on muscle mass (Yuegang et al., 2008). Creatinine is a common indicator of kidney function and is the best marker to calculate glomerular filtration rate (GFR) to estimate kidney function. Creatinine clearance testing is used to track the progression of renal disease (Miller et al., 2005). When serum creatinine levels exceed the upper limit of the “normal” interval, renal failure is usually

suspected. The excretion of creatinine by both the glomeruli and the tubules eventually decreases in chronic renal failure and uremia (Edmund & David, 2006). Creatinine levels may fluctuate because its production is influenced not only by muscle mass but also by muscle function, muscle composition, diet, activity, and health status (Banfi & Del, 2006). Creatinine tubular secretion may be increased in some patients with kidney dysfunction, resulting in a false negative value (Branten et al., 2005). Elevated values have also been observed in muscular dystrophy paralysis, anemia, and hyperthyroidism. On the other hand, glomerulonephritis, congestive heart failure, and dehydration are associated with lower values (Edmund & David, 2006).

Urea, which is a major nitrogenous end product of protein catabolism, is produced by the liver and is then distributed into the intracellular and extracellular body fluids. The kidneys filter urea in the glomeruli which is then partially reabsorbed with water (Corbett, 2008). The concentration of urea in the serum is one of the most commonly determined clinical indices for estimating renal function. It aids in the differential diagnosis of acute renal failure and pre-renal conditions in which the blood urea nitrogen–creatinine ratio is elevated (Mitchell & Kline, 2006). Increased blood urea nitrogen (BUN) levels are associated with kidney disease or failure, tubular dysfunction, congestive heart failure, dehydration, and others. However, trauma, surgery, and anabolic steroid use are all associated with low serum urea levels (Pagana & Kathleen, 2002).

The presence of significant amounts of protein in urine is one of the first clinical signs of almost all renal diseases (Gowda et al., 2010). Proteinuria estimation aids in distinguishing between tubulointerstitial and glomerular diseases, as well as tracking the

progression of renal disease and assessing the response to therapy (Helena & Jason, 2004). Most healthy adults excrete between 20 and 150 mg of protein in their urine over a 24-hour period. Proteinuria greater than 3.5 gm/day is considered diagnostic of nephrotic syndrome. Protein measurement panels containing various proteins such as albumin have been used in the differential diagnosis of prerenal and postrenal disease. The protein/creatinine ratio has been recommended as an Index of Quantitative Proteinuria in 24-hour urine collection (Jerums & MacIsaac, 2002). Semi-quantitative dipstick urinalysis is one of the proteinuria investigations because it is inexpensive and simple to perform (Sandeep et al., 2004). A dipstick proteinuria diagnosis should be confirmed by either a 24-hour urine collection or a protein-creatinine ratio (Jason et al., 2005).

1.2.5. Prevention of Drug-Induced nephrotoxicity

Some patients with certain conditions and situations may be at high risk for developing drug-induced nephrotoxicity. Thus, knowing the risk factors that a patient may have, and early intervention are keys for successful prevention of kidney injury (Schetz et al., 2005). An example of prevention strategy would be to target the used medication that can induce nephrotoxicity and monitor its levels carefully in patients who are at risk for developing kidney injury. If possible, risk factors must be corrected before administering any nephrotoxic medication (Guo & Nzerue, 2002).

1.2.6. Risk Factors

Risk factors for patients may differ depending on the used medication, but there are some common risk factors for all used nephrotoxic medications (Kaufman et al.,

1991). Those risk factors are older age (more than 60 years) (Bellomo 2006), taking more than one nephrotoxic medication, having diabetes or heart failure, sepsis (Schrier et al., 2004), or starting with an underlying renal insufficiency (e.g., glomerular filtration rate that is less than 60 ml/min/1.73 m^2) (Leblanc et al., 2005). The presence of additional risk factors increases the risk of patient for developing acute renal failure. Such patients must be monitored closely for any change in their renal function whenever a new medication is added, or a dose is increased.

1.2.7. Nephroprotective Effect of Natural Products

Recently, increased use of natural product to treat different kidney diseases is noticed. In fact, there are many reports indicating that various medicinal plants have a curative effect against nephrotoxicity associated with some drugs (Heidari-Soreshjani et al., 2017). Examples of some reno-protective plants found in the literature are Ginkgo biloba, Nigella sativa, green tea, and garlic (Hosseinian et al., 2018). When studied, such plants were able to decrease serum creatinine and urea in cisplatin-induced nephrotoxicity animal models (Rad et al., 2017). However, their effect on human still needs to be investigated. Different compound found in such plants are responsible for their medicinal benefits. Some of these natural products are alkaloids, flavonoids, terpenoids, saponins, and sesquiterpenes (Ojha et al., 2016). The medicinal benefits of such natural products are due to their anti-inflammatory, antioxidant, and anti-apoptotic properties (Gómez-Sierra et al., 2018). This is important especially in the case of cisplatin associated kidney damage as many of the nephrotoxic pathways of cisplatin are related to inflammation, increased oxidative stress, and apoptosis of kidney cells (Cox-Georgian et al., 2019). Thus, many

treatment options against cisplatin-induced nephrotoxicity can be found in natural products.

1.3. Cisplatin-Induced Nephrotoxicity

1.3.1. Properties and Uses of Cisplatin

Cisplatin, also known as diamminedichloridoplatinum (II) (Figure 1), is one of the mostly used chemotherapeutics in clinical settings as it is the most effective in treating different types of cancer, especially solid tumors. To add, it is the first chemotherapeutic medication with simple structure that contains a metal. This platinum containing medication is used for several types of solid tumors like head and neck, testicular, ovarian, cervical cancer, bladder, melanoma, lymphomas and lung cancer (Aldossary, 2019).

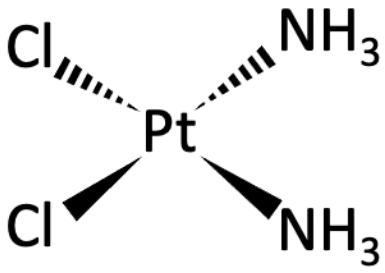

Figure 1. Chemical structure of cisplatin

This chemotherapeutic drug is a crystalline powder that has white to yellow color. It is highly soluble in water with a solubility of 2.53 g/L at 25 °C. This medication is stable in its active *cis*-isomer at normal pressure and temperature. However, with time, it can slowly transform into its inactive trans-isomer. The molecular weight of Cisplatin is 301.1 g/mol, its density is 3.74 g/cm^3, its melting point is 270 °C, and its log K_{ow} is −2.19

(Long & Repta, 1981). The time needed for half of cisplatin administered to be eliminated by the body, also known as half-life (t½), is around 36 to 47 days. This long half-life of the drug is since cisplatin has a biphasic elimination phase in the kidneys. Kidney elimination of cisplatin is mainly through glomerular filtration followed by tubular secretion (Barabas et al., 2008). M. Peyrone synthesized cisplatin compound first in 1844. In 1965, the compound started getting scientific recognition after the initial observation done by Barnett Rosenberg in Michigan State University where it was shown that *Escherichia coli* cell division was inhibited by platinum mesh electrodes through some of its electrolysis products (Dasari & Tchounwou,2014). Since then, much interest was generated to create chemotherapeutic drugs containing noble metals such as platinum.

1.3.2. Anticancer Activity of Cisplatin

The anticancer effect of cisplatin is possible due to different mechanisms. Mainly, this alkylating agent forms covalent bonds with DNA (even the mitochondrial DNA), RNA, and proteins, which cause cell cycle arrest, impairs cell function, and leads to cell death (Cepeda et al., 2007) Another mechanism for cisplatin anticancer effect is due to generating oxidative stress in cancer cells which leads to apoptosis (Ghosh, 2019).

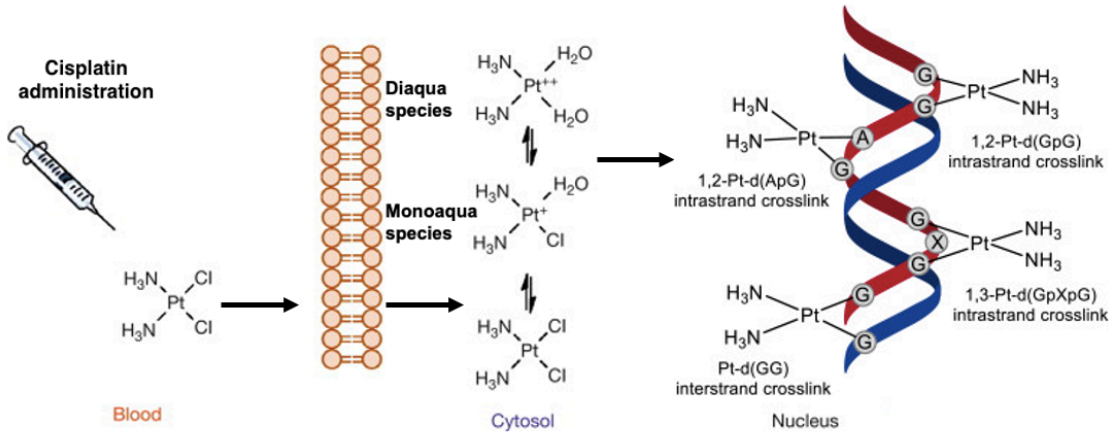

Figure 2. Cisplatin hydrolysis in the cytoplasm into monoaqua and diaqua species that can damage DNA in the nucleus through intra- and inter-strand crosslinking (Adapted from Wang & Zhu).

After administering cisplatin to patients intravenously in a sterile solution, it circulates in the blood where around 65-95% of cisplatin is strongly bound to plasma protein and albumin within 24 hours of administration. Binding of cisplatin to plasma proteins deactivates a large amount of the introduced dose, and the rest enters the cells. cisplatin enters mammalian cells through copper transporter (Ctr1), and the rest enters through the plasma membrane by passive diffusion. Thus, cells that have higher expression of CTR1 will have higher cisplatin accumulation and it will be more sensitive to cisplatin. Inside the cells, cisplatin is activated after the displacement of chloride atoms by water molecules. This results in mono-aqua $[cis\text{-}(NH)PtCI(HO)]^{+}$ and di-aqua diaquated $[cis\text{-}(NH)Pt(HO)]^{2+}$ reactive compounds formation in the cytoplasm (Figure 2). The mono-aqua and di-aqua reactive compounds are one thousand times more reactive than cisplatin itself. Now the available potent electrophile will react with any available

nucleophile like the nitrogen found in nucleic acid and the sulfhydryl Group found in proteins. Cisplatin binds to the reactive purine center N7 found in DNA that leads then to DNA damage and consequently to cell cycle interruption at different phases and apoptosis (Arnesano et al., 2013). Cell cycle arrest can occur at any phase of the cell cycle since cisplatin is not cell cycle specific, so it can arrest the cell at G1, S and even G2 phases (Aldossary, 2019). Cisplatin can form adducts with DNA, RNA and proteins (Dasari & Tchounwou, 2014).

Besides DNA damage, cisplatin can cause cell death by inducing oxidative stress in the cell. Cisplatin increase ROS levels inside the cells depending on the dose and the duration of the used medication. Molecules containing thiol Group (–SH) maintain the intracellular redox homeostasis. When thiol Group forms thiyl radicals in some circumstances, the radicals can react with oxygen molecule forming reactive oxygen species. Increased ROS in the cell causes apoptosis through different intrinsic and extrinsic pathways. To add, increased ROS levels causes lipid peroxidation, arrests cell cycle, induces p53 signaling, and down regulates both anti-apoptotic proteins and proto-oncogenes. Eventually this leads to cancer cell death (Siddik, 2003).

Also, cisplatin is able to modulate calcium signaling inside the cell as part of its cytotoxic effects. This disruption of the homeostasis of calcium, which is the result of endoplasmic reticulum (ER) stress, will trigger chain of events like enzyme inhibition and lipid peroxidation. This will damage the mitochondria, inhibit its function, and leads to the depletion of cofactors and adenosine triphosphate (ATP) inside the cell. Consequently, such events will lead to cell damage, apoptosis, followed by tissue necrosis. That is why

some clinical cytoprotective practices suggests using calcium supplements to increase calcium levels of the cancer patient receiving cisplatin since calcium can compete with cisplatin on its binding site which can prevent many cisplatin toxicities (Sun et al., 2019).

The last mechanism of action of cisplatin is the induction of cell apoptosis through the cell membrane. This pathway of apoptotic cell death is the result of type II Fas receptor activation through type II transmembrane protein as well as Fas ligand (FasL). This leads to the formation of apoptosome complex from Fas associated death domain (FADD) in addition to procaspade-8. Then, the apoptosome complex activates a series of caspases that results in the cleavage of key substrate and finally cell apoptosis (Qi et al., 2019).

1.3.4. Cisplatin-Induced Nephrotoxicity

Because cisplatin targets rapidly dividing cells, it can affect not only cancer cells but also normal cells in the body, resulting in a number of life- threatening side effects if not carefully monitored (Ciarimboli, 2014). The primary dose limiting toxicity for cisplatin is nephrotoxicity (Florea & Büsselberg, 2011). Nephrotoxicity refers to any damage to the kidneys and not only renal failure. In fact, around 20 to 30 % of patients that are already taking cisplatin with hydration face acute kidney injury (AKI). Without hydration, this percentage increases to reach around 100 % of patients (Manohar & Leung, 2018). The concentration of cisplatin in the epithelial cells of the kidneys is five times higher than in the serum. This greater accumulation of cisplatin in the kidneys contributes to its nephrotoxicity (Oun et al., 2018). Just as there are many mechanisms for the anti-cancer effects of cisplatin, there are different pathways in which cisplatin causes toxicity

to the kidneys. Different parts of the nephron are affected by cisplatin mainly by causing damage to the tubular part of the nephron, as well as inflammatory damage and vascular injury to the interstitial cells (Manohar & Leung, 2018).

1.3.5. Cisplatin Entry and Accumulation in the Cells of Kidneys

The high concentration of cisplatin accumulated in the kidneys compared to those in the blood suggest that cisplatin is actively transported into the nephrons. There are two active transporters that can allow cisplatin entry to the epithelial cells of the kidney, which are: Ctr1 and OCT2 active transporters (Filipski et al., 2009). This entry is achieved mainly through OCT2 transporter and not as much via Ctr1 transporter (Arnesano et al., 2013). OCT2 is the organic cation transporter, and Ctrl is the copper transporter (Koepsell, 1998). After the accumulation of cisplatin in the kidneys, it causes nephrotoxicity through apoptotic and inflammatory pathways (Ramesh & Reeves, 2005).

1.3.6. Nephrotoxicity Pathways for Cisplatin

Starting with the apoptotic pathways, many of the cytotoxic pathways of cisplatin that kills cancer cells affect the healthy nephrons (Figure 3). Cisplatin induces renal cell apoptosis through oxidative stress which in turn activates different intrinsic and extrinsic endonucleases and cascades responsible for cell apoptosis (Chirino et al., 2009). Thus, any strategy that is intended to protect the kidneys from cisplatin- induced nephrotoxicity must be carefully chosen so it would not decrease the anti-cancer effect of cisplatin (Ciarimboli et al., 2010).

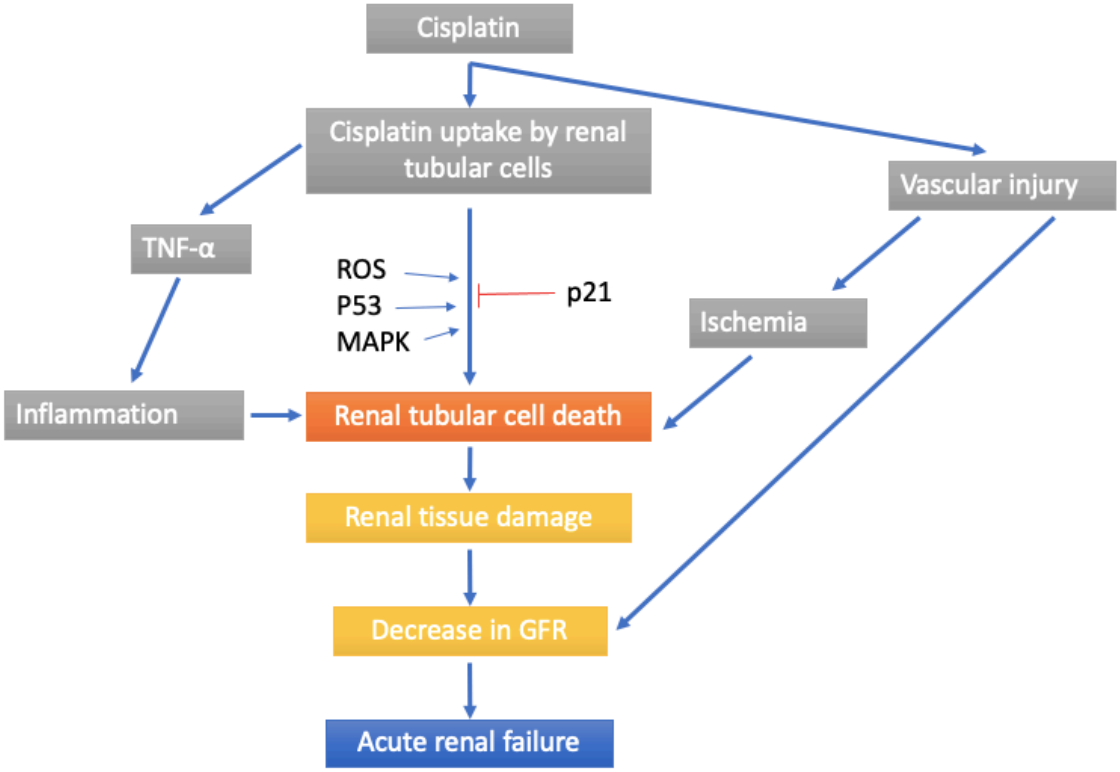

Figure 3. Schematic representation of Cisplatin nephrotoxic pathways. Cisplatin enters renal cells via the tubular cells, where it activates signaling pathways that promote cell death. Meanwhile, cisplatin stimulates TNF- production in tubular cells, resulting in a strong inflammatory response that contributes to tubular cell injury and death. Cisplatin may also cause damage to the renal vasculature, resulting in ischemic tubular cell death and a decrease in glomerular filtration rate (GFR). These pathological events, when combined, result in acute renal failure (Adapted from Fang et al).

As for the inflammatory pathway for cisplatin-induced nephrotoxicity, cisplatin induces several inflammatory mediators that causes renal injury (Miller et al., 2010). Cisplatin mainly causes damage-associated molecular pattern molecules (DAMPs) release from injured epithelial cells in the kidney which in turn can activate the

transmembrane protein Toll-like receptor 4 (TLR4). TLR4 activation results in the activation of a series of chemokines and cytokines like Tumor Necrosis Factor alpha (TNF alpha) (Figure 3). This attracts inflammatory cells to the region and increases adhesion molecules which causes injury to the cells present in this region (Kang et al., 2009). Nephrotoxicity induced by cisplatin is dose and duration dependent. Patients suffering from renal injury typically starts showing signs of renal insufficiency few days after cisplatin dose administration (Santoso et al., 2003).

The expression of cyclooxygenase-2 (COX-2) enzyme in induced in response to inflammation of the renal tissue (Bertolini et al., 2002). When such enzyme is activated, different pro-inflammatory cytokines (like TNF-α) and reactive oxygen species (ROS) are generated which play an important role in renal physiopathology (Cheng & Harris, 2004). Thus, at the molecular levels, a decrease in the generated COX-2 enzyme can decrease inflammation of the kidney and thus protects the glomerular from further damage induced by inflammation (Zarghi & Arfaei, 2011).

1.3.7. Clinical Presentation and Risk Factors for Cisplatin-Induced Nephrotoxicity

Clinical signs of cisplatin-induced nephrotoxicity are the increase in serum creatinine and urea, decrease in urine output, hypomagnesemia typically after repeated cisplatin administration, and decrease in glomerular filtration rate (GFR) (Taguchi et al., 2005). Recovery from this renal injury might take up to two to four weeks, taking into consideration that renal injury can reoccur in those patients and some patients did not recover from this injury at all as the reports indicated (Shi et al., 2018; Pabla & Dong,

2008). Some patients are at a higher risk than others for having kidney injury because of their risk factors. For example, patients that already have a kidney disease, patients taking nephrotoxic drugs (e.g., NSAID or iodine containing contrasts), patients with hypomagnesemia, or those taking initially a high dose of cisplatin (Miller et al., 2010) and even patients with specific genetic variations (Zazuli et al., 2018) are at a higher risk for developing cisplatin induced nephrotoxicity. Such patients must be carefully and routinely monitored, started with a lower dose, or even switched to a different medication.

1.4. Wild Carrot

1.4.1. Introduction to *Daucus carota* L. ssp. *carota*

Daucus carota L. ssp. *carota* also known as wild carrots or Queen Anne's lace a white-flowering, spiny-fruited herb. It grows in mainly in the moderate regions of Asia, Africa, Europe, North and South America (Mitich, 1996). It has an edible flower umbel, and its seed oil is commercially available and used as a flavoring agent in food products. (Leung, 1980). Lebanese people are used to eating the young taproot of this plant after cooking it, and they use the plant itself in the traditional medicine to treat different kind of diseases like cancer, diabetes, gastric ulcer, and as a protective medicine against liver diseases. It has been used in traditional European medicine to treat both cystitis and prostatitis (Hoffman, 1990).

1.4.2. Pharmacological properties of the wild carrot

In fact, studies have shown that this plant have an antilithic effect (Thomas et al., 2001), diuretic effect (Wyk & Wink, 2004), antifungal activity (Rossi et al., 2007), and antibacterial activity (Maxia et al., 2009). Wild carrot oil extract is shown to consist of different compounds like phenols (Gonny et al., 2004), flavonoids (Maxia et al., 2009), monoterpenes (Mockute & Nivinskiene, 2004), phenylpropanoids (Staniszewska et al., 2005), and sesquiterpenes (Shebaby et al., 2013). With different geographical area, the composition of the extracted oil from the wild carrot plant varies greatly (Shebaby et al., 2013). Studies have proven that methanolic and aqueous oil extracts of *Daucus carota* L. ssp. *carota* has a significant anti-ulcer, anti-oxidant (Wehbe et al., 2009), anti-inflammatory (Shebaby et al., 2013), anticancer activity (Zeinab et al., 2011),and it has hepatoprotective effect due to its anti-oxidant activity both in vitro and in vivo (Shebaby et al., 2015). A study done at Lebanese American University, showed that the major constituent of Lebanese DCOE is β-2-himachalen-6-ol (HC) (Figure 4) (Shebaby et al., 2013). This major sesquiterpene component is also proven to have chemotherapeutic anticancer effect (Shebaby et al., 2015a). Thus, the anti-inflammatory and antioxidant effects of DCOE, with its major component HC, makes it a good candidate for studying its reno-protective activity against cisplatin induced nephrotoxicity.

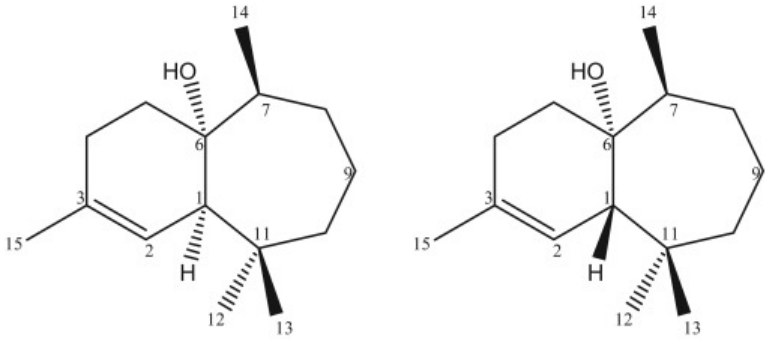

Figure 4. Chemical structure of 2-himachalene-6-ol (left) and β-2-himachalen-6-ol (right) (Taleb et al., 2016).

1.5. *Cannabis sativa*

1.5.1. Introduction to *Cannabis sativa* L. ssp. *indica*

Cannabis is the most used federally illicit drug in the United States (Rein, 2020). It is a genus of flowering plants in the family Cannabaceae. *Cannabis sativa, Cannabis indica, and Cannabis ruderalis* are the most common species recognized worldwide. *Cannabis sativa* L. (*C. sativa*), an aromatic annual herb, has been widely cultivated throughout history and harvested for its oil, seeds, and fiber, and used for medicinal and industrial purposes (Hartsel et al., 2016). It possesses numerous therapeutic properties naming diuretic, anti-epileptic, anti-emetic, and anti-inflammatory. Hence, physicians used cannabis and hemp seeds as a traditional remedy in the Arab world between the 8th and the 18th century (Clarke and Merlin, 2013; Grotenhermen and Russo, 2006; Lozano, 2001; Nahas, 1982). Physicians of the middle age such as Jabir ibn Hayyan (721-815CE), Abu Bakr Al Razi (854–925 CE) and the famous Avicenna (980–1037 CE) documented their knowledge on the medical uses of cannabis. In addition, according to a key word

"cannabis" search on the search engine PubMed, over 29,000 studies or reviews have been published in the scientific literature about cannabis and its biologically active compounds. However, its medicinal indications and therapeutic applications are still debatable till today.

1.5.2. Lebanese Cannabis Strains

The major cannabis strains found in Lebanon are *Cannabis sativa* and *Cannabis indica.* The plant was grown unlawfully since forever in the Beqaa valley, East Lebanon, even though the Lebanese government prohibited to harvest, sell, and consume cannabis and its related products until April 22, 2020. Recently, a draft law legalizing the cultivation of cannabis for medical and industrial purposes was legalized by the Lebanese parliament (Lebanese Official Gazette; issue 23; 2020). A new study that was done recently in L.A.U. by Shebaby showed that cannabis oil has been traditionally used in Lebanon to treat many ailments mainly cancer, diabetes and chronic pain associated with arthritis (Shebaby et al., 2021).

1.5.3. Cannabis Plant Composition

Cannabis plant contains different phytochemicals including cannabinoids, terpernes, and phenolic compounds (Andre et al., 2016). Several factors affect the chemical composition of cannabis strains naming age, harvest time, tissue-type, humidity, nutrition, and light levels (Keller et al., 2001; Turner et al., 2017). Tetrahydrocannabinol (THC) and cannabidiol (CBD) are the most important cannabinoids that have been thoroughly studied (Hanuˇs, Lumír Ondˇrej et al., 2016; Pellati et al., 2018). The

psychoactive cannabinoid THC has been identified as a potential drug in the treatment of pain, cancer, multiple sclerosis, and neurodegenerative disorders (Koppel et al., 2014; Sanders et al., 1979). The non-psychoactive cannabinoid CBD possesses wider pharmacological activities such as anti-inflammatory, antioxidant, antimicrobial, neuroprotective, anxiolytic, and anticonvulsant (Appendino et al., 2011; Campos et al., 2016; Sangiovanni et al., 2019). The combination of THC and CBD enhances their therapeutic effects when compared to each of the cannabinoids alone (Russo and Guy, 2006; Russo, 2011). Furthermore, the presence of other plants' constituents such as terpenes and phenols increase the therapeutic activity of CBD, known as 'entourage effects' (Gallily and Yekhtin, 2019; Gallily et al., 2015; Romano et al., 2014; Russo, 2011). Several studies have supported this synergism in chemically induced intestinal inflammation as well as in inflammatory murine models of Alzheimer and Huntington's diseases (Aso et al., 2015; Gallily et al., 2015; Pagano et al., 2016).

1.5.4. The Endocannabinoid System

The role of Endocannabinoid system (EC) has been a focus of extensive research in the central nervous system. Its presence in peripheral organs, including the kidneys, has been studied also over the course of the past two decades. The EC system plays a major role in normal renal physiology and there is evidence indicating that the alterations of these pathways lead to the pathogenesis of both acute and chronic kidney disease. Endocannabinoids exert their numerous cellular mechanisms through interaction with cannabinoid receptors, cannabinoid subtype-1 (CB_1) and subtype-2 (CB_2) (Munro et al., 1993). The EC system comprises numerous components including endogenous fatty acid-

derived ligands, their receptors, and the enzymes required for their biosynthesis and degradation (Howlett, 2002). The most well-characterized ECs are *N*-arachidonoyl ethanolamide, also known as anandamide (AEA), and 2-arachidonoyl-*sn*-glycerol (2-AG). After binding to CB1 and CB2, they are taken up by cells through a high-affinity uptake mechanism and rapidly degraded by fatty acid amide hydrolase and monoacylglycerol lipase enzymes (DiPatrizio & Piomelli, 2012).

Considerable concentrations of ECs, the machinery required for their biosynthesis and degradation, as well as CB receptors have been detected in kidney tissues (Kondo et al., 1998; Ritter et al., 2016). The presence of functional CB_1 receptor has been demonstrated in proximal convoluted tubules, distal tubules, and intercalated cells of the collecting duct in the human kidneys (Larrinaga et al., 2010). CB_1 receptor expression was found in afferent and afferent arterioles of rodents (Koura et al., 2004), as well as in thick ascending limbs of the loop of Henle (Silva et al., 2013), and glomeruli (Barutta et al., 2010). It was also found to be present in various kidney cell subtypes such as glomerular podocytes, tubular epithelial cells (Jourdan et al., 2014), and cultured mesangial cells (Deutsch et al., 1997). CB_2 receptor expression was localized in podocytes (Barutta et al., 2010), proximal tubule cells (Kenkin et al., 2010), and mesangial cells (Deutsch et al., 1997) in both of human and rat renal cortex samples.

CB_1 and CB_2 receptors were shown to play an important role in the pathogenesis of acute kidney injury. Following renal ischemia/reperfusion injury in mouse kidney model, selective CB_1 and CB_2 receptor agonists played a dose-dependent effect in preventing tubular damage (Feizi et al., 2008). Another study revealed that cannabidiol

reduced renal tubular injury in rats after bilateral renal ischemia/reperfusion by preventing the elevation of serum creatinine, nitric oxide, and renal malondialdehyde levels that are usually high in this case of impaired kidney function (Fouad et al., 2012). Furthermore, it was proven that activation of CB_2 receptor attenuated immune cell infiltrates and inflammatory cytokine release mediating anti-inflammatory effects in a mouse model of cisplatin-induced nephrotoxicity as measured by creatinine and blood urea nitrogen serum concentrations (Mukhopadhyay et al., 2016). Cannabidiol exerts its anti-inflammatory effect mainly through cell proliferation inhibition, cytokine production suppression, T cell apoptosis induction, and regulatory T cell induction. One study has revealed that post-injury treatment with CBD decreased kidney oxidative damage and inflammation in an ischemia-reperfusion animal model; CBD- treated animal Group showed a lower myeloperoxidase activity in the kidney when compared to the control Group (Soares et al., 2015).

1.6. Aim and Objectives

The aim of this study is to evaluate the nephroprotective effects of two Lebanese plants against cisplatin-induced nephrotoxicity by evaluating the following hypothesis.

1.6.1. Hypothesis

Beta-2-himachalen-6-ol, DCOE, and COE may demonstrate nephroprotective activity against cisplatin nephrotoxicity in murine models.

1.6.3. Specific Objectives

To investigate the in vivo nephroprotective effect of DCOE, HC, and COE against cisplatin-induced nephrotoxicity by assessing:

- Biochemical makers in urine and serum
- Estimated glomerular filtration rate (eGFR)
- Anti-inflammatory pathway involved

Chapter Two

Materials and Methods

2.1. Chemicals and Reagents

Primary Rabbit antibodies (COX-2), as well as horseradish peroxidase (HRP)-coupled secondary antibodies were purchased from Abcam (Cambridge, MA, USA). Fetal bovine serum (FBS), penicillin-streptomycin, 2X Laemmli buffer solution, acrylamide (30%), blotting pads (9x10.5 cm), PVDF membranes, Precision Plus Protein ladder, Bio-Rad Protein Assay, and enhanced chemiluminescence (ECL) substrate kit were purchased from Bio-Rad (Hercules, CA, USA). Insight Urinalysis Reagent Strips was purchased from a local pharmacy. Cremophor, glycine, Tris-base, Tris-HCl, NaOH, NaCl, ammonium persulphate, 2-mercaptoethanol, 2-propanol, methanol, Tween 20, Tween 80, Coomassie Brillant Blue, trypan blue, sodium dodecyl sulfate (SDS), well plates, Dulbecco's phosphate-buffered saline (PBS), bovine serum albumin (BSA), TEMED, Trypsin EDTA with phenol red 1X, cisplatin 50mg/50ml was provided from Benta Pharma Industries (Dbayeh, Lebanon). The GCMS solvents: dichloromethane (DCM), ethyl acetate, and methanol were purchased from Sigma-Aldrich (St. Louis, MO, USA).

The following solutions and buffers were prepared:

- Buffer for separating gel (500 mL): 90.75 g Tris-base, 500 mL distilled H_2O, pH=8.8
- Buffer for stacking gel (200 mL): 12 g Tris-base, 200 mL distilled H_2O, pH=6.8

- Ammonium persulphate (APS 10%): 10g ammonium persulphate, 100 mL distilled H_2O
- Sodium dodecyl sulfate (SDS 10%): 10g sodium dodecyl sulfate, 100 mL distilled H_2O
- Separating gel (10%), for 2 gels: 6 mL distilled H_2O, 4.95 mL acrylamide (30%), 3.75 mL buffer (pH=8.8), 150 µL SDS (10%), 150 µL APS (10%), 7.5 µL TEMED
- Stacking gel (5%), for 2 gels: 2.8 mL distilled H_2O, 850 µL acrylamide (30%), 1.25 mL buffer (pH=6.8), 50 µL SDS (10%), 50 µL APS (10%), 5 µL TEMED
- Running buffer 5X (1 L): 15 g Tris-base, 75 g glycine, 25 mL SDS (20%), pH=8.3
- Tris Base Saline TBS 10 X (1 L): 24.2 g Tris-base, 80 g NaCl, 25 mL SDS (20%), pH=7.6
- Washing buffer/TBST (400 mL): 360 mL distilled H_2O, 40 mL TBS (10X), 0.1% Tween 20
- Transfer buffer 5X (500 mL): 7.6 g Tris-base, 37.5 g glycine, pH=8.5
- Transfer buffer 1X (100 mL): 20 mL Transfer buffer (5X), 60 mL distilled H_2O, 20 mL methanol
- Blocking buffer (5%): 5g bovine serum albumin (BSA) in 100 mL TBST

2.2. Animals

For the HC experiment, six to eight weeks old white (Albino) male rats were obtained from the animal facility of the Biology Department at the Lebanese American University (L.A.U). Animals were kept in an environment of controlled temperature with

12 hours per day light to dark cycle. All animals had free access to food and water at all the experimental time. National Institutes of Health guidelines were followed for animal care and handling.

For the cannabis oil experiment, six to eight weeks old black male mice were obtained from the animal care facility labs at Lebanese American University in Byblos. Animals were kept in an environment of controlled temperature with 12 hours per day light to dark cycle. All animals had free access to food and water at all the experimental time. National Institutes of Health guidelines were followed for animal care and handling.

2.3 Experimental design

For the HC and DCOE treatment, rats were divided into six Groups, each Group containing 7 rats where rats were injected for ten days before sacrifice according to the following design:

- **Normal control:** A mixture of Ethanol: Cremophor: PBS (1:1:18) administered intraperitoneal (i.p.) for 10 days and a single i.p. injection of 1.4 ml PBS on the 5th day.
- **Cisplatin control:** A mixture of Ethanol: Cremophor: PBS (1:1:18) administered intraperitoneal (i.p.) for 10 days and a single dose of cisplatin (7.5 mg/kg, i.p.) was administered on the 5th day.
- **HC control:** Rats are to be treated with HC (25 mg/ kg/day) dissolved in a mixture of Ethanol: Cremophor: PBS (1:1:18) i.p. for 10 days and a single i.p. injection of 1.4 ml PBS on the 5th day.

- **HC (25 mg/kg) + Cisplatin:** Rats are to be treated with HC (25 mg/kg/day) dissolved in a mixture of Ethanol: Cremophor: PBS (1:1:18) i.p. for 10 days and a single dose of cisplatin (7.5 mg/kg, i.p.) on the 5th day, 90 minutes after the HC doses.
- **HC (50 mg/kg) + Cisplatin:** Rats are to be treated with **HC (50 mg/kg/day)** dissolved in a mixture of Ethanol: Cremophor: PBS (1:1:18) i.p. for 10 days and a single dose of cisplatin (7.5 mg/kg, i.p.) on the 5th day, 90 minutes after the HC doses.
- **DC (100 mg/kg) + Cisplatin:** Rats are to be treated with **DC (100 mg/kg/day)** dissolved in a mixture of Ethanol: Cremophor: PBS (1:1:18) i.p .for 10 days and a single dose of cisplatin (7.5 mg/kg, i.p.) on the 5th day, 90 minutes after the HC doses.

Rats were scarified on the day 11 of the experiment. All animals were weighed on days 1, 5, and 10 of the experiment. Urine collection is done on day 10 of experiment for all of the Groups of rats. The sources of all reagents used in this experiment are mentioned wherever it is appropriate. The schematic diagram of experimental design was shown in Figure 5.

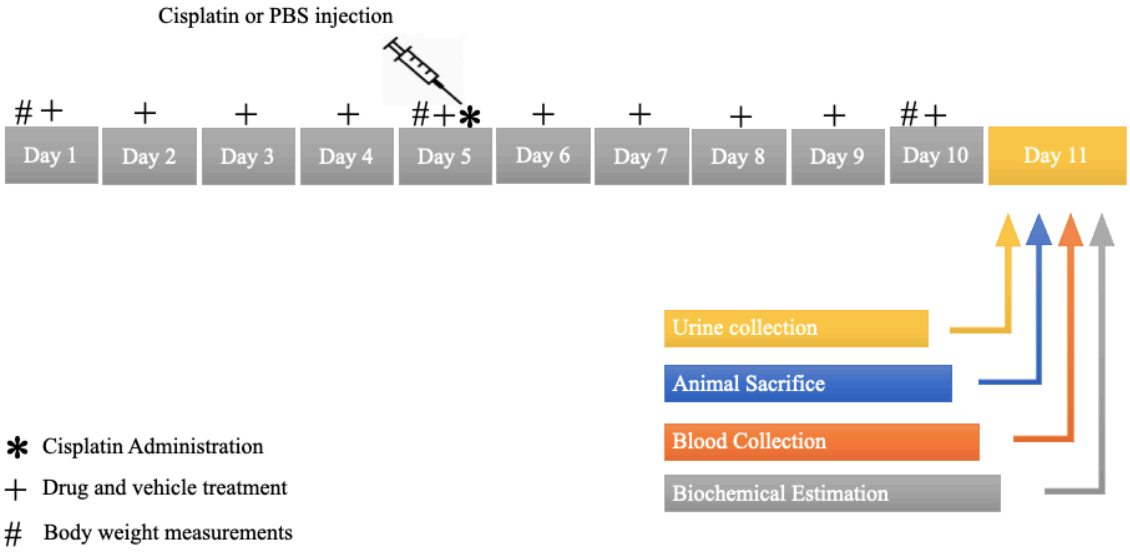

Figure 5. Experimental design for the HC and DCOE treatment

For the COE treatment, mice were divided into seven Groups, each Group containing 7 mice where mice were injected for three days before sacrifice according to the following design:

- **Normal control:** A mixture of Ethanol: Tween80: PBS (1:1:18) 0.1ml administered i.p. for 3 days and a single i.p. injection of PBS (0.4ml) on the first day 90 minutes after vehicle injection.
- **Cisplatin control:** A mixture of Ethanol: Tween80: PBS (1:1:18) 0.1ml administered i.p. for 3 days and a single dose of cisplatin (20 mg/kg, i.p.) is administered on the first day 90 minutes after vehicle injection.
- **Cannabis oil control:** Mice are to be treated with Oil (10 mg/ kg/day b.w.) 0.1ml dissolved in a mixture of Ethanol: Tween80: PBS (1:1:18) i.p. for 3 days and a

single i.p. injection of 0.4ml PBS on the first day 90 minutes after the oil doses.

- **Cannabis oil (2.5 mg/kg) + Cisplatin:** Mice are to be treated with oil (2.5 mg/kg/day) 0.1ml dissolved in a mixture of Ethanol: Tween80: PBS (1:1:18) i.p. for 3 days and a single dose of cisplatin (20 mg/kg, i.p.) 0.4ml on the first day, 90 minutes after the oil doses.
- **Cannabis oil (5 mg/kg) + Cisplatin:** Mice are to be treated with oil (5 mg/kg/day) dissolved in a mixture of Ethanol: Tween80: PBS (1:1:18) 0.1ml i.p. for 3 days and a single dose of cisplatin (20 mg/kg, i.p.) 0.4ml on the first day, 90 minutes after the oil doses.
- **Cannabis oil (10 mg/kg) + Cisplatin:** Mice are to be treated with oil (10 mg/kg/day) dissolved in a mixture of Ethanol: Tween80: PBS (1:1:18) 0.1ml i.p. for 3 days and a single dose of cisplatin (20 mg/kg, i.p.) 0.4ml on the first day, 90 minutes after the oil doses.
- **Cannabis oil (20 mg/kg) + Cisplatin:** Mice are to be treated with oil (20 mg/kg/day) dissolved in a mixture of Ethanol: Tween80: PBS (1:1:18) 0.1ml i.p. for 3 days and a single dose of cisplatin (20 mg/kg, i.p.) 0.4ml on the first day, 90 minutes after the oil doses.

Mice were scarified 72 hours after cisplatin injections. Urine collection was done for Groups I, II only. The sources of all reagents used in this experiment are mentioned wherever it's appropriate. The schematic diagram of experimental design was shown in Figure 6.

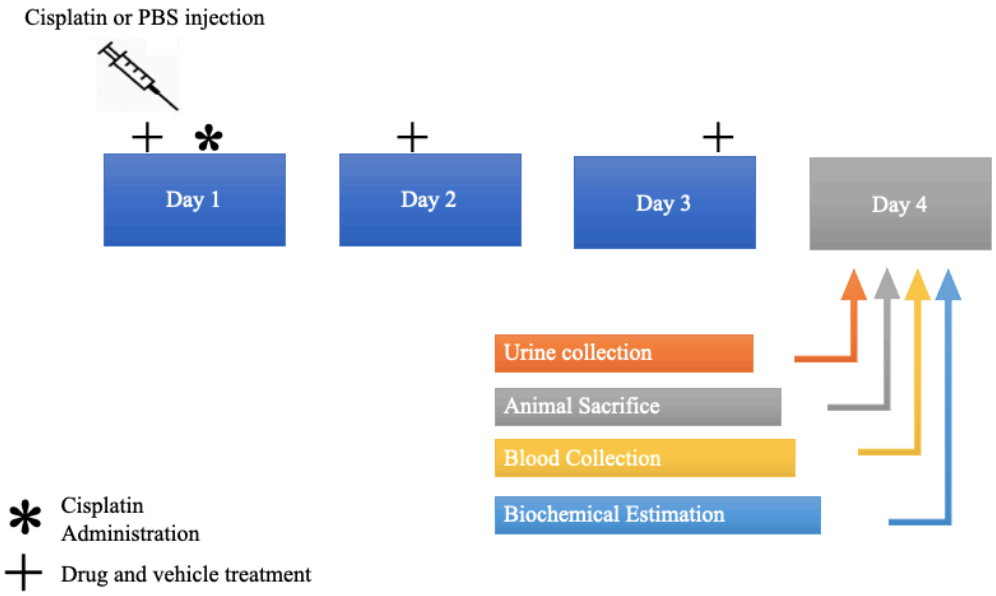

Figure 6. Experimental design for the COE treatment

2.4. Plant collection and oil extraction

2.4.1. Daucus carota

Mature umbels of *Daucus carota* ssp. *carota* were collected at the post flowering period between August and September from their natural habitat in Lebanon. The plant was identified according to the characteristics described in "Handbook of Medicinal Herbs" (Van Wyk and Wink, 2004) and confirmed by Dr. A. Houri, a Lebanese plant expert at the Lebanese American University. The extraction procedure was carried out according to the method described by (Zeinab et al., 2011). Briefly, 1 kg of air-dried umbels were cut into small pieces and soaked in 5 liters of methanol/acetone (1:1) for 72 h. The extraction was repeated twice, and the extracts were collected, filtered and evaporated to dryness under reduced pressure. The residue was centrifuged at 500 rpm and the oil was removed and then dried over anhydrous sodium sulfate to give at the end 34.7g of crude oil extract. It was kept in a closed

amber bottle at 4°C until use. The major component of Lebanese wild carrots which is β-2- himachalen-6-ol was isolated and purified according to the protocol described by Taleb et al, (2016). Briefly,

DCOE was further fractionated into four fractions using column chromatography. The pentane:diethyl ether (50:50) fraction of DCOE was further fractionated into three different sub-fractions of oil with yellow, dark green, and dark oil colors using column chromatography with silica gel. These sub-fractions were additionally analyzed using TLC with a mobile phase of hexane/ethyl acetate. One of the sub-fractions was chromatographed one more time resulting with β-2- himachalen-6-ol (98% purity).

2.4.2. Cannabis Sativa

Dried samples of Lebanese cannabis strain (*Cannabis sativa* L. ssp. *indica*) were provided by drug Enforcement Office in Zahle, Beqaa Governorate. The samples were collected from Deir El Ahmar This Lebanese village is located 22 km to the northwest of Baalbek and 100 km from Beirut. Cannabis plant material were securely stored in special storage facility on campus of L.A.U, Byblos. The plant material was stored in a secure storage facility on campus premises. As described by Shebaby et al. (2021), around 1.2 g of cannabis oil was extracted from 10 g of cannabis flowers that were air-dried. Extraction was performed using ethanol for 48 hours. The obtained natural extract was then filtered and concentrated at temperature 45 ◦C with reduced pressure to obtain the pure COE.

2.5. GC-MS analysis

GC-MS was used for the analysis of DCOE and its fractions as well as the isolated β-2-himachalene-6-ol compound. The used carrier gas was helium, and the settings of the flow rate were fixed to 1.2 ml/min. More details of the used setting for GC-MS are available in previous study done by Taleb et al., (2016).

GC-MS analyses for compounds identification of COE were also performed using a Shimadzu GCMS-QP2020NX. More details of the used setting for GC-MS are available in previous study performed by Shebaby et al., (2021).

2.6. Biochemical analysis

2.6.1. Urine Analysis

Urine collected from rats of control, cisplatin, HC and DCOE treated Groups were tested for proteins using urine dipstick kit for qualitative detection of proteins. 5 µL of urine was added on the paper strip for 2 min before reading the results from the reference provided by the kit (Insight Urinalysis Reagent Strips).

For the cannabis oil experiment, urine samples collected from mice of control, cisplatin and 10mg/kg COE treated Groups were further diluted in 2X Laemmli Sample Buffer containing 10 % β-mercaptoethanol. Equal volumes of each sample were then loaded and resolved under reducing conditions by SDS-PAGE using 5% stacking gel, 10% separating gel and 1X running buffer (0.3% Tris Base, 1.4% glycine, 20% SDS, pH = 8.3) at 90 V for 30 min and then at 120 V for 2 h. A molecular weight protein marker (10–250 kD) *(Precision Plus Protein™ Kaleidoscope™ Prestained Protein Standards #1610375)* was loaded in parallel to verify the size of the amplicons. After migration, the gel was stained by

Coomassie Brillant Blue for two hours and then distained by distilled water overnight. The bands were visualized on Chemidoc bioimaging system.

2.6.2. Serum Analysis

Blood samples from both experiments were collected immediately after the sacrifice of the animals. EDTA was added to prevent clotting of the samples in a ratio of 7 µL for each 1ml of blood collected. Then serum was separated using centrifugation at 3000 rpm for 15 min at 4◦C temperature. Serum creatinine and urea were analyzed using a clinical chemistry analyzer system and kits.

2.7. Tissue Preparation

Kidneys were removed from mice Groups of the cannabis oil experiment and homogenized (10% w/v) in ice cold lysis buffer (50mM Tris–HCl adjusted to a pH=7.4 (0.5ml of 1M), 1% Triton-X, 0.2% Sodium deoxycolate, 1mM disodium EDTA, 0.2% SDS) using a homogenizer (or mini-homogenizer if the tissue sample is small, a sonicator–homogenizer can be used as well) until the pieces are fully homogenized and a homogenous solution is obtained. The mixture was then centrifuged at 4000 × *g* for 5 min at 4°C. The supernatant was collected and an equal volume of 2X Laemmli buffer was added to each sample. Total protein concentrations were then determined using nanodrop.

2.8. Western Blot

Equal protein concentrations of kidney cell lysate samples were subjected to 10% SDS-PAGE (5% stacking gel, 10% separating gel and 1X running buffer: 0.3% Tris Base, 1.4% glycine, 20% SDS, pH = 8.3) at 90 V for 30 min and then at 120 V for 2 h. Proteins were then transferred to PVDF membrane using a semi-dry electro blotter (PEQLAB, Erlangen, Germany) at 10V for 30 min. The blots were incubated with blocking buffer (1 × TBS, 0.1% Tween-20, 5% BSA) for 2 h and then probed with primary antibodies against COX-2, iNOS and actin at 4 ◦C overnight. The blots were washed with TBST for 30 min and incubated with horseradish peroxidase (HRP)- coupled secondary antibodies for 1 h, and then washed away with TBST. Proteins were visualized using the chemiluminescence ECL kit and blot images were obtained using the ChemiDoc imaging 3.8 instrument (Bio-Rad, Hercules, CA, USA) and analyzed with ImageLabTM Software (BioRad, Hercules, CA, USA).

2.9. Statistical Analysis

One-way analysis of variance (ANOVA) was used to assess statistical significance of the data. Within each Group, the values of the various tested parameters are presented as mean SEM. All data were analyzed using the statistical software package SPSS 18, and differences between Groups were considered statistically significant if the p-value was less than 0.05.

Chapter Three

Results

3.1. Chemical composition of Lebanese COE

Different chemical constituents of cannabis oil extracted from Lebanese *Cannabis sativa* L. were analyzed using GC-MS according to Shebaby et al. 2021. The results of this analysis showed that the major constituent of COE is cannabinoids accounting for 85.15% from the total identified components (Figure 7). The oil consisted mainly of cannabinoids accounting for 85.15 % from the total identified components. From these cannabinoids, the one with highest percentage was cannabidiol (CBD) with 59.1%, followed by tetrahydrocannabinol (THC) with a percentage of 20.2%. Other cannabinoids were present with lower percentages such as cannabinol (CBN) with 3.63% and cannabichromene (CBC) with 2.22% only. A summary of the identified components is present in Figure 7.

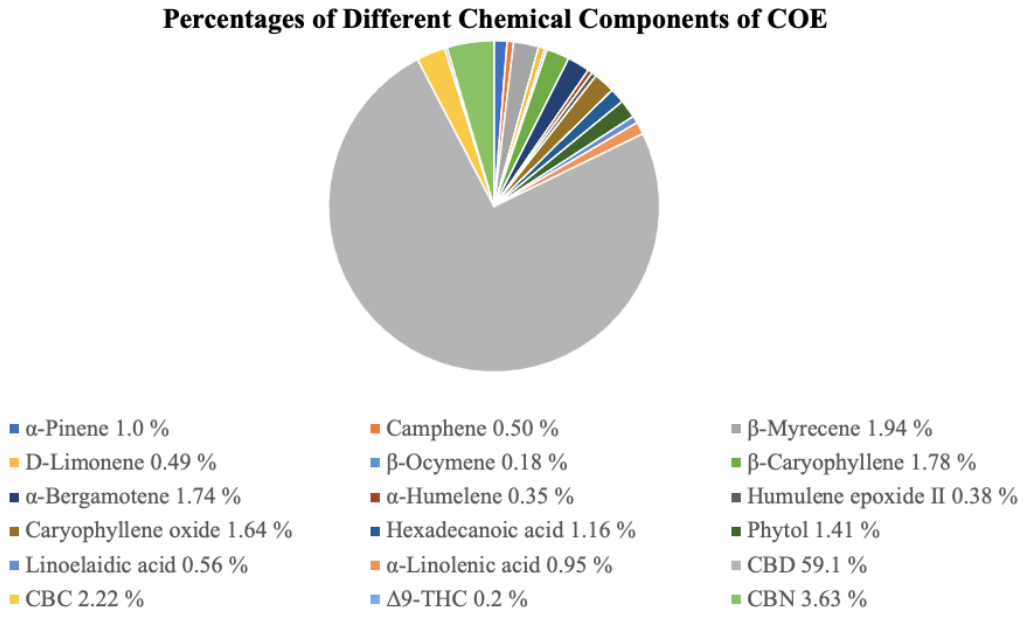

Figure 7. Chemical components of COE from Lebanese *Cannabis sativa* plant with their respective percentages after GC-MS analysis of the extracted oil (Shebaby et al. 2021).

3.2. Protective Effect of HC, DCOE and COE Against Cisplatin-Induced Renal Toxicity

To investigate the effect of HC, DCOE, and COE on cisplatin-induced renal toxicity in animal models, levels of serum urea and creatinine were measured 5 days after cisplatin injection in *Daucus carota* experiment and 72 hours after cisplatin administration in the COE experiment.

As shown in Figures 8 and 9, intraperitoneal injection of cisplatin produced an elevation of both serum creatinine and urea levels. A marked increase in the serum markers was observed at the fifth day after cisplatin administration of a dose of 7.5 mg/kg

in rats and 20mg/kg in mice compared to normal control Groups. None of the animals was dead before the designated sacrifice day of the experiment.

Treatment with a high dose of HC (50 mg/kg - Group V) or with DCOE (100mg/kg - Group VI) was able to decrease serum creatinine levels in rats compared to cisplatin Group (Group II), but this decrease was insignificant (Figure 8A). However, these treatments were able to significantly decrease serum urea levels (Figure 8B) when given every day 5 days before and 5 days after cisplatin injection (n= 7/ each Group; $P < 0.05$). HC alone with a dose of 25 mg/kg had no effects on serum creatinine levels but significantly decreased serum urea levels compared to the vehicle-treated Group (n =7; $P < 0.05$; Figure 8B).

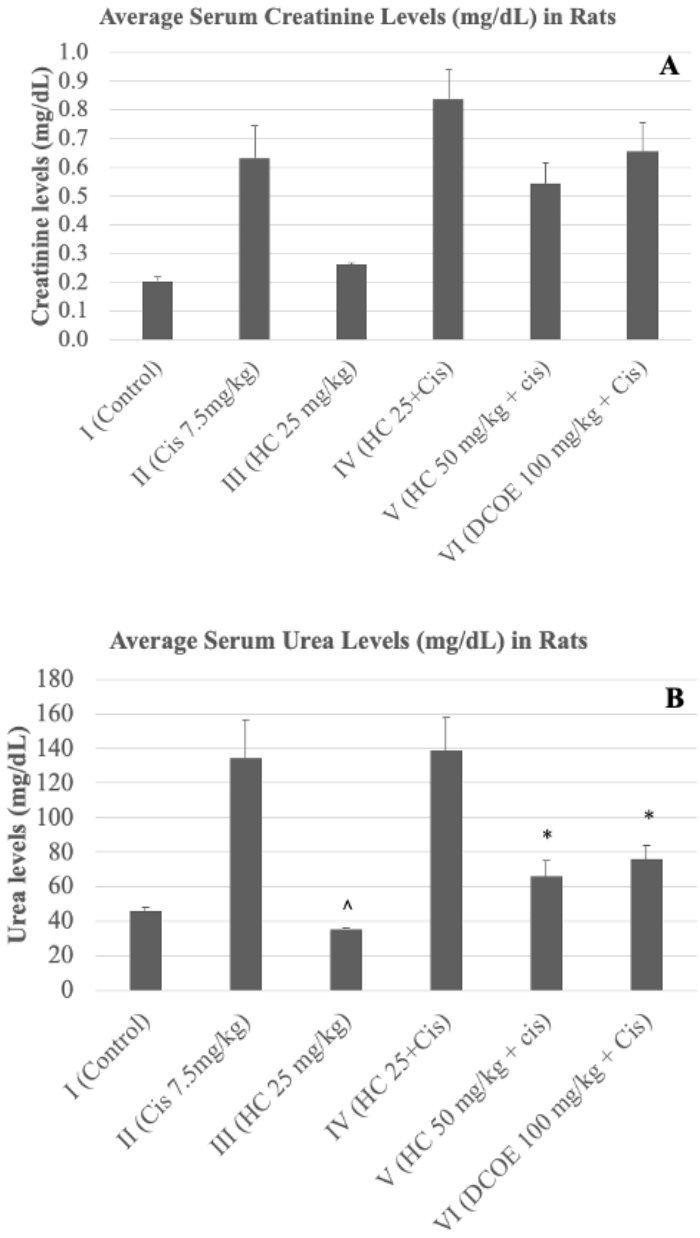

Figure 8. Average serum creatinine levels (mg/dL) (**A**) and average serum urea levels (mg/dL) (**B**) in different Groups of rats in *Daucus carota* experiment. HC/ DCOE was injected i.p. once a day for ten days and 90 minutes before cisplatin injection (7.5 mg/kg, i.p.) on day five of the experiment. Serum urea and creatinine levels were measured five days after cisplatin injection. Each column represents the mean ± SEM of seven animals. * $P < 0.05$ vs. cisplatin, ^ $P < 0.05$ vs. control.

As for the *Cannabis sativa* experiment, COE at 20 mg/kg administration for three days after cisplatin (20 mg/kg) injection in mice was able to significantly decrease both serum creatinine and urea levels (n= 7; $P < 0.05$; Figure 9A and B). The administration of lower doses of 2.5 or 5 mg/kg of COE significantly decreased serum urea levels only as shown in Groups IV and V in Figure 9 (n= 7/ each Group; $P< 0.05$). COE alone with a dose of 10 mg/kg significantly decreased serum creatinine and urea levels compared to the vehicle-treated Group (n =7; $P < 0.05$; Figures 9A and B).

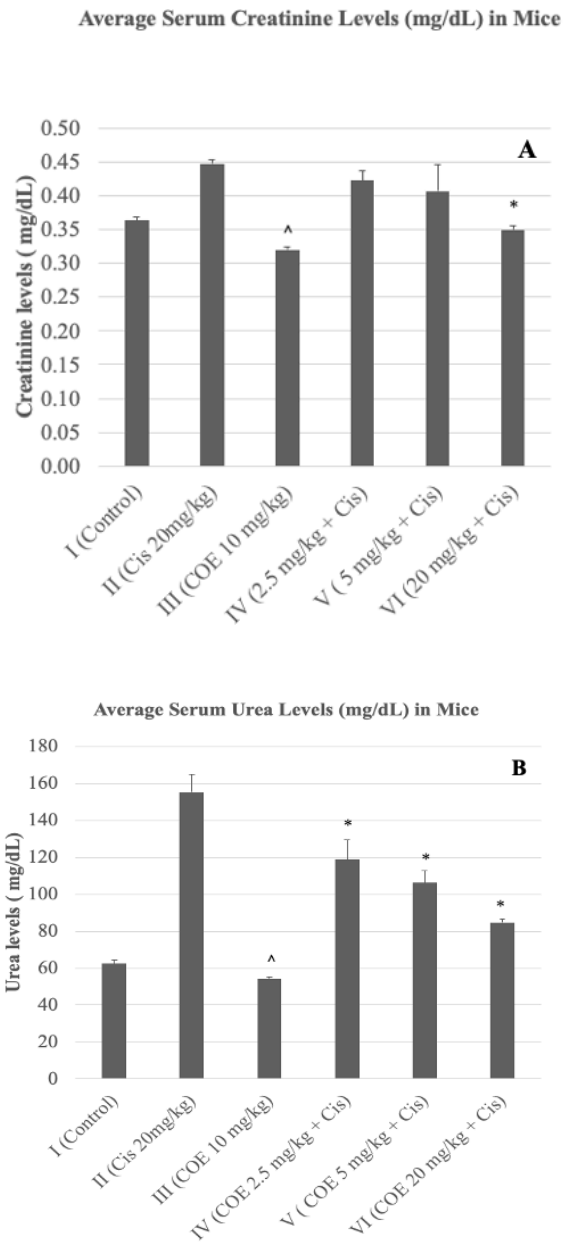

Figure 9. Average serum creatinine levels (mg/dL) (A) and average serum urea levels (mg/dL) (B) in different Groups of mice in *Cannabis sativa* experiment. COE was injected i.p. once for three days. On day one, COE was injected 90 minutes before cisplatin injection (20 mg/kg, i.p.). Serum urea and creatinine levels were measured 72 hours after cisplatin injection. Each column represents the mean ± SEM of seven animals. * $P < 0.05$ vs. cisplatin, ^ $P < 0.05$ vs. control.

3.3. Attenuation of Cisplatin-Induced Increased Albumin Secretion in Urine

Urine samples from rats treated with cisplatin, HC, and DCOE were analyzed for proteins using a urine dipstick kit for qualitative protein detection. Cisplatin treated Groups has an increased percentage of 100 mg/dL levels of proteins detected in the urine samples of 43 % compared to 29 % in the vehicle-treated Group. Treatment with HC (25 or 50 mg/kg) or with DCOE (100 mg/kg) resulted in a marked decrease in the levels of 100 mg/dL compared to cisplatin treated Groups. HC alone given at a 25 mg/kg was able to decrease the percentage of 100mg/dL proteins in urine to 14 % compared to the normal control Group which had 29 % of that level of proteins in the urine.

Table 1. Percentages of rats with low (50mg/dL) and high (100mg/dL) urine protein levels in each Group (n=7).

Groups of rats	**Percentages of protein levels in urine**	
	50 mg/dL	100 mg/dL
I (Control)	71	29
II (Cisplatin 7.5 mg/kg)	57	43
III (HC 25 mg/kg)	86	14
IV (HC 25 mg/kg + Cis)	100	0
V (HC 50 mg/kg + Cis)	100	0
VI (DCOE 100 mg/kg + Cis)	86	14

In the *Cannabis sativa* experiment, urine samples were collected from control, cisplatin and 10mg/kg COE treated groups. These samples were analyzed by SDS-PAGE (Figure 10). Three gels were prepared for the three Groups and each gel contained six urine samples from mice. The control Groups had very faint bands at 65 KDa which indicated the minimal presence of albumin in the collected urine. Cisplatin Group had a much larger and more stained albumin bands indicating the nephrotoxic effect of cisplatin on the kidneys which leads to increased proteinuria. Treatment with COE (10 mg/kg) showed marked decrease in the intensity of albumin bands compared to cisplatin group which indicates that COE (10 mg/kg) had nephroprotective effect and decreased albumin secretion in urine induced by cisplatin.

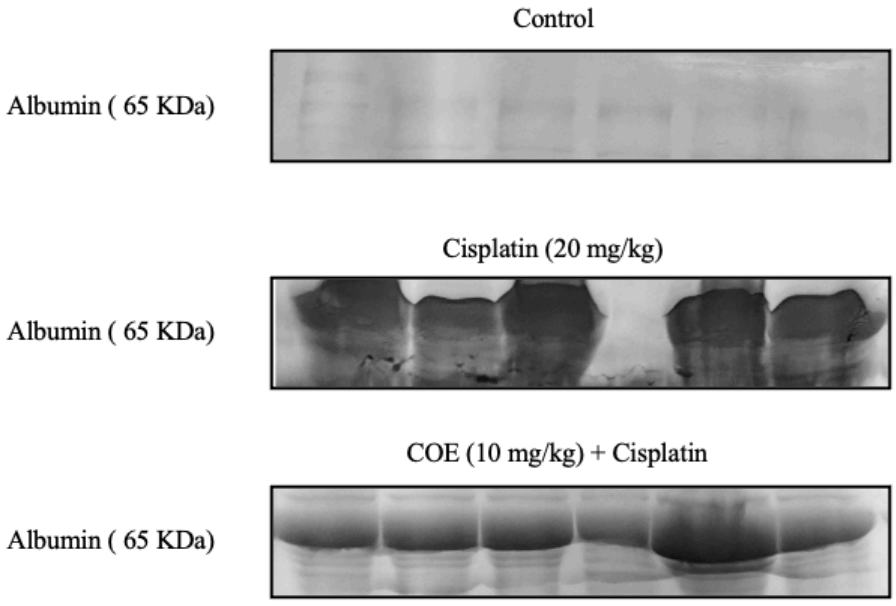

Figure 10. SDS-PAGE study for albumin detection in urine samples of mice.

The samples were run on a Bio-Rad protein electrophoresis equipment and staining was performed with Coomassie blue. Three Groups were tested which are the control, Cisplatin (20 mg/kg), and the Group treated with COE (10 mg/kg) and was injected with cisplatin. The bands at 65 KDa represent albumin. The control Group had very faint bands, cisplatin Group had large bands, and the COE treated Group had moderate bands staining.

3.4. Attenuation of Cisplatin-Induced Decrease in GFR

In order to monitor kidney function, estimated glomerular filtration rate (eGFR) was calculated using the following equations (Besseling et al., 2021):

- Plasma creatinine < 52 μmol/L→ $eGFR = 880 * W^{0.695} * C^{-0.660} * U^{-0.391}$
- Plasma creatinine ≥ 52 μmol /L→ $eGFR = 5862 * W^{0.695} * C^{-1.150} * U^{-0.391}$

Where:

- eGFR: Estimated GFR (μL/min)
- W: Weight (g)
- C: Creatinine concentration (μmol/L)
- U: Urea (mmol/L)
- 52 μmol/L= 0.937 mg/dL

Cisplatin markedly decreased eGFR compared to vehicle-treated Group when given to rats at a dose of 7.5 mg/kg (Figure 11 A) and to mice at a dose of 20 mg/kg (Figure 11 B). This decrease in eGFR represents a decrease in kidney function caused by cisplatin which was attenuated by HC, DCOE and COE. HC (25 mg/kg) treatment for 10 days,

including 5 days after cisplatin injection, significantly improved eGFR compared to cisplatin Group (n=7/ each Group; P< 0.05). In *Cannabis sativa* experiment (Figure 11 B), COE treatment improved kidney function, and the doses of 5 and 20 mg/kg (Groups V and VI respectively) significantly increased eGFR when COE was injected in mice once a day for three days after cisplatin injection (n=7; P< 0.05).

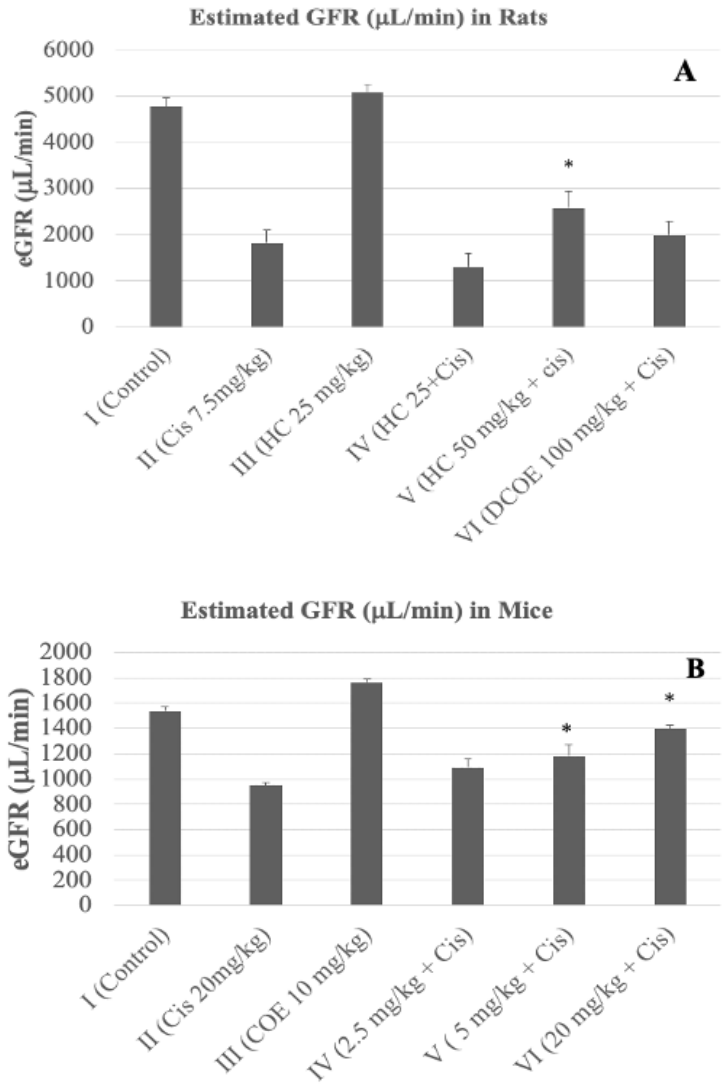

Figure 11. Estimated GFR (µL/min) for different Groups of rats (A) in *Daucus carota* experiment and of mice (B) in *Cannabis sativa* experiment. Each column represents the mean ± SEM of seven animals. * P < 0.05 vs. cisplatin.

3.4. Attenuation of Cisplatin-Induced Inflammation in Kidneys of Mice

Cisplatin markedly increased the expression of COX-2 (molecular weight of 70 KDa) in kidneys of mice (Figure 12) indicating enhanced inflammatory response. COE treatment decreased COX-2 expression in a dose dependent manner with minimal decrease with 2.5 mg/kg and marked decrease with 20 mg/kg in COE treated mice. This indicated that COE attenuates kidney inflammation caused by cisplatin with the dose 20 mg/kg being the most effective among the tested doses of COE.

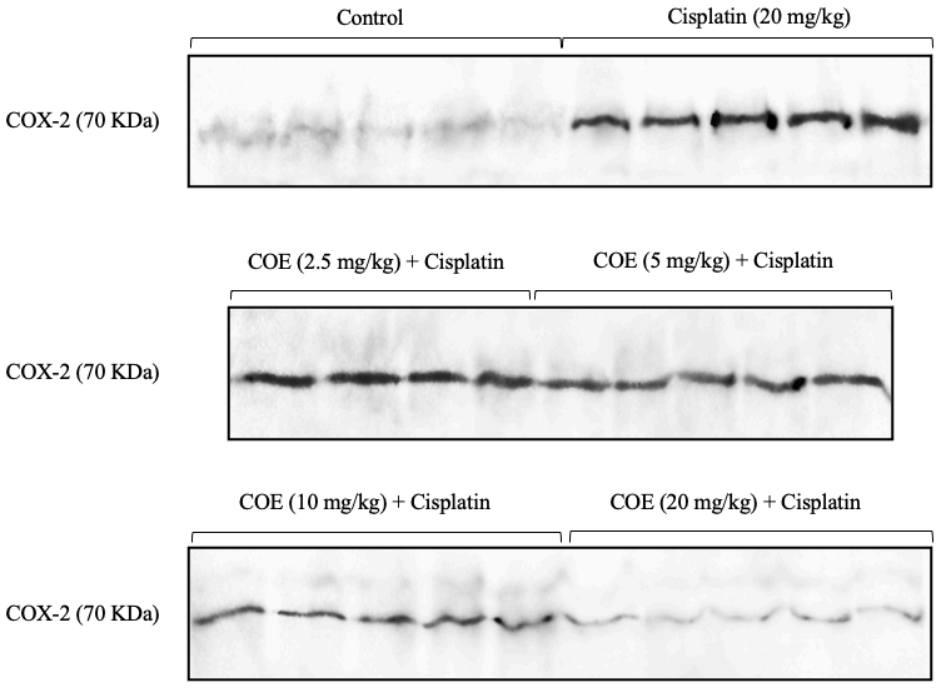

Figure 12. Representative western blots showing COX-2 protein from kidneys of mice. Each membrane represents different Group of mice (control, cisplatin, and different doses of COE treated Groups).

Chapter Four

Discussion

Despite the fact that cisplatin induces kidney damage that is cumulative, dose and time dependent, it is one of the most active cytotoxic drugs used in the treatment of cancer. The main pathway involved in nephrotoxicity is oxidative stress, inflammation, and apoptosis (Baek et al., 2003). Thus, any agent that may protect renal tissues from oxidative stress with its exogenous antioxidants, in addition to its anti-inflammatory or cytoprotective properties, should be used in conjunction with chemotherapy treatments to lessen the nephrotoxic and damaging effects of drugs like cisplatin (Husain et al., 1998).

In this study, the nephroprotective effects of two native Lebanese plants, *Daucus carota* and *Cannabis sativa*, in animal models were investigated. Previous studies in our laboratory showed that *Daucus carota* oil extract (DCOE) possesses antioxidant, anti-inflammatory, and anticancer properties (Wehbe et al., 2009; Shebaby et al., 2013; Zeinab et al., 2011). To add, the major component of DCOE, β-2-himachalen-6-ol (HC), which comprises 33 % of Lebanese DCOE, has been isolated and found to be a potential safe and potent chemotherapeutic agent (Taleb et al. 2016; Shebaby et al., 2013). As for *Cannabis sativa* plant, recent studies showed that COE demonstrated potent *in vitro* and *in vivo* anti-inflammatory activities (Shebaby et al., 2021). Previous report revealed that CBD displayed a significant nephroprotective effect against cisplatin-induced nephrotoxicity (Pan et al., 2009) where it caused a decrease in serum creatinine and urea in cisplatin-treated mice. Cannabinoids exert their effects by interacting with the receptors

of the endocannabinoid system. In fact, the potential role of EC system in treating various kidney diseases has been an emerging area of research, specifically in the context of cannabinoid receptors (Nettekoven et al., 2016). The two types of receptors (CB_1 and CB_2) that are activated by the pharmacologically active ingredients of cannabis are found in numerous tissues, including the kidneys (Park et al., 2017). Cannabidiol, which acts as an antagonist/ inverse agonist by weakly binding to both CB1 and CB2 receptors, was found to partially prevent renal tubular injury after bilateral renal ischemia/reperfusion (Nettekoven et al., 2016). Another series of studies using cisplatin-induced renal injury yielded similar results where blocking the CB1 receptor or activating the CB2 receptor has been shown to protect against tubular damage by reducing oxidative stress and inflammation in the kidney (Horváth et al., 2012; Mukhopadhyay P et al., 2016; Mukhopadhyay P et al., 2010). Alterations of cannabinoid receptors have been involved in different renal diseases such as acute kidney injury, chronic kidney disease, and diabetic nephropathy (Park et al., 2017). Hence, targeting the EC system in treating nephrotoxicity may present some therapeutic value. As for the other constituents of DCOE and COE, both extracts contain several common terpenes, mainly β-caryophyllene (Shebaby et al., 2013; Shebaby et al., 2021). Previous study showed that the natural product, β-caryophyllene, which can act as a full CB_2 agonist, could protect the kidney from the harmful effects of cisplatin through decreasing inflammation and oxidative stress (Horváth et al., 2012; Nuutinen, 2018).

Furthermore, COE is expected to be more potent than using a cannabinoid alone due to the synergism of its various cannabinoids and terpenes, a phenomenon known as the "entourage effect" (Gallily and Yekhtin, 20119). Because Lebanese COE contains

approximately 59 % CBD (Shebaby et al., 2021), the 2.5, 5, 10, and 20 mg/kg doses of COE used in our study were nearly equivalent to 1.5, 2.95, 5.9, and 11.8 mg/kg of CBD, respectively. All these COE doses significantly reduced serum urea and were shown to be reno-protective in mice; however, when CBD was used alone in a similar study done on mice, only 5 and 10 mg/kg doses significantly decreased serum urea with no significance for 2.5 mg/kg dose (Pan et al., 2009). Thus, the fact that low COE doses (corresponding to 1.5 and 2.95 mg/kg doses of CBD) were more effective than similar dose of CBD used alone (2.5 mg/kg) supports the "entourage effect" phenomenom.

In order to assess kidney function, serum creatinine and urea, eGFR, proteinuria, and inflammatory markers were evaluated. The amount of urea in serum measures how much of protein waste product are present in blood. Serum urea increases when the kidneys are less able to remove urea from the bloodstream due to a certain disease or damage Gounden & Jialal, 2018). In addition, the kidneys are responsible for removing creatinine from the blood. Creatinine blood levels rise when renal filtering is inadequate (Schock-Kusch et al., 2011). As a result, the creatinine clearance, which reflects the glomerular filtration rate (GFR), may be calculated using blood creatinine levels. Since it is a marker of renal function, the GFR is clinically significant (Besseling et al., 2021). Following cisplatin administration, a reduced glomerular filtration rate as well as an increased plasma creatinine levels have been observed (Baliga et al., 1998). In the present study, cisplatin treatment caused significant increase in functional nephrotoxicity markers such as serum creatinine and urea levels, indicating intrinsic acute renal failure. Treatment with 50 mg/kg of HC, 100 mg/kg of DCOE, or any of the used doses of COE significantly decreased serum urea, COE a 5 mg/kg also showed significant increase in eGFR, and only

COE at a dose of 20 mg/kg significantly reduced serum creatinine and improved eGFR. In fact, a study conducted to investigate the nephroprotective effects of CBD on mice showed that both 5 and 10 mg/kg doses of CBD significantly decreased serum creatinine and urea (Pan et al., 2009). This indicates that HC (50 mg/kg), DCOE (100 mg/kg), and COE provided significant protection against cisplatin-induced nephrotoxicity mainly on the tubular level which is the site of creatinine and urea excretion.

Another parameter used to assess kidney damage is the presence of proteins in urine. In the *Daucus carota* experiment, cisplatin caused an increase in protein levels in urine which was decreased in the HC and DCOE treatment Groups indicating their potential renal protective effect. For the cannabis oil experiment, gel electrophoresis results revealed a clear increase in albumin present in the collected urine after cisplatin treatment compared to the control. However, in the10mg/kg COE treated Groups the albumin level was markedly decreased compared to that in the cisplatin Group which reflects the reno-protective effect of COE. In fact, small amounts of Albumin can be due to stress and diet, however increased levels of Albumin indicate kidney damage (Gounden & Jialal, 2018).

Western blot experiment was used to detect COX-2 antibodies in the kidneys of mice from the Cannabis oil experiment. Cyclooxygenase (COX) is a rate-limiting enzyme in the generation of prostaglandins from arachidonic acid in the cell (Masfferer et al., 1992; Sirois J et al., 1992). Furthermore, COX-2 is an inducible COX isozyme that is produced in response to inflammatory stimuli and acts as a cell growth regulator (Singh J et al., 1997; Tao L et al., 2002). In this investigation, the overexpression of COX-2 in the

kidney of the cisplatin-treated Group was used as an indicator to describe renal interstitial inflammation. Reduced expression of COX-2 in the COE treated Groups was observed, mainly the 20 mg/kg dose. This result is consistent with previous study where it was shown that ischemia can induce a subsequent cascade of events that are responsible for kidney damage, but CBD inhibited this signaling pathway which consequently suppressed COX-2 expression and other inflammatory genes (Fouad et al., 2012). Furthermore, several reports indicated that both THC and CBD exhibited anti-inflammatory activities through the reduction of COX-2 expression (Duangnin et al., 2017; Costa et al., 2004). Thus, COE could prevent renal damage and protect the glomerular possibly via modulating COX-2 expression.

Chapter Five

Conclusion

To the best of our knowledge, this is the first study that investigates the nephroprotective effects of *Cannabis sativa* oil, *Daucus carota* extract, and β-2-himachalen-6-ol against cisplatin-induced nephrotoxicity in animal models. The current findings demonstrate that HC (50 mg/kg) and DCOE (100 mg/kg), and all used doses of COE could be a promising approach to protect against cisplatin-induced nephrotoxicity, partially by ameliorating inflammatory response. Thus, these agents may be of significant therapeutic benefits against cisplatin renal toxicity, especially that these are natural products with good safety and tolerability profiles, as well as their reported anticancer, anti-inflammatory and antioxidant properties. Future studies are required to examine higher doses of DCOE and investigate the signaling pathways and the mechanisms of actions underlying the nephroprotective effect of HC, DCOE, and COE against cisplatin toxicity without compromising its anticancer effects.

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