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Efficacy of Lebanese Cannabis Oil Extract in the Treatment of Folic Acid-Induced Renal Fibrosis in Rats

By

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Efficacy of Lebanese Cannabis Oil Extract in the Treatment of Folic Acid-Induced Renal Fibrosis in Rats

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ABSTRACT

Kidney disease is a main contributor to mortality and morbidity worldwide. Renal fibrosis is the most common and final manifestation of chronic kidney disease. To date, there are no medications that target kidney cells or kidney fibrosis. The aim of this study is to evaluate the effect of Cannabis oil extract in the management of renal fibrosis in Folic acid-induced renal injury model in Sprague Dawley rats. Folic acid is known to accumulate easily in large amounts in the kidney at the proximal tubule segment when compared to other tissues due to the high content of its receptors in the kidneys. Folic acid was administered as a single dose of 250 mg/kg to induce nephrotoxicity in rats. Cannabis oil extract was administered at increasing doses of 5, 10, or 20 mg/kg to the Folic acid-treated groups for 2 weeks. Another group of rats was given 20mg/Kg Cannabis oil for 5 days followed by Folic acid administration on day 6. Body weight of rats were monitored during the study, serum creatinine, urea, and electrolytes were measured as well as pathological examination of the kidney and heart. Rats that were injected with Folic acid showed a marked reduction in body weight and increase in serum creatinine in comparison to the control group. Treatment with 20mg/Kg Cannabis oil caused a significant increase in body weight compared to the Folic acid treated group. In addition, a significant decrease in serum creatinine was observed in groups receiving cannabis extract at the doses of 5 and 10 mg/kg. Serum sodium was significantly reduced in all the groups receiving Cannabis oil extract. Cannabis oil ameliorated renal and cardiac pathology abnormalities induced by Folic acid in a dose dependent manner. In conclusion, the current results reveal a potential therapeutic and protective effect of Cannabis oil extract in renal fibrosis.

Keywords: Nephrotoxicity, Renal fibrosis, Cannabis oil extract, *Cannabis Sativa,* Folic acid.

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LIST OF ABBREVIATIONS

ACEI	angiotensin-converting enzyme inhibitor
AKI	acute kidney injury
Ang II	angiotensin II
ARBs	angiotensin II receptor blockers
BUN	blood urea nitrogen
CB1	cannabinoid receptor type 1
CB2	cannabinoid receptor type 2
CBD	cannabidiol
CKD	chronic kidney disease
COX-2	cyclooxygenase-2
CRS	cardiorenal syndrome
CTGF	connective tissue growth factor
ECM	extracellular matrix
ECS	endocannabinoid system
FA	folic acid
FGF-23	fibroblast growth factor 23

GFR	glomerular filtration rate
I.p	intra-peritoneal
IL-6	interleukin-6
iNOS	inducible nitric oxide synthase
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemoattractant protein-1
NGAL	neutrophil gelatinase-associated lipocalin
PAI1	plasminogen activator inhibitor-1
PBS	phosphate buffered saline
ROS	reactive oxygen species
STAT	signal transducer and activator of transcription
TGF-β	transforming growth factor-β
THC	tetrahydrocannabinol
TNF-α	tumor necrosis factor alpha

CHAPTER ONE

INTRODUCTION

1.1 Renal Disease

1.1.1 Prevalence of Kidney Disease

The world's disease profile and pattern changed drastically throughout the years. Infectious diseases were the prominent cause of disability and mortality back in the twentieth century. However, currently, non-infectious and non-communicable diseases are the main reason behind mortality and morbidity around the world (Atkins, 2005). Kidney disease is a key contributor and main cause of mortality and morbidity resulting from non-communicable diseases. In 2017, there were 697.5 million cases of chronic kidney disease (CKD) globally. This means that there are more people suffering from kidney diseases than from diabetes, osteoarthritis, asthma, chronic obstructive pulmonary disease, or depressive disorders (Purcell et al., 2020). According to the World Health Organization (WHO), it is estimated that 5 to 10 million people die annually from different types of kidney disease. Furthermore, it is said that almost as many people die from kidney disease as from cancer, diabetes, or respiratory diseases. In 2010, 2.62 million people received dialysis globally and the need for dialysis is projected to be doubled by 2030. It is important to note that kidney disease poses a tremendous economic and financial burden. Two to three percent of the annual health care budget is spent by high income countries on the treatment of patients with end stage renal disease even though they represent only 0.03% of the overall population (Luyckx et al., 2018). The prevalence of

dialysis in Lebanon is among the highest in the world. It estimated at 777 patients per million population compared to 410 dialysis patients per million population worldwide (Aoun et al., 2022).

1.1.2 Acute and Chronic Renal Disease

Around one quarter (25%) of the total blood supply passes through the kidneys every minute. The main function of the kidneys is to eliminate waste products and toxins from the body. They also help in the regulation of osmolarity, maintenance of ionic balance and acid-base homeostasis, and production of hormones such as renin, erythropoietin, prostaglandins and kinins (Marieb, 2009).

Acute kidney injury (AKI) formerly referred to as acute renal failure is a clinical syndrome characterized by a rapid and abrupt decrease in kidney excretory function along with the accumulation of nitrogen metabolism products such as urea and creatinine. Also, decreased urine output, metabolic acids accumulation and increase in potassium and phosphate may be observed (Bellomo et al., 2012). Acute kidney injury is a common complication observed in hospitalized patients and those who are admitted to the intensive care unit. The rapid and early diagnosis and management of AKI is crucial (Ronco et al., 2019).

On the other hand, chronic kidney disease is a progressive disease marked by functional and structural changes to the kidneys (Kalantar-Zadeh et al., 2021). CKD patients are most often asymptomatic. Therefore, screening and early detection of the disease are of a high importance especially in patients who are at high risk. The major risk factors of chronic kidney disease are the following: diabetes, hypertension, age more than 60 years, smoking,

familial history of kidney disease and history of AKI (Chen et al., 2019). Concerning the management of chronic kidney disease, the main target is to manage the underlying conditions such as hypertension, diabetes mellitus, and cardiovascular diseases. In addition, all CKD patients should be advised to avoid nephrotoxic drugs. Also, the dosing of all drugs should be adjusted in accordance with the estimated GFR. Dietary management, more specifically protein intake is also crucial to prevent the progression of CKD. In advanced stages of CKD, renal replacement therapy or kidney transplantation may be warranted based on the individual cases, the presence of symptoms and the eGFR (Chen et al., 2019).

1.1.3 Renal fibrosis

1.1.3.1 Overview of Renal Fibrosis

Renal fibrosis characterized by tubulointerstitial fibrosis and glomerulosclerosis is one of the final and most common manifestation of chronic kidney disease. It is the principal process underlying the progression of CKD to end stage renal disease also known as ESRD (Cho, 2010). By definition, glomerulosclerosis is the accumulation of matrix proteins such as collagens I, III, IV, and fibronectin in the glomerulus. On the other hand, tubulointerstitial fibrosis is characterized by the replacement of the tubules and/or the surrounding interstitium by matrix proteins (Gewin, 2018). The two main causes of chronic kidney disease are hypertension and diabetes that cause glomerular expansion leading to hemodynamic changes and endothelial dysfunction. Renal tissues react to the damage in a similar way to the process of wound healing that occurs in other tissues in the body. However, dysregulated wound healing process whereby there is an imbalance

between the excessive synthesis and the reduction in the breakdown of extracellular matrix will result in the development of glomerulosclerosis and tubulointerstitial fibrosis.

Thoroughly, the process of fibrosis starts by the production of angiotensin II (Ang II) that up-regulates the expression of many other factors including TGF-β, CTGF, PAI1, and NFκB which lead to the recruitment of neutrophils. The neutrophils are then substituted by macrophages and T-lymphocytes that trigger an immune response and cause interstitial nephritis. Tubular cells respond to this inflammatory process by forming a lesion of the basal membrane and by epithelial– mesenchymal transition transformed into interstitial fibroblasts. The resulting fibroblasts produce collagen that lead to an imbalance between the excessive synthesis and diminished breakdown of the extracellular matrix. This will eventually cause damage of the blood vessels and renal tubules and consequently the development of a cellular scar and renal fibrosis. In addition, endothelial and mesangial oxidant stress can impair the production of nitric oxide in the glomerulus thus reducing its protective effect in glomerulosclerosis. Renal sclerosis can also arise from non-hemodynamic origins such as proto-oncogenes, growth factors, infiltrating macrophages, vasoactive substances…. Thus, developing renal fibrosis along with chronic kidney disease is strongly associated with renal failure and poor long term prognosis that can eventually lead to renal collapse (Nogueira et al., 2017).

1.1.3.2 TGF-β Role in Renal Fibrosis

The main pathway involved in renal fibrosis is the TGF-β/Smad signaling pathway. The activity of TGF-β is highly dependent on TGF-β receptors and Smad transcription factors. During fibrosis, Smad3 becomes highly activated while the inhibitory Smad7 is

downregulated. The imbalance between Smad3 and Smad7 activates the myofibroblasts that lead to the excess synthesis of extracellular matrix and a decrease in its breakdown (Meng et al., 2015).

1.1.3.3 Management

Currently, there are no medications used clinically that could target specifically renal fibrosis or kidney cells (Klinkhammer et al., 2017). Controlling blood pressure and glycemia are very important factors to preserve the renal function. The standard therapy to slow down the progression of CKD and fibrosis is the blockage of the Renin-Angiotensin-Aldosterone system using ACEI or ARBs (Nogueira et al., 2017). As mentioned previously there is no approved treatment for renal fibrosis, however several drugs targeting renal fibrosis are still under clinical studies. For instance, *Rehmannia glutinosa*, a traditional Chinese herbal medicine, reduces the expression of TGF-β mRNA and the accumulation of type IV collagen leading to the inhibition of the progression of fibrosis (Lee et al., 2009). An anti-fibrotic agent called Pirfenidone has shown to attenuate renal fibrosis in animal models that have undergone unilateral ureteral obstruction (UUO) by decreasing the synthesis of collagen and TGF-β in renal tissue (Shimizu et al., 1998). Although Pirfenidone was shown to be effective in animal models, it had mixed results in humans with CKD (Liu & Zhuang, 2019). Moreover, mesenchymal stem cells based therapy was shown to be beneficial in renal fibrosis (Zhuang et al., 2019).

1.1.4 Renal Function Test and Biomarkers of Kidney Disease

Renal function tests are used to identify the presence of kidney disease, monitor the kidneys' response to therapy, and determine renal disease progression. Creatinine is

commonly used as an indicator of renal function. Urea is mostly excreted by the kidneys. Therefore, an increase in serum urea levels can be due to a decrease in renal clearance. However, these two tests are not fully reliable since they can be affected by different factors such as age, muscle mass, concomitant drug use, diet… (Gounden et al., 2022). It is suggested that GFR is the best estimate of chronic kidney disease (Wasung et al., 2015). Cystatin C is a non-glycosylated protein that is fully catabolized in the proximal tubule after glomerular filtration and is not returned to the blood. Unlike creatinine, its concentration is not affected by age, gender, race, protein intake and muscle mass. Cystatin C levels increase in a proportional manner with the decrease in GFR (Murty et al., 2013). Therefore, the use of creatinine and Cystatin C together provides the most accurate estimate of GFR (Wasung et al., 2015). Moreover, electrolyte panel is often used to detect acid-base and electrolyte imbalances. The electrolyte panel usually tests the levels of sodium, potassium, chloride and bicarbonate in the blood. Hyperkalemia is a major complication of renal failure whereby there is a decrease in the filtration and secretion of potassium in the distal tubule (Gowda et al., 2010).

1.1.5 Cardiorenal Syndrome

In the XIX century, it was firstly reported that patients with advanced kidney disease had structural changes in their hearts. This is known as cardiorenal syndrome (CRS) which is defined as disorders of the heart and kidneys wherein acute or chronic dysfunction in one organ may induce acute or chronic dysfunction of the other. This is due to the fact that the heart and kidney are both crucial for maintaining cardiovascular homeostasis and initial organ injury during a disease state can cause structural and functional changes in the other. CRS is classified into subtypes depending on the primary organ dysfunction and whether

the condition is acute or chronic. CRS type 1, also known as acute cardiorenal syndrome, refers to cardiac dysfunction that leads to acute kidney injury. CRS type 2 or chronic cardiorenal syndrome is characterized by chronic cardiac dysfunction leading to the appearance of renal impairment that progresses into chronic kidney disease. CRS type 3 or acute reno-cardiac syndrome refers to the acute cardiac injury and/or dysfunction secondary to acute renal dysfunction. CRS type 4 or chronic reno-cardiac syndrome is defined as cardiac damage in patients with any stage or chronic kidney disease. Finally, CRS type 5 also known as secondary cardiorenal syndrome is characterized by the concurrent injury and/or malfunction of both the heart and kidney secondary to a systemic condition such as drug toxicity, sepsis, cirrhosis… (Delgado-Valero et al., 2021).

1.2 Folic Acid Induced Nephrotoxicity

1.2.1 Animal Models of Kidney Disease

Animal models have been used extensively to study the pathogenesis and mechanisms of renal disease. Rats and mice are the most commonly used animals to study kidney disease (Bao et al., 2018). There are numerous chemical, surgical, genetic, and in vitro models to study renal fibrosis. Some of the chemical agents that can be used include the following: mercuric chloride, vanadate, uranyl nitrate, folic acid, Adriamycin, cyclosporine A (Nogueira et al., 2017). In our study, the folic acid model was chosen since it comprises numerous advantages: the induction of kidney disease is fairly simple and straightforward and does not require surgical intervention (Yan, 2021), the model is highly reproducible and mimics the clinical symptoms of kidney disease in humans (Gupta et al., 2012), folic acid is considered to be safe and does not cause any hazards on the environment and on

the persons handling it, and folic acid only causes injury to the kidneys without affecting other organs in the body (Rattanasinganchan et al., 2016).

1.2.2 Folic Acid Properties and Uses

Folic acid, also known as vitamin B9, is a water-soluble type of vitamin. It is naturally available in many types of food, and it is also available as dietary supplement. Folic acid is a coenzyme or co-substrate that plays an important role in the synthesis nucleic acids, in other terms DNA and RNA, and in the metabolism of the different amino acids (McPartlin, 2005). Folic acid is usually indicated for the treatment and prevention of folic acid deficiency anemia and for the prevention of neural tube defects and other types of birth defects in pregnant women and in women planning to become pregnant (Minigh, 2007). Deficiency in folate can lead to megaloblastic anemia, initiation of cancer, cognitive and neurological impairment, hyperhomocysteinemia (risk factor for vascular disease), and birth defects (Miller, 2013).

1.2.3 Folic Acid Induced Nephrotoxicity

Folic acid is freely filtered by the glomerulus since it is a water-soluble compound of a small molecular size. Within normal folate concentrations, minimal renal excretion is observed, however, nearly 100% of the folate undergoes renal reabsorption. The reabsorption process is mediated by high affinity folate receptors that are found on the luminal side of the proximal tubule epithelial cells (Samodelov et al., 2019). Therefore, folic acid can accumulate easily in large amounts in the kidneys when compared to other tissues due to the high content of its receptors in the kidneys (Nazki et al., 2014).

When administered at low doses, folic acid has beneficial effect against oxidative stress (Schneider et al., 2011). However, high doses of folic acid are considered to be toxic. Folic acid induced renal injury was firstly reported in Germany in 1969. In the 1970s, the concepts of "folate nephropathy" and "renal folate toxicity" started to emerge (Yan, 2021).

To induce kidney injury, folic acid is given as a single intravenous or intraperitoneal injection at a dose of 250mg/kg of body weight in male mice or rats (Dai et al., n.d). Acute kidney injury is observed with a maximal increase in BUN, serum creatinine and urine glucose after two days. These parameters return back to normal levels within 10 to 15 days, however, decreased urine osmolality and persistent polyuria suggest the occurrence of permanent tubular damage even after recovery of global renal function. Following folic acid administration, crystals are formed in the tubular lumen, but it is not the only mechanism causing nephrotoxicity because alkalization of urine decreases the deposition of crystals but lesions in the tubules remain apparent. Elevation in fibrosis markers is observed after 6 days and tubulointerstitial fibrosis is seen after 14 days (Rabe & Schaefer, 2016).

1.2.4 Mechanisms of Folic Acid Induced Nephrotoxicity

The precise mechanism of renal damage caused by folic acid remains unclear. Remarkable inflammatory cell infiltrations were detected in injured kidneys of this model. These infiltrating inflammatory cells will contribute to the renal damage through reactive oxygen species (ROS) generation, further leukocytes recruitment, and proinflammatory and profibrotic cytokines production (Doi et al., 2006).

In fact, the underlying molecular and biochemical mechanisms of injury are multifaceted and complex. Several mechanisms are suggested to be involved in the process of FA induced renal damage (Figure 1). The first major mechanism is renal oxidative stress (Yan, 2021). Oxidative stress is defined as a disturbance in pro-/antioxidant balance that leads to an excessive production of reactive oxygen and nitrogen species that harm the cells and cause the oxidation of biological molecules like proteins, DNA and lipids. The second mechanism involved in the FA induced kidney injury is ferroptosis (Yan, 2021). Ferroptosis is considered as a newly discovered type of cell death characterized by the accumulation of large amounts of iron and lipid peroxidation (Li et al., 2020). The third proposed mechanism is the impairment in mitochondrial bioenergetics (Yan, 2021). Stage 3 respiration was diminished in the isolated mitochondria during the acute stage of renal injury and returned back to normal after 7 and 14 days respectively (Aparicio-Trejo et al., 2020). β-oxidation was impaired during this process which further contributes to the transition from acute to chronic renal injury and renal fibrosis (Yan, 2021). Hence, mitochondrial dysfunction contributes to AKI and its progression to CKD and fibrosis (Stallons et al., 2014). Another contributor to renal injury is fibroblast growth factor 23 (Yan, 2021). FGF-23 is responsible for the regulation of vitamin D and phosphate homeostasis (Shimada et al., 2004). This protein is observed to be increased in animal models of FA induced kidney injury (Christov et al., 2013). The overexpression of FGF-23 in both AKI and CKD is mediated by interleukin-6 since the inhibition of IL-6 prevented the increase of FGF-23 protein (Durlacher-Betzer et al., 2018). Impaired mitophagy is also considered to be involved in F.A induced renal injury (Yan, 2021). In fact, the kidney is an organ rich in mitochondria and requires a lot of energy. Renal function highly depends on mitophagy (Pickles et al., 2018). By definition, mitophagy is

a process by which damaged or dysfunctional mitochondria are selectively removed by autophagy. It is the main mechanism that regulates the quality and quantity of mitochondria (Pickles et al., 2018). Impaired mitophagy is associated with different diseases in humans such as cancer, aging, neurodegenerative and cardiovascular diseases (Zuo et al., 2020).

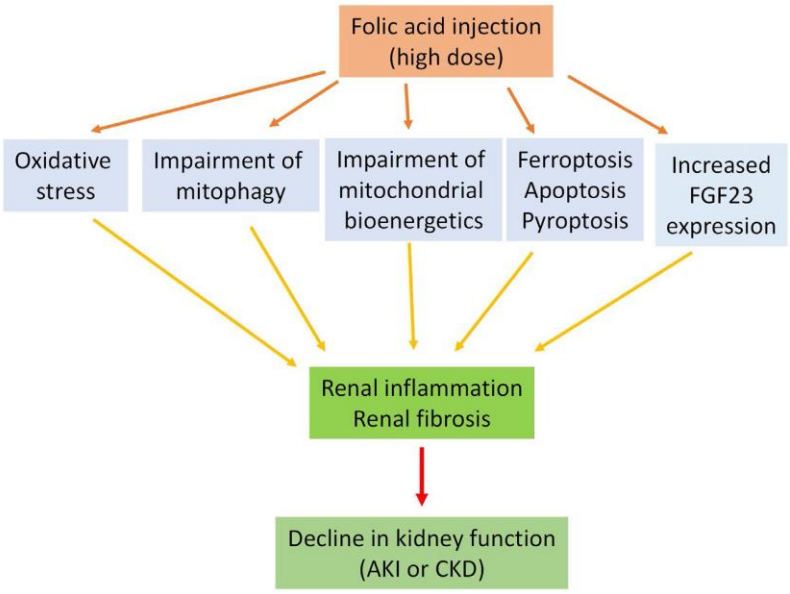


Figure 1. Major pathological mechanisms of folic acid induced kidney damage. Adapted from (Yan, 2021)

1.3 Cannabis

1.3.1 Plant Based Medicine

Humans have utilized natural products to manage and treat different diseases and illnesses since the prehistoric eras (Yuan et al., 2016). The use of plants as medicinal agent dates back to 60,000 years ago (Fabricant & Farnsworth, 2001). For instance, Paclitaxel and

Vinca alkaloids are widely used anti-cancer drugs that are derived from plants (Yuan et al., 2016).

Hence, it can be said that plant derived products play an important role in the management of different disease conditions. However, plant-derived remedies require a deep evaluation and assessment of their pharmacological effects and safety aspects (Firenzuoli & Gori, 2007). In our study, *Cannabis sativa* L. ssp. indica, a well-known Lebanese plant, is chosen to evaluate its effect against folic acid induced nephrotoxicity.

1.3.2 Cannabis Overview

Cannabis is one of the most cultivated and trafficked illicit drugs in the world (Bridgeman & Abazia, 2017). It is among the oldest plants in the world that belongs to the Cannabaceae family. The most common cannabis species are *Cannabis sativa, Cannabis indica* and *Cannabis ruderalis*. Among these species, *Cannabis sativa* (*C. sativa*) has been of interest to scientists and to the public (Hartsel et al., 2016). Cannabis was used as a medicinal agent ages ago. For instance, between the 8th and 18th century, Arab physicians used cannabis as a traditional medicine for different diseases due to the fact that it has anti-emetic, diuretic, anti-inflammatory and anti-epileptic properties (Shebaby et al., 2021).

In Lebanon, the cultivation of cannabis goes back as far as the Roman times. The cultivation, selling and consumption of cannabis was prohibited in Lebanon. However, the cannabis plant was cultivated in an illegal manner in the Beqaa valley for a long time. As of April 22, 2020, the Lebanese parliament approved a draft law that states upon the legalization of the cultivation of cannabis in the context of medical and industrial use. *C.*

sativa L.ssp. sativa, C. sativa L. ssp. indica (Lam.), and the dominant hybrid strain are the main cannabis strains found in Lebanon. Moreover, Cannabis oil extract has been widely used in Lebanon in the management of certain medical conditions mainly cancer, pain and diabetes. However, it is important to note that studies about the therapeutic effect of Lebanese cannabis are very limited and need to be explored thoroughly (Shebaby et al., 2021).

1.3.3 Cannabis Plant Composition

The phytochemistry of the cannabis plant is considered to be complex and challenging since over 480 compounds have been found (Flores-Sanchez & Verpoorte, 2008). It contains numerous phytochemicals such as terpenes, cannabinoids and phenolic compounds are present in the cannabis plant (Andre et al., 2016). Cannabinoids are the mostly studied compounds of cannabis (Flores-Sanchez & Verpoorte, 2008). Extreme environmental conditions including humidity, temperature, soil nutrients, radiation and parasites have a great impact on the phytocannabinoids content of the cannabis plant (Bonini et al., 2018). Among the cannabinoids group, cannabidiol (CBD) and tetrahydrocannabinol (THC) are the most prominent and most studied phytocannabinoids (Hanuš et al., 2016). THC has psychoactive properties and is mainly used for neurodegenerative diseases, pain, multiple sclerosis, and cancer, whereas CBD does not have psychoactive activity and possesses anti-inflammatory, anxiolytic, antioxidant, neuroprotective, antimicrobial properties (Shebaby et al., 2021).

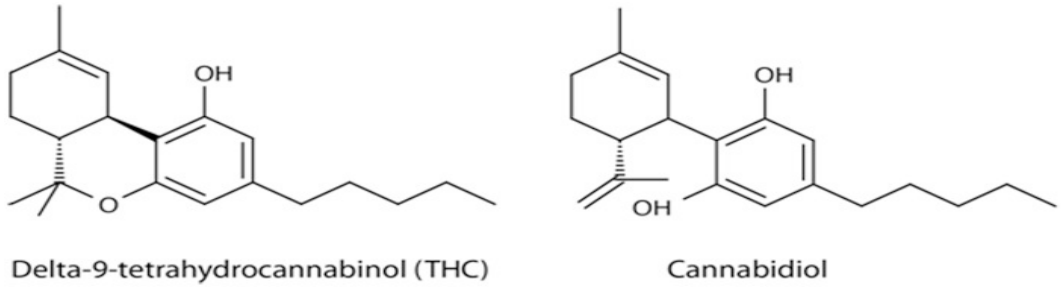


Figure 2. Chemical structure of delta-9-tetrahydrocannabinol and cannabidiol (Atakan, 2012)

The combination of CBD and THC has been shown to have improved therapeutic outcomes when compared to the effect of each cannabinoid alone (Shebaby et al., 2021). In fact, CBD has been shown to antagonize some adverse effects that accompany THC such as sedation, tachycardia, and intoxication while having anti-emetic, anti-carcinogenic, and analgesic properties. These findings allow the use of higher doses of THC in the management of certain medical conditions such as multiple sclerosis, neuropathic pain, sleep disturbances, intractable pain in cancer patients, and rheumatoid arthritis (Russo & Guy, 2006). Furthermore, other plant constituents such as phenols, terpenes and minor phytocannabinoids have been shown to enhance and improve the activity of CBD, this is known as the "entourage effect" (Gallily & Yekhtin, 2019; Gallily et al., 2015; Romano et al., 2014; Russo, 2011).

1.3.4 The Endocannabinoid System

Most of the biological activities and properties exerted by cannabinoids in humans are highly dependent on their interactions with the endocannabinoid system (ECS) in the body (Andre et al., 2016). The two main cannabinoid receptors are G-protein coupled receptors

known as cannabinoid receptor type 1 (CB1) and cannabinoid receptor type 2 (CB2) (Di Marzo & Piscitelli, 2015). CB1 receptors are mainly found in the central nervous system (CNS) and the brain, they can also be found to a lesser extent in the periphery including retina, sperm cells, peripheral neurons, testicles (Reggio, 2010). On the other hand, CB2 receptors are mostly expressed in immune cells and peripheral tissues (Zou & Kumar, 2018). Moreover, there are transient receptor potential (TRP) channels, and peroxisome proliferator activated receptors (PPAR's) that are engaged by certain cannabinoids (Lu & Mackie, 2016). The ECS also comprises of the endogenous ligands: N-arachidonoyl-ethanolamine, also known as anandamide (AEA) and 2-arachidonoylglycerol (2-AG) (De Petrocellis et al., 2011). The endocannabinoid system also includes enzymes used for the synthesis and inactivation of endogenous cannabinoids (Di Marzo et al., 2005). CBD acts as CB1 antagonist and as a negative allosteric modulator at CB2 receptors. When compared to THC, CBD has a lower level of activity at these receptors (Levinsohn & Hill, 2020). On the other hand, THC is considered to be a partial agonist at the CB1 and CB2 receptors (Pertwee, 2008).

Both CB1 and CB2 receptors are expressed in the kidneys but the effects of the ECS are still not very well understood in the kidneys (Ho et al., 2019). Also, the enzymes used in the synthesis and degradation of endocannabinoids are shown to be expressed in the kidneys of rodents (Deutsch et al., 1997). CB1 receptors are found in different parts of the kidneys more specifically in the distal tubules, proximal convoluted tubules, and intercalated cells of the collecting ducts in humans (Larrinaga et al., 2010). In rodents, CB1 receptors were found to be expressed in the afferent and efferent arterioles, glomeruli, thick ascending limbs of Henle, podocytes, mesangial cells, and tubular

epithelial cells (Chua et al., 2019). As for CB2 receptors, they are mainly localized in the podocytes, mesangial cells, and proximal tubule cells (Barutta et al., 2011; Deutsch et al., 1997; Jenkin et al., 2010).

The modulation of cannabinoids receptors has been suggested in different types of renal disease, nephropathies, and renal fibrosis (Hryciw & McAinch, 2016). A study showed the upregulation of CB1 receptors in mice after undergoing unilateral ureteral obstruction which is used as a model for renal fibrosis. The mice treated with rimonabant, a CB1 antagonist, showed a reduction in macrophage infiltration (Lecru et al., 2015). Another study showed that the injection of XL-100, an inverse CB2 agonist, improved the kidney injury, inflammation, and fibrosis in ischemia/reperfusion and obstruction animal models suggesting that CB2 receptors are a mediator in renal fibrosis and targeting them may present a potential strategy to treat fibrosis (Zhou et al., 2018). A recent study published in February 2021 confirms the emerging role of the endocannabinoid system in renal fibrosis: the novel CB2/β-Catenin pathway promotes renal fibrosis which implicates that the inhibition of the CB2 or β-arrestin 1 might have a beneficial role in protecting against renal fibrosis (Zhou et al., 2021). However, the role of CB2 receptors in renal fibrosis remains controversial since it was discovered a drug called celastrol can inhibit the progression of kidney fibrosis by upregulating the expression of CB2 receptors (Tang et al., 2018). Furthermore, several studies have explored the role of the most common cannabinoids in renal disease. For instance, in a study by Soares et al., protective effects against inflammatory and oxidative damage in renal ischemia/reperfusion models were observed after cannabidiol treatment (Soares et al., 2015). Another study published in the Journal of Pharmacology and Experimental Therapeutics showed that the non-

psychoactive cannabinoid CBD has a significant role against cisplatin-induced nephrotoxicity in murine models. The results revealed that CBD improves renal function and attenuates the oxidative/nitrosative stress, cell death, and inflammation in the kidneys (Pan et al., 2009). Moreover, it was discovered that cannabidiol possesses anti-inflammatory, antioxidant, and anti-apoptotic properties allowing it to have nephroprotective effective against ischemia/reperfusion kidney injury model in rats (Fouad et al., 2012). In addition to CBD and THC, there are different components present in the Lebanese cannabis oil extract such as β-myrcene, cannabinol (CBN), and limonene that possess anti-inflammatory properties. According to Shebaby et al., COE exerts its anti-inflammatory effects through the inhibition of different pro-inflammatory factors such as COX- 2, TNF-α, and i-NOS through MAPK pathway blockade (Shebaby et al., 2021).

Emerging studies have identified a critical and important role of the endocannabinoid system in kidneys' function. Thus, therapeutic approaches that target CB1 and CB2 receptors in renal dysfunction could be of great clinical relevance and impact and should be studied thoroughly and deeply.

1.3.5 Adverse Effects of Cannabinoids

CBD exhibits pharmacokinetic and pharmacodynamic properties that can lead to drug-drug interactions (DDI) and adverse events. In fact, CBD and other components that can be found in cannabis have known Cytochrome P450 (CYP 450) and other enzymes system activity which make these compounds susceptible to drugs interactions (Jiang et al., 2011). CBD also presents with several adverse effects such as elevation in transaminase levels,

sleep disturbances, infect ion, sedation, and anemia (Brown & Winterstein, 2019). As for THC, its short-term use is associated with side effects such as cognitive and memory impairment, psychosis, and motor coordination impairment. The long-term use of THC can lead to cognitive impairment, higher risk of addiction, higher risk of chronic psychosis, and problems in brain development when it is used during the early adolescence phase (Andre et al., 2016). The concomitant use of CBD with THC has a protective role and can alleviate the side effects associated with the use of THC alone (Iseger & Bossong, 2015).

1.4 Hypothesis

Cannabis oil extract may demonstrate potential protective and preventive effects on folic acid induced renal fibrosis.

1.5 Objectives

The main objective is to investigate the Lebanese cannabis oil extract as a potential treatment and/or prevention of folic acid induced kidney fibrosis through the evaluation of:

- Biochemical markers in the serum (serum creatinine, urea, and electrolytes).
- Histopathological evaluation of the kidneys.

Also, the study aims to investigate the effect of the administration of high dose folic acid and cannabis oil extract on cardiac tissues through the histopathological evaluation of the rats' hearts.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Reagents and Chemicals

Folic acid was provided by Mediphar Laboratories (Lebanon).

The following buffers and solutions were prepared:

- **Sodium bicarbonate solution 0.3M (30 ml):** 0.756g sodium bicarbonate, MilliQ water to reach 30 ml.
- **Folic acid solution (25 mg/ml):** 50mg of folic acid, 2 ml of 0.3M sodium bicarbonate solution.
- **PBS (500 ml), pH = 7.4:** 400 ml distilled water, 4g sodium chloride (NaCl), 100 mg potassium chloride (KCl), 0.75 g sodium phosphate dibasic (Na_2HPO_4), 122.5 mg potassium phosphate monobasic (KH_2PO_4), add distilled water to reach 500 ml.

2.2 Animals

Eight weeks old male Sprague Dawley rats were acquired from the animal care facility laboratories at the Lebanese American University after the approval of the Animal Care and Use Committee. The animals had free access to food and water and were kept at 12 hours light to dark cycle.

2.3 Experimental Protocol

Rats were divided randomly into six groups.

All injections were administered via the intra-peritoneal route.

Sodium bicarbonate (300 mM) was used as a vehicle to dissolve folic acid. The dose of folic acid is 250 mg/kg given by a single intra-peritoneal injection on day 6 of the experiment.

Cannabis oil was dissolved in a mixture of Ethanol: Tween80: PBS at a 1:1:18 ratio respectively and administered eight days after the FA injection daily for 8 days (from day 14 till day 21 of the experiment) then every other day for one week (from day 22 till day 30 of the experiment) thereafter.

The duration of the experiment is one month.

- **Group 1 (**normal control, n = 5): i.p injections of vehicle (day 1-5 and day 14-30) and i.p injection of sodium bicarbonate (300 mM) vehicle at day 6.
- **Group 2** (FA control, n = 5): i.p injections of vehicle (day 1 till 5 and day 14 till 30) and at day 6 folic acid is injected.
- **Group 3** (FA + 5 mg/kg of body weight COE, n = 7): i.p injections of vehicle solution from day 1 to 5, at day 6 folic acid is administered as single i.p injection, and from day 14 to 30 cannabis oil extract is administered at a dose of 5 mg/kg of body weight.
- **Group 4** (FA + 10 mg/kg of body weight COE, n = 6): i.p injections of vehicle solution from day 1 to 5, at day 6 folic acid is administered as single i.p injection, and from day 14 to 30 cannabis oil is administered at a dose of 10 mg/kg of body weight.

- **Group 5** (FA + 20 mg/kg of body weight COE, n = 7): i.p injections of vehicle solution from day 1 to 5, at day 6 folic acid is administered as single i.p injection, and from day 14 to 30 cannabis oil is administered at a dose of 20 mg/kg of body weight.
- **Group 6** (20 mg/kg of body weight COE + FA, n = 7): i.p injections of 20 mg/kg of body weight cannabis oil, at day 6 folic acid is administered as single i.p injection, and i.p injections of vehicle solution from day 14 till day 30 of the experiment.

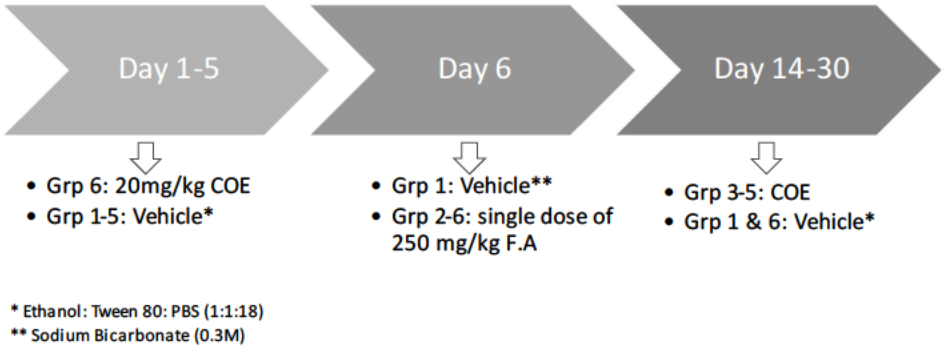


Figure 3. Schematic representation of the experimental protocol

Animals were sacrificed one day after the last injection.

Blood and urine samples were collected from each rat. The kidneys and hearts were also harvested for histopathological examination.

2.4 Biochemical Tests

The blood specimens were immediately collected, placed along with EDTA to prevent clotting, and centrifuged at 3000 rpm for 20 minutes at 4°C to obtain the serum. The

samples were then stored at -80^{o}C until analysis. Serum creatinine, urea, and electrolytes were analyzed.

2.5 Histopathology

Harvested kidneys and hearts were placed in formalin until the analysis. For the histopathological examination, renal and cardiac tissues were sectioned and stained by hematoxylin-eosin (HE).

2.6 Statistical Analysis

IBM SPSS statistics 26.0 software was used for data analysis. Comparison of data between groups was done using one-way analysis of variance (ANOVA). Data is presented as mean +/- SEM. A P-value of less than 0.05 was considered to be statistically significant.

CHAPTER THREE

RESULTS

3.1 Effect of COE on Serum Creatinine and Urea Levels

To investigate the potential protective effect of COE on folic acid induced nephrotoxicity, serum creatinine and urea were measured at the end of the experiment.

As shown in the figure 4 below, folic acid administered at a dose of 250 mg/kg significantly increased serum creatinine levels when compared to the control group. Treatment with cannabis oil extract at a dose of 5 mg/kg ($n = 7$, $P < 0.05$) and 10 mg/kg ($n = 6$, $P < 0.05$) significantly decreased the serum creatinine levels. However, COE at a dose of 20 mg/kg of body weight administered as prevention and treatment for groups 5 and 6 ($n = 7$ in each group, $P > 0.05$) decreased serum creatinine but not significantly.

As for the serum urea levels, the lower doses of COE (5 and 10 mg/kg of body weight) did not affect the urea levels. However, groups 5 ($n = 7$) and 6 ($n = 7$) receiving 20 mg/kg of COE had higher serum urea levels than the rats in the folic acid control groups ($n = 5$, $P < 0.05$) (Figure 5).

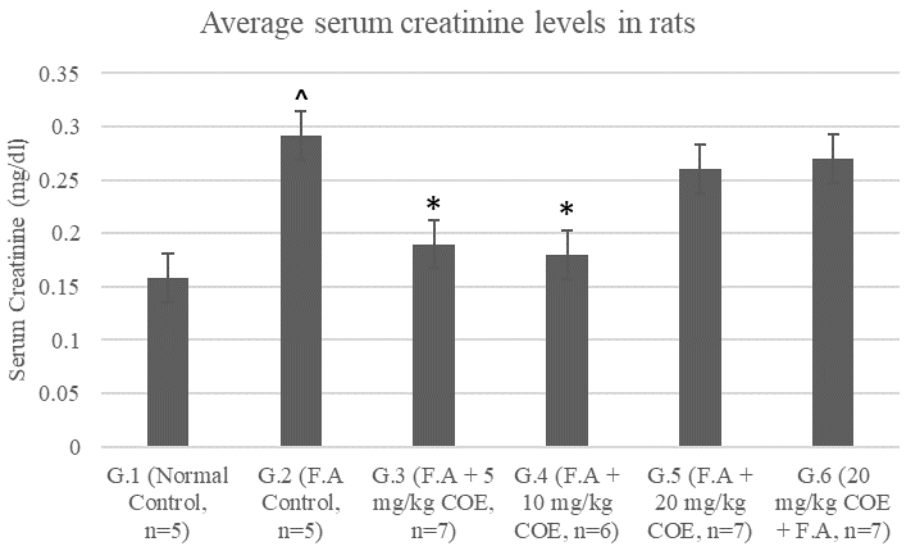


Figure 4. Serum creatinine levels in different rat groups. Each column denotes the mean +/- SEM. ^ $P < 0.05$ vs. normal control, * $P < 0.05$ vs. F.A control.

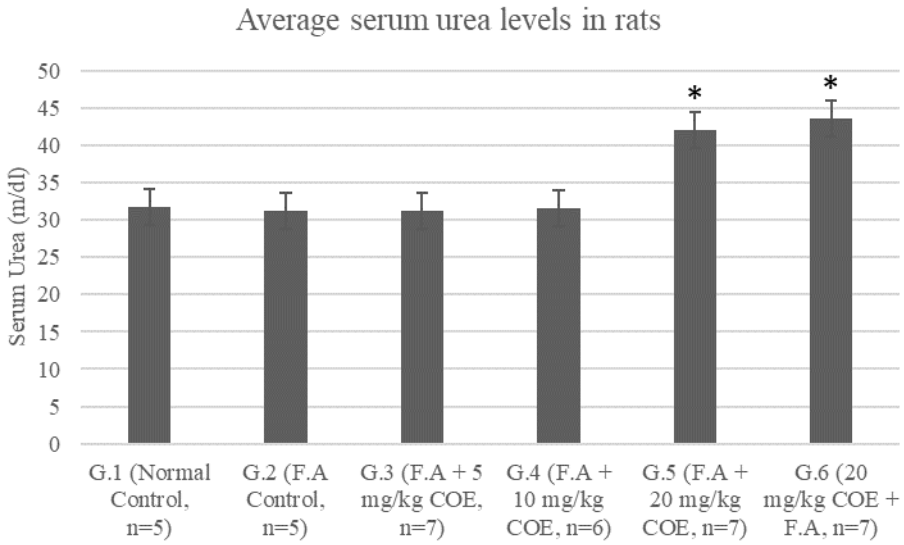


Figure 5. Serum urea levels in different rat groups. Each column denotes the mean +/- SEM. * $P < 0.05$ vs. F.A control.

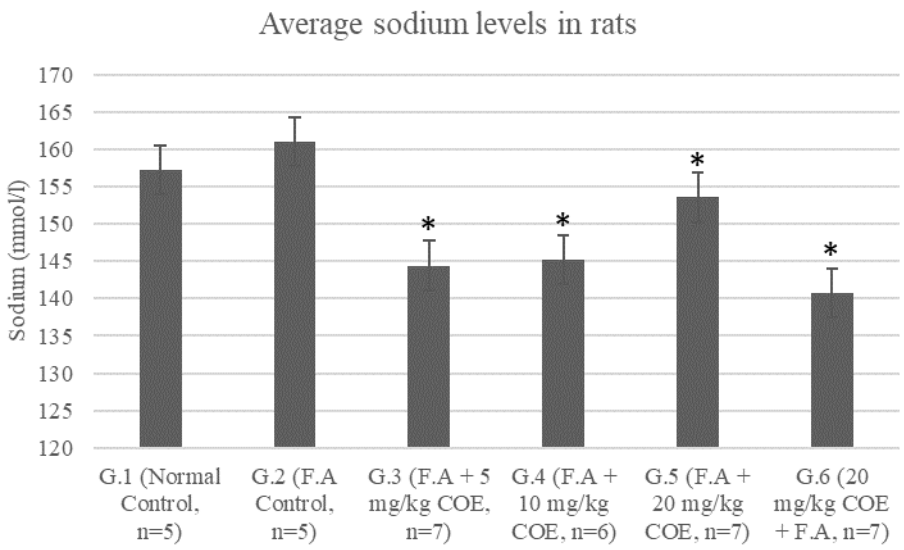
3.2 Effect of COE on Electrolytes Levels

The sodium levels (Figure 6.A) for all the groups receiving cannabis oil extract at the different doses (5, 10, 20 mg/kg of body weight) are significantly lower than those in the folic acid control group ($P < 0.05$).

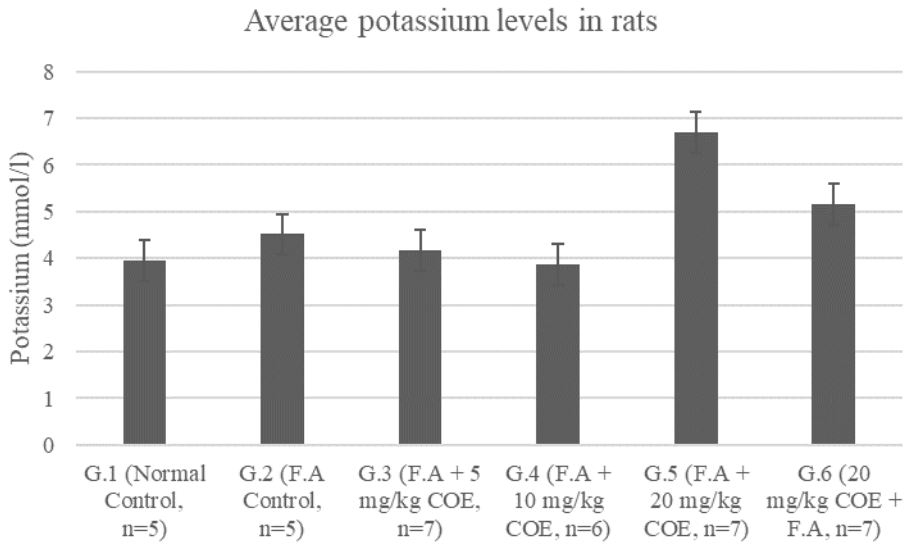
No changes in potassium levels was observed between the different groups (Figure 6.B)

As for the chloride levels shown in figure 6.C, no significant difference was detected between the different groups.

A.



B.



C.

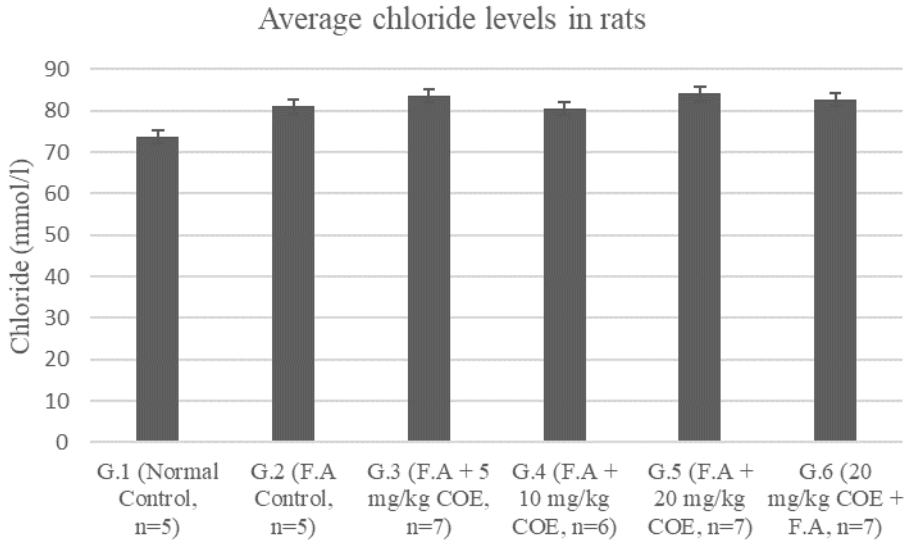


Figure 6. Levels of (A) sodium, (B) potassium, (C) chloride levels in different rat groups. Each column denotes the mean +/- SEM. * $P < 0.05$ vs. F.A control.

3.3 Organs and Body Weights

3.3.1 Kidney and Heart Weights

The average kidneys weights of the different studied groups are represented in table 1. Rats in group 2 who received a single i.p injection of 250 mg/kg of folic acid have a significant lower kidney weight than the rats in the normal control group that only received the vehicle ($n = 5$, $P < 0.05$). The rats in group 5 ($n = 7$) and group 6 ($n = 7$) receiving 20 mg/kg of COE as treatment and prevention respectively have higher kidney weight when compared to the folic acid control group ($P < 0.05$) (Table 1).

There is no significant difference in the heart weights of the rats in the six different groups (Table 1).

Table 1: Kidney and heart weight of rats. Values are reported as mean +/-SEM. *P-value<0.05 vs control, +P-value<0.05 vs F.A group.

	Group 1 (Normal control, n=5)	**Group 2 (F.A control, n=5)**	**Group 3 (F.A + 5mg/kg COE, n=7)**	**Group 4 (F.A + 10mg/kg COE, n=6)**	**Group 5 (F.A + 20mg/kg COE, n=7)**	**Group 6 (20mg/kg COE + F.A, n=7)**
Kidney weight (g)	0.98±0.03	0.81±0.05*	0.82±0.04	0.85±0.03	1.04±0.03+	0.95±0.04+
Heart weight (g)	0.86±0.05	0.74±0.04	0.79±0.04	0.82±0.05	0.86±0.03	0.80±0.04

3.3.2 Body Weight

The average body weight of the rats in group 2 (n =5) receiving only folic acid at day 6 is significantly lower than the average body weight of the rats in the normal control group only receiving the vehicle (n = 5, P < 0.05).

The average body weight of the rats receiving 20 mg/kg of COE as treatment in group 5 (n = 7) is higher when compared to the average body weight of the rats in the folic acid control group (n= 5, P < 0.05) (Figure 7).

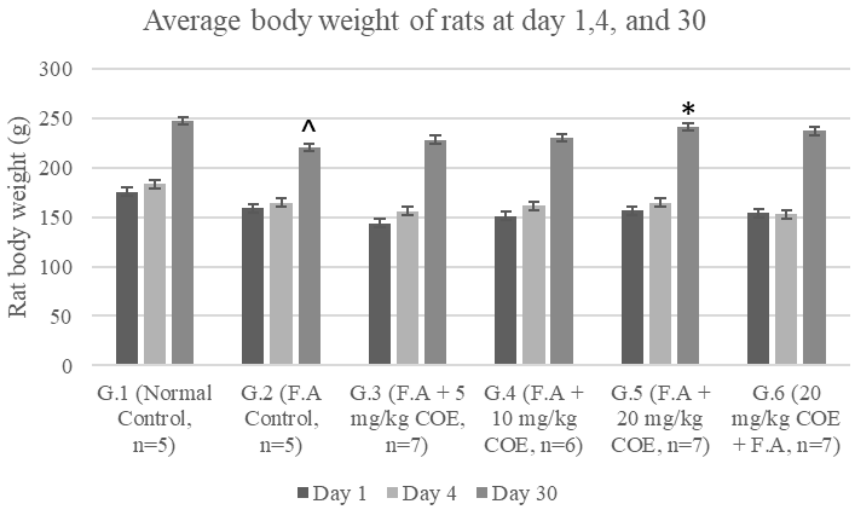


Figure 7: Body weight of rats. Each column denotes the mean +/-SEM. ^P<0.05 vs normal control, *P <0.05 vs F.A control.

3.4 Histopathological Findings

3.4.1 Kidney Findings

The results of the pathological examination are represented in figure 8 below. In the control group that had only received empty vehicle, histopathological examination

showed no specific changes or damage in kidney tissues. However, 40% of rats in group 2 which received only folic acid injections presented with black pigment in renal tubules and interstitial inflammation. Rats receiving 5mg/kg of cannabis oil extract did not show a remarkable amelioration whereby interstitial inflammation, tubular distention and vascular dilatation were still available. Moreover, cannabis oil extract treatment at 10 and 20mg/kg showed a remarkable improvement in kidney structure whereby there were no significant findings of damage. As for the rats that have received a dose of 20mg/kg of cannabis oil extract as prevention, 50% of them showed normal kidney structure and the rest presented with resolution of interstitial inflammation but tubular distention and vascular dilation were still present.

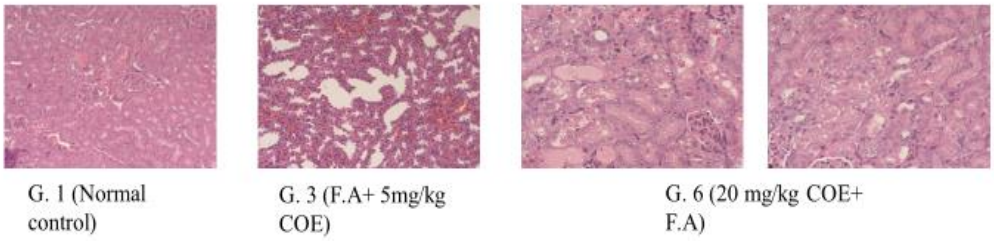


Figure 8: Renal pathological findings in different groups

3.4.2 Cardiac Findings

The results of cardiac pathology are represented in figure 9 below. Upon histopathological examination, the heart sections of rats in group 1, which is the control group showed no signs of damage or injury to the cardiac tissues. On the contrary, 60% of the samples from group 2 which received only folic acid showed chronic inflammation and scattered lymphocytes present among myocytes. Treatment with 5mg/kg cannabis oil extract did

not cause any major improvement in cardiac structure whereby 75% of the samples showed the presence of chronic inflammation. All the rats in group 4 receiving 10mg/kg of cannabis oil extract showed a normal cardiac structure with no inflammation. Treatment with 20 mg/kg of cannabis oil extract improved the cardiac structure in 50% of the rats, whereas the dose of 20mg/kg of COE given as prevention showed a remarkable improvement whereby 75% of the samples presented with normal cardiac structures.

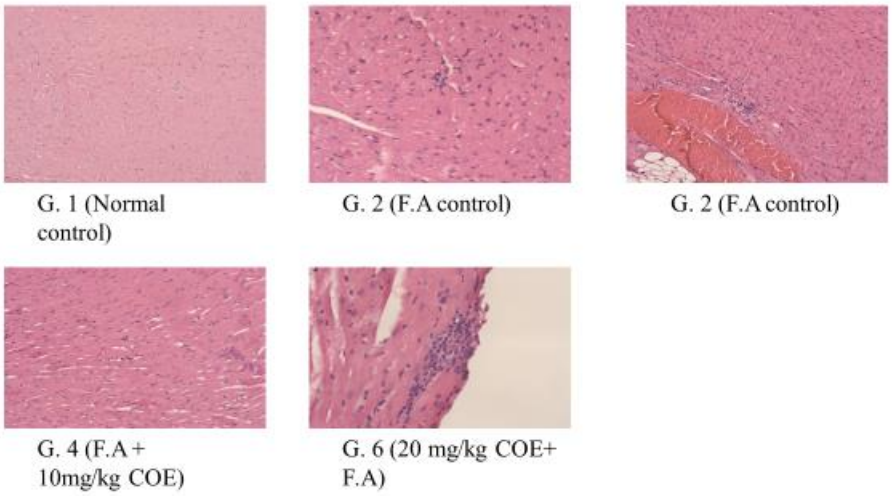


Figure 9: Cardiac pathological findings in different groups

CHAPTER FOUR

DISCUSSION

Inflammation is the first, healthy, and protective response against the occurrence of renal injury (Meng, 2019). However, ongoing and prolonged inflammation processes will lead to the progression of renal fibrosis. In addition to renal inflammation, reactive oxygen species, advanced glycation end-products, and certain conditions such as hyperglycemia, hypertension, proteinuria, and hypoxia have been related to the development of renal fibrosis (Meng et al., 2014). Hence, agents capable of restoring the redox balance and possessing anti-inflammatory properties can be beneficial in the management of renal fibrosis (Sun et al., 2017).

In our current study, the effect of the Lebanese cannabis oil extract on animal models of renal fibrosis was investigated. In vitro and in vivo studies have demonstrated that COE possess potent anti-inflammatory effects (Shebaby et al., 2021). Moreover, CBD which is a major component in COE is shown to have antioxidant activity (Atalay et al., 2019). Cannabinoids exert their effect through the interaction with the receptors of the endocannabinoid system in the human body (Andre et al., 2016). Both CB1 and CB2 receptors are expressed in the kidneys (Ho et al., 2019). In various forms of renal diseases, nephropathies, and renal fibrosis, the regulation of cannabinoids receptors has been proposed (Hryciw & McAinch, 2016). For instance, a study showed that cannabidiol therapy had protective benefits against inflammatory and oxidative damage in renal ischemia/reperfusion models (Soares et al., 2015). Another study showed that CBD reduces oxidative/nitrosative stress, cell death, and inflammation in the kidneys in murine

models of cisplatin induced nephrotoxicity (Pan et al., 2009). Also, besides THC and CBD, cannabis oil extract contains different components such as β-myrcene, cannabinol (CBN), and limonene that possess anti-inflammatory properties (Shebaby et al., 2021). For instance, a study showed that myrcene which is present at a proportion of 1.94% in cannabis oil extract was able to improve the function of the kidneys through the downregulation of oxidative stress and inflammation (Shebaby et al., 2021; Islam et al., 2020). Thus, targeting the endocannabinoid system is considered to be a potential therapeutic approach in the management of nephrotoxicity.

In order to assess renal function, serum urea, creatinine and electrolytes were evaluated. Creatinine is the most widely used endogenous marker to assess glomerular function. It is entirely eliminated from the body by the kidneys. An elevation in serum creatinine indicates a significant decrease in glomerular filtration or an obstruction in urine elimination. On the other hand, 85% of urea is excreted via the kidneys. A rise in serum urea can be indicative of a decrease in renal clearance (Gounden et al., 2022). Studies have shown that following folic acid administration in mice, serum creatinine and urea increased (Liao et al., 2021). In our current study, folic acid administration significantly increased serum creatinine but however, it failed to significantly increase serum urea levels. Treatment with cannabis oil extract at a dose of 5 and 10mg/kg significantly decreased serum creatinine, on the other hand, the dose of 20mg/kg of cannabis oil extract given either as treatment or prevention did not significantly decrease serum creatinine. These findings indicate that COE at doses of 5 and 10mg/kg could be a potential agent used to reverse the elevation of serum creatinine in renal disease. In fact, a study exploring the nephroprotective effect of cannabidiol in cisplatin induced nephrotoxicity showed that

treatment with 5 and 10mg/kg of CBD decreased serum creatinine and urea significantly in mice (Pan et al., 2009). In addition, a study revealed that exposure to high doses of marijuana (25 and 50mg/kg) in which THC was the major constituent led to a decrease in plasma sodium levels in Wistar rats (Taiwo et al., 2021). In our study, all doses of cannabis oil extract led to a significant decrease in plasma sodium levels.

The current study showed that the animals receiving folic acid alone had lower body weights at the end of the experiment. Only the animals receiving 20mg/kg of COE as treatment had higher body weights at the end of the experiment when compared to the folic acid treated rats. As for the kidney weights, F.A treated rats had lower kidney weights than normal ones while rats receiving 20 mg/kg of COE as treatment and prevention showed normal kidney weights. A study showed that the administration of folic acid at lower doses (100 mg/kg) to induce acute kidney injury caused significant weight loss in mice but the single administration of 250mg/kg of folic acid did not affect the weight. It was also observed that following folic acid administration renal hypertrophy occurred and the weight of the kidneys increased as a compensatory mechanism (Gupta et al., 2012).

As for the kidney structure, histological examination showed changes in renal structure such as interstitial inflammation, tubular distention and vascular dilatation in the kidneys of rats receiving either folic acid alone or folic acid followed by COE at a dose of 5mg/kg suggesting the presence of renal injury. These findings are in accordance with another study that demonstrated that a single dose of 250mg/kg of folic acid in rats had a toxic effect on the kidneys such as widening of Bowman's urinary space and dilatation of the tubules which indicate the occurrence of tubular necrosis (Mahmoud et al., 2014). Also, the persistent inflammation triggers the activation of wound healing process but if it is not

eliminated rapidly abnormal wound healing and scarring occur indicating the occurrence of fibrosis (Delgado-Valero et al., 2021). Moreover, our results showed that treatment with higher doses of COE (10 and 20mg/kg) caused improvement in renal structure whereby there were no significant findings of damage in renal tissues. COE given as prevention in a dose of 20mg/kg was able to resolve interstitial inflammation completely, but tubular distention and vascular dilatation were still present in half of the samples while the other half showed complete recovery of renal structure. Hence, these findings suggest that treatment with COE at a dose of 10 and 20mg/kg can be a promising agent for the reversal of structural renal damage in nephrotoxicity. Whereas the dose 20mg/kg of COE given as prevention needs further research in order to better explore and understand its effects. The resolution of inflammation in the groups receiving cannabis oil extract could be explained by the fact that its components including CBD, β-myrcene, cannabinol (CBN), and limonene possess anti-inflammatory properties (Shebaby et al., 2021).

The beneficial effect of cannabis oil extract on renal structure and function is not well understood yet. The modulation of cannabinoid receptors, more specifically CB1 and CB2 receptors play an important role in renal disease models (Hryciw & McAinch, 2016). In fact, CBD is considered as a CB1 antagonist and as a negative allosteric modulator at CB2 receptors (Levinsohn & Hill, 2020). On the other hand, THC acts a partial agonist at the CB1 and CB2 receptors (Pertwee, 2008). Previous studies have shown that the activation and upregulation of CB1 receptors contribute to the development and worsening of kidney disease (Lecru et al., 2015). Whereas CB2 agonists reduce inflammation and improve kidney disease and fibrosis (Zhou et al., 2018). Moreover, other studies have shown that the blockage of CB1 receptors or the activation of CB2 receptors protected against tubular

damage by decreasing renal inflammation and oxidative stress (Mukhopadhyay et al. 2016; Mukhopadhyay et al, 2010). Hence, in our case, the antagonism of CB1 receptors and the activation of CB2 receptors could explain the improvement of renal function and structure following Lebanese cannabis oil extract administration.

In addition, the current study investigated the relationship between renal damage and cardiac damage. Cardiorenal syndrome (CRS) is defined as disorders of the heart and kidneys wherein acute or chronic dysfunction in one organ may induce acute or chronic dysfunction of the other. CRS type 4 denotes cardiovascular damage in CKD patients at any stage. In CKD, volume overload and pressure cause left ventricular hypertrophy that is accompanied by histological alterations and fibrosis. The structural modifications further lead to diastolic dysfunction and oxygen demand is increased (Delgado-Valero et al., 2021). To the best of our knowledge, a lot of studies investigated the effect of acute kidney injury on cardiac function. For instance, a study showed alterations in cardiac function and structure following folic acid induced acute kidney injury (Nikolic et al., 2020). However, few studies have explored the effects of CKD and renal fibrosis induced by nephrotoxic agents on the heart. This study revealed that high doses of folic acid either given alone to induce renal fibrosis or followed by 5mg/kg of COE led to damage in cardiac structure represented as chronic inflammation. This study is the experimental confirmation of cardiorenal syndrome type 4. The administration of 10mg/kg of COE improved the cardiac structure in all the samples. These findings are novel and promising however, they need further studies in order to better understand the effect on cardiac function, the mechanism of cardiotoxicity, and the mechanism of action of cannabis oil extract on the heart.

CHAPTER FIVE

CONCLUSION

The findings of this study demonstrate that COE at doses of 5 and 10 mg/kg was able to significantly reduce serum creatinine. Kidney and cardiac structures were both improved after administration of COE at doses of 10 and 20 mg/kg. The effect of cannabis oil extract on renal and cardiac structure was dose dependent. This study suggests that the optimal dose of COE that was able to improve both serum creatinine, kidney and cardiac structure is 10 mg/kg. Presumably, this is the first study to investigate the role of COE in the management of renal fibrosis. Cannabis oil extract is a promising preventive and treatment agent against folic acid induced nephrotoxicity. However, future studies are needed for the better understanding of the side effect profile, the mechanisms of action, and signaling pathways involved in the nephroprotective effect of cannabis oil extract.

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