Attenuation of carbon tetrachloride induced nephrotoxicity in rats by *Indigofera Cordifolia*

By

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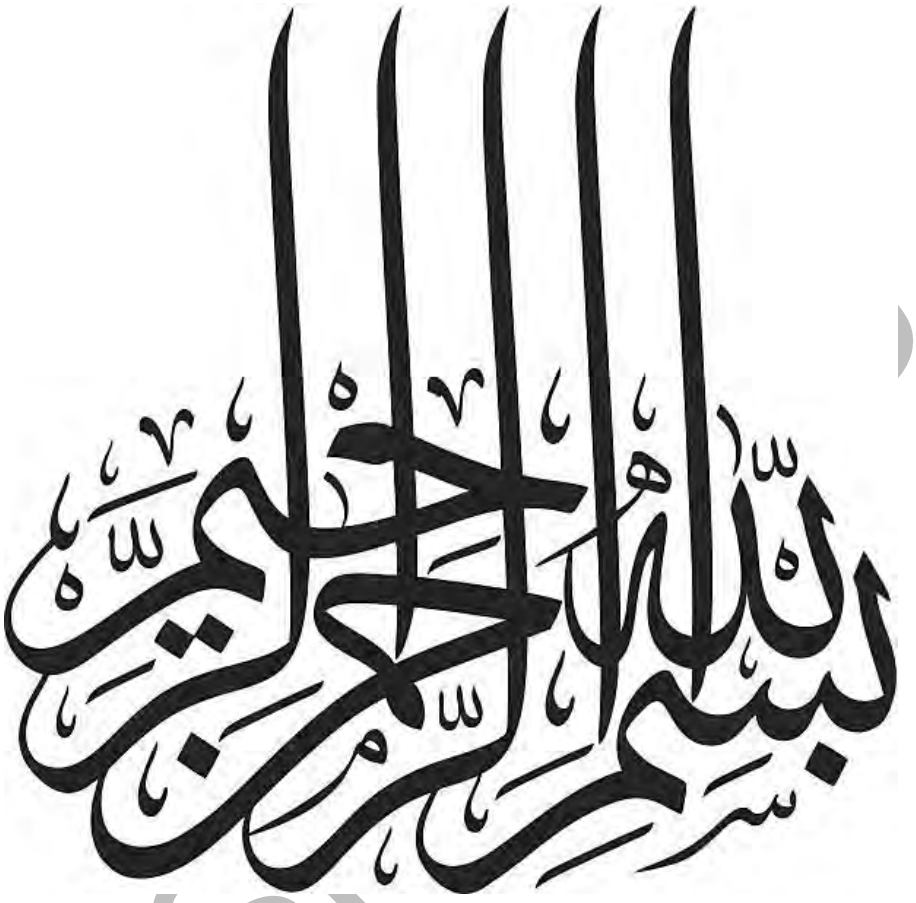

DECLARATION

I **Zunaira Tehseen** hereby declare that this dissertation titled **"Attenuation of carbon tetrachloride-induced nephrotoxicity in rats by *Indigofera Cordifolia*"** is not plagiarized and all work was carried out in accordance with the regulations set by Quaid-I-Azam University Islamabad. No component of this thesis has previously been presented for any other degree. If found anything contrary at any time, I shall be held responsible.

Zunaira Tehseen

Dedication

This study is wholeheartedly dedicated to my beloved parents, who have been my source of inspiration and gave me strength when I thought of giving up, who continually provide their moral, spiritual, emotional, and financial support.

Acknowledgment

All glories are to **Almighty Allah**, the most beneficent, the most Merciful, WHO bestowed me with the potential and ability to complete my research work. All respects to the **Holy Prophet (SAW)**, source of knowledge and blessings for entire creation, who has guided his Ummah to seek knowledge from cradle to grave and enabled me to win honor of life.

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Zunaira Tehseen

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List of Abbreviations

OH	Hydroxyl
µg	Microgram
µl	Microliter
µM	Micro molars
A	Alpha
B	Beta
AKI	Acute kidney injury
AOP	Antioxidant potential
CAT	Catalase
CCl_4	Carbon tetrachloride
cDNA	Complementary DNA
CKD	Chronic kidney disease
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenylpicrylhydrazyl
EDTA	Ethylenediamine tetraacetic acid
ER	Endoplasmic reticulum
GFR	Glomerular filtration rate
GPA	Granulomatosis with polyangiitis
GSH	Glutathione
GSSG	Glutathione disulfide
H_2SO_4	Sulphuric acid
HCL	Hydrochloric acid
$HgCl_2$	Mercuric chloride
HOBR	Hypobromous acid
HOCL	Hypochlorous acid
IC	Indigofera cordifolia
IL	Inter-leukin
Mg	Mili-gram
Ml	Milli-liter

NADPH	Nicotinamide Adenine Dinucleotide phosphate
NaOH	Sodium hydroxide
NO	Nitric oxide
NSAID	Non-steroidal anti-inflammatory drug
pH	Potential of Hydrogen
PI3K	Phosphoinositide 3Kinase
POD	Peroxidase
PPAR	Peroxisome proliferator-activated receptors
Ppt	Precipitates
PUFA'S	Polyunsaturated fatty acids
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Reverse Transcription polymerase chain reaction
SD	Standard deviation
SOD	Superoxide dismutase
TBAR'S	Thiobarbituric acid reactants
TFC	Total flavonoid content
TLR's	Toll-like receptors
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
TPC	Total phenolic content
WHO	World Health Organization
Wnt.	Wingless

ABSTRACT

Plants have been used to treat a variety of illnesses since ancient times because of their therapeutic and medicinal capabilities. Secondary metabolites such as alkaloids, flavonoids, phenolic compounds, terpenoids, and others, are thought to be the cause of their pharmacological potential. Both qualitative and quantitative investigation of the methanolic extract of *Indigofera cordifolia* was performed *in-vitro* and *in-vivo*. The presence of important phytochemicals such as flavonoids, coumarins, alkaloids, saponins, tannins, quinones, glycosides, and many others was observed through qualitative analysis of the samples. These findings were then supported by *in-vitro* antioxidant assays, which showed the ICM to be an effective root of antioxidant and anti-inflammatory agents. For *in-vivo* investigation, rats with CCl_4 induced toxicity get an oral dose of methanolic extract. The impact of the dose on rat models was then investigated at three different levels, depending on the dose administered: biochemically, histologically, and molecularly. In tissue homogenate, the amount of antioxidant enzymes, protein content, and oxidative species were evaluated. Serum biomarkers for renal functionality were also assessed. To examine morphological changes, the tissue sections of the kidney were examined under a light microscope. The expression of selected genes (XBPu, XBPt, XBPs, IL-6, Bcl2, TNF-a, TGF-β , Chop, Casp-3, B-actin) crucial in preserving the physiological and structural integrity of nephrons was then investigated. By enhancing renal functionality, as seen by upsurge in albumin levels and a decline in urea and creatinine levels in serum, ICM demonstrates its nephroprotective action, and it's also efficient in restoring the biological system's antioxidative status by balancing the levels of antioxidant enzymes (CAT, SOD, POD, and GSH reductase) respectively, protein content, and oxidative species (TBARs, nitrite, and hydrogen peroxide). Additionally, it reversed the levels of expression of afore mentioned genes that had been changed by CCL_4-induced toxicity and oxidative stress. Following study, it was shown that ICM is one of the sources of antioxidants and has nephroprotective qualities against both acute and chronic kidney disorders.

Keywords: CCl_4, Nephrotoxicity, *Indigofera Cordifolia*, Methanolic extract, Nephroprotection

Introduction

Plant based medicines are used throughout the world for the cure of various ailments. From the beginning of human civilization herbs are used for different pathogenic and infectious disorders. For thousands of years, plants have been at the heart of many traditional medicine systems around the world, and they continue to give humans with novel treatments (Nwonu *et al*., 2019). There are around 4.2 million plant species on the planet, but there is a paucity of information on their medicinal properties, which must be explored. Some plant species have been examined pharmacologically to some degree. For therapeutic and pharmaceutical uses, around 35,000 species are researched (Khan *et al*., 2015). According to the WHO, herbal medicines are used as basic healthcare by 4 to 4.7 billion people around the world, while traditional medicines developed from plant extract are known as "modern herbal medicines." (Pan *et al*., 2013). Plants' pharmacological action is attributed to the presence of secondary metabolites that contain bioactive molecules that defend against infections and are used to treat diseases. Many Secondary Metabolites have multitarget actions, which explains why complex extracts from medicinal plants are used to treat more health problems with multiple targets. Herbal therapy is a pragmatic medication, not a placebo, and scientific trials have demonstrated efficacy for numerous of them (M. Wink, 2015) because it is socially acceptable, compatible with human biology, and has no negative side effects, herbal medicine remains the primary health care choice for around 85% of the world's population, particularly in underdeveloped and developing countries (Sam, 2019).

1.1. Importance of medicinal plants

Traditional medicinal plant use has been a long-standing practice among human groups, with traditional knowledge being passed down from generation to generation and community to community (Pieroni and Quave., 2005: Samy and Ignacimuthu, 2000 Vitalini *et al*., 2013). Therapeutic potential of plants is because of the presence of secondary metabolites. The pharmacological action of secondary metabolites varies. Terpenoids, phenolics, flavonoids, alkaloids, and glycosides are some of the most important secondary metabolites, and they serve as a source of single bioactive components in nutraceuticals and modern pharmaceuticals. Secondary metabolites

have excellent antioxidant properties and can be employed in nutraceuticals as a natural source of antioxidants (Velu *et al*., 2018).

Phytochemicals have ring structures, substitutions, and functional groups that work together to protect the body from disease. Anti-inflammatory, antioxidant, antiallergic, antithrombotic, antidiabetic, anticarcinogenic, hepatoprotective, and antiviral properties are all known. These are also renowned for their ability to scavenge free radicals and chelate metals. Free radicals are produced in a variety of metabolic pathways and during aerobic respiration (Barry & Gutteridge, 1990). Due to excessive concentrations of free radicals and reactive oxygen species (ROS), free radicals react with biomolecules and cause oxidative damage to cells, resulting in oxidative stress. Strong antioxidant activity in medicinal plants scavenges ROS or free radicals, protecting cells from oxidative stress and strengthening the biological system with minimal negative effects (Krishnaiah, Sarbatly & Nithyanandam, 2011).

1.2. Fabaceae

Leguminosea, Legumes, which are plant fruits, are members of the plant family Fabaceae, and the plant is known as a bean or pea plant. The third-largest family of flowering plants is called Leguminosae, with 18,500 species divided into 750 genera (Ahmad *et al*., 2016). Astragalus (over 2,500 species), Acacia (over 960 species), Indigofera (about 710 species), Crotalaria (approximately 780 species), and Mimosa (approximately 510 species) are the largest genera, accounting for 9.6 percent among all flowering species of the plant (Magallon *et al*., 2001). These are Prostrate annual trees, shrubs, vines, and herbs with branching, pubescent stems. Compound leaves and the production of legume-like fruits distinguish members of the family, which are found all over the world (Fernandes *et al*., 2014). Fabaceae contains many Phyto-constituents with broad pharmacological actions, including antioxidant, anticancer, anti-asthmatic, antimicrobial, antiviral, antipyretic, cardiotonic, anti-inflammatory, and in the medication of various skin diseases like eczema according to several phytochemical studies. They are also used as food, in cosmetics and textile industries (Krishna *et al*., 2012).

1.3. *Indigofera*

Indigofera is the third-largest Fabaceae genus, with about 750 species (Gerometta *et al*., 2020). They can be found in all the world's tropical and subtropical regions (Su *et al*., 2018). Most of them have pinnate leaves. Red-flowered racemes occur in the leaf axils, although there are a few white- and yellow-flowered species as well (Tan *et al*., 2020). *Indigofera* species have been reported in Pakistan's mountainous areas, including the Northwest Frontier Province, Azad Jammu and Kashmir, and the Northern Areas of Dir, at elevations ranging from 1500 to 3000 meters (Shinwari *et al*., 2006). The phytoconstituents found in the root, stem, bark, leaves, fruit, flower, and seeds are responsible for their biological activities and are responsible for their therapeutic characteristics (Rahman *et al*., 2018).

1.3.1. *Indigofera cordifolia*

Indigofera cordifolia is a member of the Fabaceae family (Papilionaceae). 'Heart leaf indigo' is a frequent name for it. It is a wild herb with nitrogen-fixing bacterial nodules in the lateral roots of most plants. It's a low-growing annual that grows prostrate on the ground and have hairy leaves. It has a dark green color and clumps of little pink blooms that are used to make indigo dye commercially. The leaves are simple, small, green in color, phyllotaxy opposite decussate, form lanceolate to ovate, leaf base symmetrical, smooth appearance, veinationreticulate, marginentire, odour is distinctive, and flavour is caustic. 1-1.7 cm in length and 1.2.5 cm in breadth is the average leaf size (Pande *et al*., 2017).

1.3.2. Agroecosystem of *Indigofera cordifolia*

It is native to Afghanistan, Assam, China Southeast, Socotra, Egypt, Ethiopia, Gulf States, Myanmar, India, Mali, Mauritania, Niger, Oman, Pakistan, Sudan, West Himalaya. It thrives in both sunny and partly shady locations with well-drained soil. It forms mat-like structure (El-Ghani *et al*., 2017).

1.3.3. Ethnopharmacology of *I. cordifolia*

Throughout the world, *Indigofera* species are frequently used in traditional medicine to treat a variety of diseases. In order to evaluate the pharmacological activities and chemical makeup of these species, numerous investigations have been carried out

based on their therapeutic qualities (Gerometta *et al.,* 2020). 60 chemicals have so far been isolated from different *Indigofera* species. Terpenoids, flavonoids, and chemicals containing nitro groups make up the majority of the chemical components, along with steroids and other substances. The genus *Indigofera's* metabolites and crude extracts have been reported to have a variety of bioactivities, including phytotoxic, hepatotoxic, and cytotoxic effects (Rahman *et al.,* 2018). The methanolic extract of the plant has antioxidant as well as anti-inflammatory activities.

1.3.4. Taxonomical Categorization

According to the Global Taxonomic Information System, *I. cordifolia* has the following taxonomic grade:

Table 1.1 Taxonomic classification of *I. cordifolia*

Kingdom	**Plantae**
Subkingdom	Viridaeplantae (green plants)
Infrakingdom	Streptophyta (land plants)
Super division	Embryophyta
Division	Tracheophyta (vascular plants)
Subdivision	Spermatophytina (seed plants)
Infra division	Angiospermae
Class	Magnoliopsida
Order	Fabales
Superorder	Rosanae
Family	Fabaceae
Subfamily	Faboideae
Genus	*Indigofera*
Specie	*Indigofera cordifolia Roth*
English name	Heart-leaf indigo

1.3.5. Synonyms

- *Anil cordifolia Kuntze*
- *Heylandia cordifolia*

Figure 1.1. *Indigofera cordifolia Roth*

Figure 1.2. *Indigofera Cordifolia Roth.* Herbarium specimen submitted to Herbarium of Pakistan, QAU, Islamabad.

1.4. Phytochemicals

Phytochemicals are chemical substances found naturally in plants, such as beta-carotene. The phrase is typically used to describe substances that could have a negative impact on health but have not yet been proven to be important nutrients. The government and scientific community both strongly encourage diets high in fruits and vegetables, but there is little proof that specific phytochemicals are responsible for the health advantages. There is scant evidence that certain phytochemicals are responsible for the health benefits. Since ancient times, phytochemicals have been employed as medicines (Tyagi *et al*., 2010). The majority of phytochemicals are antioxidants which shields our cells from oxidative damage and lowers the possibility of getting some cancers. They lessen osteoporosis and menopausal symptoms. Allicin, a phytochemical found in garlic, has antibacterial effects. Phytochemicals are endogenous compounds that give plants the ability to resist intermittent or persistent environmental stressors while also regulating vital growth and reproductive processes (Molyneux *et al*., 2007). While each of these functions normally benefits the generating species, the biological activity of these elements often has severe negative effects on other creatures that encounter them. However, when the process of bioactivity can be identified, such effects may be the key indicator of desired qualities, such as therapeutic potential (De Silva *et al*., 2017). Plant secondary metabolites have long piqued man's curiosity because of their medicinal importance. Higher plants are known to manufacture biologically active secondary metabolites with a variety of structural variations that have demonstrated diverse medicinal potency as well as anti - carcinogenic characteristics. Even though it is unclear what exactly they do in the plant, they are crucial to humanity (Brielmann *et al*., 2006).

Customers who are concerned about their health today are getting more and more interested in plant-based antibacterial and antioxidant compounds as a substitute for synthetic preservatives as green consumerism expands. Plants are the best source of phytochemicals, which are chemical compounds with potent antibacterial and antioxidant properties. Examples of phytochemicals include polyphenols, alkaloids, glycosides, and flavonoids (Prakash *et al*., 2020). Since classical times, phytochemicals have been employed as medicines. Hippocrates, for instance, might

have recommended willow tree leaves to treat fever. Salicin, a substance with anti-inflammatory and analgesic qualities, was first isolated from the white willow tree's bark before being synthesized and becoming the common over-the-counter medication known as aspirin. Some phytochemicals with health consequences might not even be complex organic compounds at all but rather simple elements. Selenium, for instance, which is present in large quantities in many vegetables, is essential for the metabolism of many hormones (thyroid) and immune system function. It serves as a cofactor and necessary component for the enzymatic synthesis of the endogenous antioxidant glutathione (Papp *et al*., 2007).

1.5. Oxidative Stress

When the balance of ROS and antioxidant scavenging are out, oxidative stress results. Reactive oxygen (RO) intermediates including SO*, H_2O_2, and OH* can degrade proteins, nucleic acids, and cell membranes, which is what causes oxidative stress. More and more evidence points to the fact that ROS accumulated impact plays a part in many disorders (Aruoma *et al*., 1998). Because hydrogen peroxide and superoxide anion are produced every time molecular oxygen chemically oxidizes electron transporters, oxidative stress is an unavoidable side effect of an aerobic lifestyle. Cardiovascular diseases including hypertension and atherosclerosis, neurological diseases like Parkinson's disease and Alzheimer's dementia, and other major illnesses have all been associated to oxidative stress (Valko *et al*., 2007). A comprehensive defense mechanism called the antioxidant system is needed by an organism to reduce these negative consequences. Catalase, glutathione peroxidase, and superoxide dismutase are examples of antioxidant enzymes that are part of this system (e.g., vitamin E, vitamin A, vitamin C, glutathione, and uric acid) (Finaud *et al*., 2006).

Biomolecules may be oxidized by increased ROS or proteins and genes may undergo structural changes which can kick off signaling cascades that can cause inflammatory disorders to start and spread. Inflammation begins because of ROS-induced activation of transcription factors and pro-inflammatory genes. Any chemical species with unpaired electrons is a free radical, according to the definition. An atom or molecule's chemical reactivity is increased by unpaired electrons. Free radicals can be directly ingested as oxidizing pollutants like ozone and nitrogen dioxide, or they can be

produced in the body because of electromagnetic radiation from the environment. Destruction may take place in several tissues if antioxidant defenses are inadequate (Betteridge, D. J. 2000). Increased ROS production can lead to lipid peroxidation, mutagenesis, and carcinogenesis by inflicting oxidative damage on biomolecules. Numerous cell-damaging mechanisms can start when ROS from the mitochondria or other intracellular or extracellular sites damage cells (Olinski *et al*., 2007).

1.5.1. Tetrachloromethane (CCl_4)

Tetrachloromethane, often known as carbon tetrachloride, is an organic molecule from the group of chlorinated hydrocarbons. It is a by-product of the manufacture of various chlorinated components and is a colorless, volatile, very poisonous, and non-flammable liquid. It can be used to clean surfaces, as a solvent, to put out fires, and as a substrate for the synthesis of fluorocarbon and other laboratory and industrial reagents (McCarty, P. L, 2016). Numerous investigations have shown that the extremely reactive tri-chloromethyl free radical and its ROO* are its metabolic products, which start the toxicity (Stoyanovsky, D. A., & Cederbaum, A. I, 1999).

1.5.2. Bioprocessing/ Bioconversion of CCl_4

The well-known hepatotoxin CCl_4 causes liver damage by generating free radicals. Acute and long-term renal damage are also brought on by CCl_4 exposure. Free radical production is induced by drug use, ionizing radiation, and oxidizing contaminants in the environment. Free radical-induced lipid peroxidation is thought to be harmful for cell membranes and has been linked to several disease conditions. Industrial solvent carbon tetrachloride (CCl_4) is a known hepatotoxin (Szymonik-Lesiuk *et al*., 2003). Numerous studies have revealed that carbon tetrachloride has other targets besides the liver, causing free radical production in tissues like the kidneys, brain, and blood (Ozturk *et al*., 2003). Additionally, it has been documented that exposure to CCl_4 causes both acute and long-term kidney damage. Numerous studies have shown that the metabolic activation of CCl_4 causes the formation of $^{\bullet}CCl_4$ and $^{\bullet}Cl$, which starts the lipid peroxidation process (Tirkey *et al*., 2005).

1.5.3. Action mechanism of CCl_4

After entering the body, carbon tetrachloride (CCl_4) is metabolized by cytochrome P450 2E1 to produce the hazardous free radical metabolites trichloromethyl radical and trichloromethyl peroxyl radical, which are utilised to cause kidney and liver damage in animals. CCl_4 causes significant liver and kidney damage and oxidative stress (Moneim *et al*., 2012). The liver and kidney are severely damaged by CCl_4. Free radical production, oxidative stress, and the inflammatory process all affect toxicity. One dose of CCl_4 results in oxidative stress and lipid peroxidation, which damage cells and allow cellular enzymes to seep into the blood. The free radicals $CCl_3^{\bullet}$ and $CCl_300^{\bullet}$ are among the metabolites of CCl_4 that cause toxicity (Burk *et al*., 1984).

Cytochrome P450 system is bioactivated by CCl_4 to produce reactive metabolic ($^{\bullet}CCl_3^-$) and ($^{\bullet}OOCCl_3^-$), which have been shown to cause acute and chronic tissue damage (Szymonik-Lesiuk *et al*., 2003). Covalent bonds between these free radicals and macromolecules like proteins, lipids, and nucleic acids can form. Polyunsaturated fatty acids (PUFA) have double allylic hydrogen bonds that can be abstracted by free radicals. Carbon tetrachloride submission results in upsurge in lipoperoxide and free peroxide radical concentrations, which are super reactive and can result in damage or necrosis (Miyazaki *et al*., 2009). Beside oxidative DNA damage induction, which includes DNA adduct formation, genetic mutations, strand breaks, and chromosomal changes (Jia *et al*., 2002). These free radicals have been shown to raise oxo8dG levels in experimental animal tissues and to decrease CYP2E1 activity (Fahmy *et al*., 2009).

1.6. Plants are potential sources of antioxidants

Numerous chronic diseases have oxidative stress as a contributor to their development. Oxidants come from a wide range of sources. The majority come from enzyme- or chemical-driven processes that result in SO*, H_2O_2, or NO. Secondary highly reactive oxygen species (ROS) and reactive nitrogen species (RNS), such as the hydroxyl radical (OH) and peroxynitrite, are created from these species ($ONOO^-$), after they have been created. ROS and RNS play a crucial part in the regulation of protein synthesis and signal transduction at the cellular level. Higher levels of ROS or RNS, however, have the potential to harm important cellular elements such membrane

lipids, structural and regulatory proteins, and DNA if left unchecked. Enzymes that change oxidants into less harmful or innocuous species and tiny molecules that act as oxidant sinks or scavengers are examples of antioxidants that balance off surplus oxidant generation (Moylan *et al*., 2007).

Natural antioxidants can be found in abundance in food and medicinal plants. The polyphenols and carotenoids are these natural antioxidants., have a wide spectrum of biological effects, including those that are anti-inflammatory, anti-atherosclerotic, and anticancer. Increased intake of exogenous antioxidants would lessen the effects of free radicals by preventing the start or spread of oxidative reactions chain, functioning as scavengers of RO*, quenchers of singlet oxygen, and reducing agents (Xu *et al*., 2017). Most exogenous antioxidants come from food and medical plants, including fruits, vegetables, cereals, mushrooms, drinks, flowers, spices, and conventional medicinal herbs (Li *et al*., 2013). Additionally, enterprises that process agricultural by-products could be significant sources of natural antioxidants (Deng *et al*., 2012). These naturally occurring antioxidants are mostly composed of polyphenols (phenolic acids, lignans, flavonoids, anthocyanins), carotenoids and vitamins (vitamins C and E) derived from plant materials. Common biological effects of these natural antioxidants include anti-inflammatory, anti-aging, antiviral, and anticancer properties. This is notably true of polyphenols and carotenoids (Manach *et al*., 2004).

In medicinal plants, there is a vast variety of naturally occurring antioxidants that vary in composition, physical and chemical capabilities, and location of action. Phenolics and flavonoids are said to be potent antioxidants that have consistently provided protection by scavenging a variety of ROS, including OH and ROO*, HClO, SO*, and ONOO in various *in vitro* cellular models (Halliwell, B. 2007). It is generally known that polyphenols have antioxidant activity in cardiovascular disorders, hepatoprotective, anticarcinogenic, antibacterial, antiviral, and anti-inflammatory properties (Serrano *et al*., 2009). Anthocyanins are the antioxidants that have been shown to prevent chemically caused cancer and to switch off genes that promote angiogenesis, inflammation, and proliferation (Karlsen *et al*., 2007). In a trial involving more than 4,000 doctors and a mean treatment period of 18 years, β - carotene, one of the carotenoids, was found to prevent cognitive decline (Artemis and

Gopalan 2003). Ascorbic acids are crucial in reducing the oxidative damage caused by photosynthesis. It also plays several other roles in protein modification and cell division. In addition to acting as an antioxidant, ascorbic acid also acts as a prooxidant. The antioxidant properties of vitamin C make it essential for the treatment and prevention of scurvy. In addition, plants contain a variety of phytochemicals that work in different ways to prevent and treat disease (Bhatt *et al*., 2013). Classification of natural antioxidants and cell responses are shown in Figures 1.2 and 1.3.

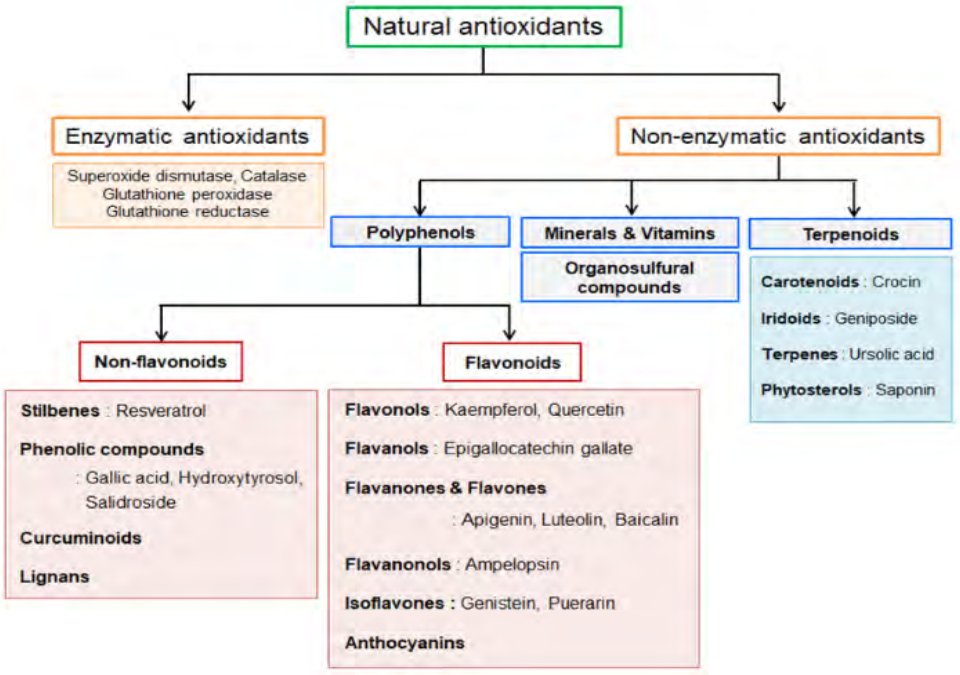


Figure 1.3. Classification of different natural antioxidants (Lee *et al*., 2021)

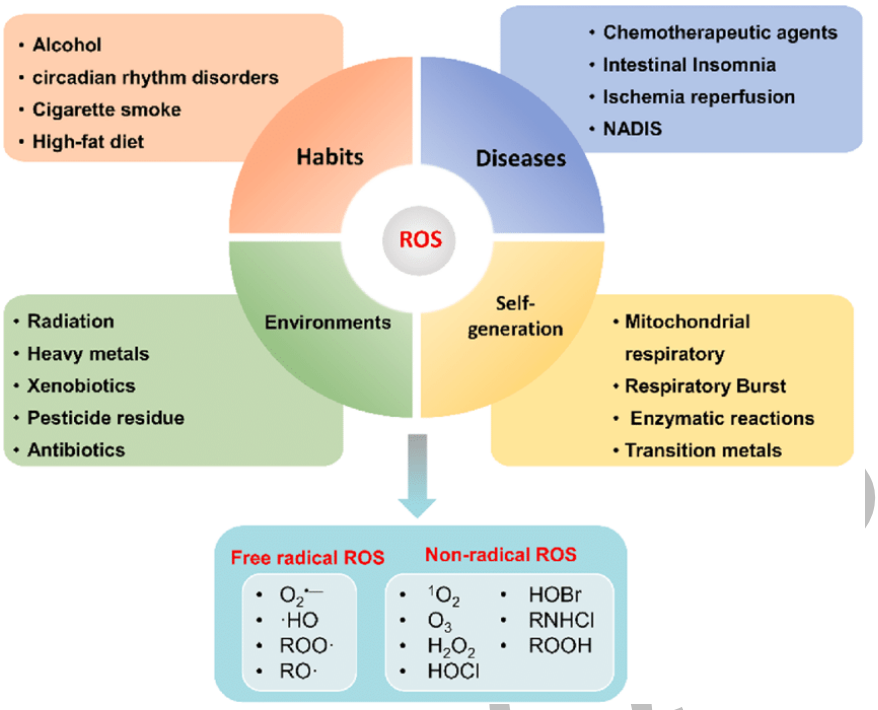


Figure 1.4. Sources and Cell responses to ROS (Wang *et al*., 2020)

1.7. Defense mechanism; Inflammation

According to definitions given by various authors, when a living tissue is harmed, inflammation is defined as "the series of changes that occur in the tissue, provided that the injury is not severe enough to quickly destroy the tissue's structure and vitality" (Sanderson, J. B, 1871). In the past, infections and the immune system have been linked to inflammation. However, more recent research indicates that a much wider range of disorders may exhibit telltale signs of inflammation. Inflammation is regarded as the foundation of pathology since the alterations it causes are a sign of both illness and injury. The primary mechanism for tissue repair following an injury is inflammation, which is made up of a series of cellular and microvascular responses that function to eliminate injured tissue and produce new tissue. The cascade comprises increased permeability in microvessels, circulating cells adhering to vessels close to the damaged area, cell migration, cell death, and the development of new tissue and blood vessels (Schmid-Schönbein, G. W, 2006). The inflammatory cascade starts at the cellular and tissue level with dilated arterioles and venules, enhanced blood vessel penetrability, and blood flow, and is commonly preceded by stasis and thrombosis, leukocyte infiltration into the tissue, plasma depart into the tissue, tissue

breakdown by proteolytic activity and oxygen free radical formation, necrosis and apoptosis, removal by phagocytic cells, and the growth of new tissue (Teder *et al*., 2002).

1.7.1 Inflammatory mediators

Leukocyte cells, commonly referred to as inflammatory cells, include macrophages, neutrophils, and lymphocytes in this intricate reaction. These cells respond to the inflammatory process by releasing specialized substances, such as eicosanoids, vasoactive amines and peptides, and eicosanoids which regulate inflammation by stopping additional tissue damage and eventually promoting healing and the return of tissue function, mediate the inflammatory process (Abdulkhaleq *et al*., 2018). As part of the system that may be involved in the case of inflammation, immune defense cells predominantly release a range of secreted mediators and other signaling molecules (such as histamine, prostaglandins and serotonin) (Anwikar, S., & Bhitre, M, 2010).

The principal mediators of the acute phase response, a complicated response of the mammalian organism to infection and injury, are the pro-inflammatory cytokines IL-1 and TNF-α. TNF- α stimulated gene 6 is one of the genes that TNF- α and IL-1 activate in different types of cells (TSG-6t). The TSG-6 cDNA encodes a secreted 35 kDa glycoprotein that can be found in high concentrations in the synovial fluids of people with different types of arthritis and in the serum of people with various inflammatory or autoimmune diseases. Two structural domains make up the TSG-6 protein: the hyaluronan-binding link module, a member of the hyaladherin family of proteins, and the C-terminal CUB domain, which can be found in a wide range of proteins (Wisniewski *et al*., 1997). IL-1 and TNF- α activate or modify many genes that code for cytokines, cellular adhesion receptors, serine- and met-alloproteinases, growth factors, and other proteins involved in host defense, inflammation, hemostasis, and tissue repair (Krönke *et al*., 1992).

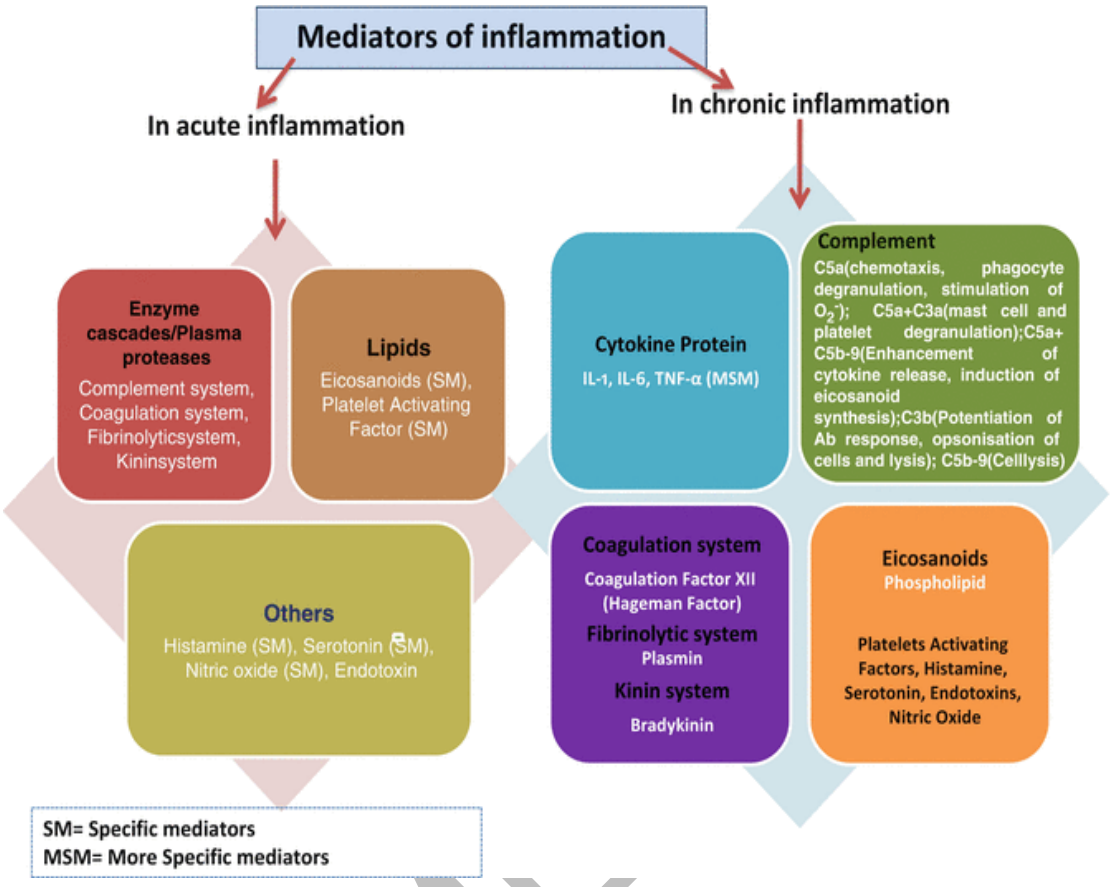


Figure 1.5. Types of inflammatory mediators in acute and chronic inflammation (Ansar & Ghosh, 2016)

Acute renal injury is still a standalone risk factor for mortality and morbidity. Today, it is thought that inflammation is a key factor in the path pathophysiology of AKI. In ischemia and nephrotoxic models, it is postulated that the first shock causes alterations in the morphology and functionality of vascular endothelial cells and tubular epithelium. The damaged kidneys are then invaded by leukocytes (neutrophils, macrophages, natural killer cells, and lymphocytes). Leukocytes are drawn into the kidneys because of the injury, which causes tubular and endothelial cells to produce inflammatory mediators such cytokines and chemokines (Akcay *et al*., 2009). For the early detection of AKI, urine biomarkers such as neutrophil gelatinase-associated lipocalin (NGAL), kidney injury molecule-1 (KIM-1), and interleukin-18 (IL-18) have been employed (Goldstein *et al*., 2008).

1.7.2. Natural therapeutics against inflammation

Non-steroidal anti-inflammatory drugs (NSAIDs) are frequently given medications, and because of the ageing population, usage is expected to rise. Although only a tiny percentage of users of NSAIDs develop negative side effects, the widespread usage of these medications has led to a significant total number of affected individuals who suffer from serious gastrointestinal issues (Lazzaroni *et al*., 2004). Although NSAIDs are mostly safe, gastrointestinal toxicity restricts their use. NSAID users frequently experience mild adverse gastrointestinal effects as dyspepsia, heartburn, nausea, or vomiting (Singh, G, 1998). New strategies to combat diseases linked to inflammation are desperately needed. Natural remedies have been used as conventional medicine for various inflammatory illnesses for ages. Herbal medications can target several aspects of the complex cellular process since they contain pleiotropic chemicals and operate according to established principles (Ustyol *et al*., 2017). Numerous phytochemicals have been found to have anti-inflammatory properties, either by directly inhibiting TNF- α or by blocking its binding and action. Most of these phytochemicals prevent TNF- α generation by preventing NF-β -mediated transcription that is controlled by MAPK or PI3K signaling. TNF- α secretion is prevented by butein and hesperetin (Leiherer *et al*., 2013).

Aims and objectives

This investigation looked at *I. cordifolia* potential to reduce CCl_4-induced nephrotoxicity.

Work plan

Phase I

- Collection, authentication, and shade drying of the *I. cordifolia* aerial parts after washing
- Creating crude extract and fractionating it to remove the active ingredients and maximize potential activity
- Evaluation of phytochemicals both qualitatively and quantitatively to support several chemical types

Phase II

- Evaluation of *in vitro* anti-inflammatory and antioxidant properties using several assays
- *In vivo* evaluation of *I. cordifolia* possible nephroprotective activity against CCl_4-induced damage in rat kidney
- Histological analysis to determine the extract's ability to protect damaged tissues
- Estimation of total proteins and kidney markers (albumin, urea, creatinine) in serum. Evaluation of the total amount of proteins in the tissue as well as the pursuit of various antioxidant enzymes (CAT, SOD, POD, and GSH), free radical species, nitrite, H_2O_2, and TBAR's in tissue homogenates

Phase III

- RNA extraction from rat's renal tissues
- Qualitative and quantitative analysis of extracted RNA
- cDNA synthesis from extracted RNA
- RT-PCR analysis to clarify the expressions of pro-apoptotic, inflammatory mediators, and anti-apoptotic markers

Literature Review

Humans have always depended on nature to provide for their necessities, including food, clothes, housing, transportation, fertilizers, tastes, and fragrances not to mention, medicines. The foundation of sophisticated traditional medical systems, which have been around for thousands of years and are still giving humankind new treatments, is plants (Gurib-Fakim, A, 2006). Numerous plants have long been valued as a primary source of effective anti-diabetics, anti-inflammatory, and antipyretic drugs. Particularly in underdeveloped nations, medicinal plants are utilised to treat illnesses to alleviate the financial burden that the expense of traditional medicines places on the populace (Sharma *et al.,* 2014). The earliest known written record of the use of medicinal herbs for medication manufacture was discovered on a Sumerian clay slab from Nagpur that is thought to be about 5000 years old. It had 12 drug production techniques referencing more than 250 different plants, some of which contained alkaloids including poppy, henbane, and mandrake (Petrovska, B. B, 2012). For their daily health care requirements, most people on this planet continue to use their indigenous materia medica (medicinal plants and other materials) (Gurib-Fakim, A. 2006).

2.1. Worldwide Importance of Medicinal Plants

Because they are seen as green medicines and are always assumed to be safe, there is a resurgence of interest in pharmaceuticals with a natural origin nowadays. The occurrences of the dangerous nature of synthetic pharmaceuticals, which are seen as adverse to humans and the environment, are another aspect that stresses this concern. Natural medicines have the benefits of being readily available, affordable, and having few or no side effects; however, this is offset by the fact that they are frequently contaminated. The likelihood of a natural medication not being available rises with its effectiveness. The natural medication is easily falsified with poor grade ingredients to satisfy the rising demand (Chanda, S, 2014). The discovery of morphine from opium by Serturner in 1803 marked the beginning of natural products chemistry (Marderosian *et al.,* 2004: Siddiqui *et al.,* 2014).

There is still interest in looking to nature for possible chemotherapeutic medicines. More than half of all medications used in clinical settings worldwide are made from

natural materials and their derivatives. No less than 27% of the total is provided by higher plants (Gurib-Fakim, A, 2006). The Papaw (Asimina spp.), the Western Yew Tree (Taxus brevifolia), effective against ovarian cancer, and the Mayapple (Podophyllum peltatum), used to combat leukemia, lymphoma, lung, and testicular cancer, were all derived from North American plants used medicinally by Native Americans. These three sources of anti-cancer drugs were only accidentally discovered in a laboratory.

2.1.1. Medicinal Plants and fate of Pakistan

Across the world, medicinal plants are crucial for the survival of underdeveloped populations. Flowers make up most medicinal plants. More than 10% of the 32000 species of higher plants (Prance, 2001) are used medicinally. By 2050, it is predicted that the global market for medicinal plants would grow to $5 trillion (US) (Zabta, 2010). Pakistan has a wide range of climatic zones and a distinctive biodiversity due to its altitude, which ranges from 0 to 8611 m. Higher plant species number roughly 6,000. 700 to 800 species are reportedly used for therapeutic purposes. Additionally, it has been calculated that 30% of all species are bi- or pluri-regional, and that 70% of all species are uni-regional. The traditional applications of the plants found in their localities are known to the local inhabitants of different parts of Pakistan for millennia. This innate understanding of plants has been passed down from one generation to the next. These plants are used to treat a wide range of illnesses, including headaches, stomachaches, and cuts and wounds (Bhardwaj and Gakhar, 2005).

Pakistan has 40,584 hakims and 455 vaids registered, and 470 Tibbi dispensaries and clinics offer the public medication. 98 dispensaries have been created under the provincial Local Bodies and Rural Development departments. Around 300 enterprises manufacture homoeopathic drugs, while between 300 and 350 companies produce herbal products. Pakistan is one of the top exporters of medicinal herbs (Hussain *et al.,* 2009). The tribal groups in northern Pakistan have a wealth of traditional knowledge about medicinal plants and their distribution. In Pakistan's north-western regions, numerous ethnobotanical studies have been carried out, and many of them have gathered data on the use of medicinal plants throughout the country (Abbasi *et*

al., 2012). In Pakistan, ethnomedicinal plant study is still being documented, and phytomedicinal investigation is a relatively new endeavor (Shinwari, 2010).

2.1.2. Involvement of Fabaceae family in therapeutics

One of the most significant families in terms of ethnopharmacology is Fabaceae. This botanical category gives rise to significant chemical elements that help cure and/or mend many physiological systems. The plants from this family stand out for their therapeutic qualities and are utilised as herbal remedies by the local traditional communities to cure a variety of illnesses. The pharmacological effects of these plants included analgesic, anti-inflammatory, anticancer, antidiabetic, anti-inflammatory, antimicrobial, and cytotoxic properties. Due to the Cerrado's high levels of biodiversity and endemism, studies of this kind have helped identify viable active principles for the creation of novel medications (Macêdo *et al.,* 2018).

Memory enhancement, abnormalities of the central nervous system, inflammation, cough, asthma, renal problems, diabetes, muscle discomfort, and microbiological infections are among the most frequent ethnomedicinal uses of Fabaceae. Numerous preclinical and clinical investigations have supported some of the benefits of plants that have been used empirically (Bora *et al.,* 2011). Family Fabaceae includes *Acacia catechu*. Bark is the component that is utilised medicinally. It has anti-inflammatory properties, hypoglycemic, antipyretic, antidiarrheal, and hepatoprotective action (Ray *et al.,* 2006). *Acacia nilotica* bark is employed in medicine. It contains antifungal, antispasmodic, anti-inflammatory, and antihypertensive properties (Pai *et al.,* 2010). *Acacia modestaWall* leaves and gums are used medically. It has analgesic, anti-platelet, anti-platelet aggregation, antibacterial, phytotoxic, and insecticidal properties (Bashir *et al.,* 2011). Members of this family that are medicinally used include *CajanusCajan, Caesalpiniapulcherrima, Pisumsativum, Delonixregia, Glycyrrhizaglabra, Desmodiumgangeticum, Sutherlandia frutescens, Tamarindusindica,* and *Cicerarietinum L*.

2.2. Oxidative stress initiators; free radicals

An essential element for life is oxygen. Free radicals are produced when cells use oxygen to produce energy, because of the mitochondria's creation of ATP (adenosine triphosphate). These by-products, which come from the cellular redox process, are

often reactive oxygen species (ROS) and reactive nitrogen species (RNS). They serve as both harmful and advantageous substances, playing a dual role. It is obvious that a crucial feature of existence is the careful balance between their two opposing impacts. ROS and RNS have positive effects on cellular reactions and immunological function at low to moderate levels. They produce oxidative stress, a detrimental process that can harm all cell structures, when present in excessive amounts. Free radicals and other non-radical reactive derivatives, generally known as oxidants, are collectively referred to by the acronyms ROS and RNS (Halliwell *et al.,* 2015: Young *et al.,* 2001).

A molecule that has one or more unpaired electrons in its outer shell is referred to as a free radical (Dröge, 2002). Free radicals are created from molecules by redox reactions, the cleavage of one radical into another, and the breaking of a chemical bond so that each fragment retains one electron (Halliwell *et al.,* 2015). $OH^{\bullet}$, $O_2^{\bullet-}$, $NO^{\bullet}$, $NO_2^{\bullet}$, $ROO^{\bullet}$, and LOO• are examples of free radicals. Additionally, although LOOH, H_2O_2, O_3, singlet oxygen ($^1O_2$), HOCl, HNO_2, $ONOO^-$, N_2O_3, and ozone (O_3) are not free radicals, they can easily result in free radical reactions in living organisms. These substances are known as oxidants (Genestra, 2007). The very unstable molecules that are biological free radicals can therefore interact with a variety of organic substrates, including lipids, proteins, and DNA (Pham-Huy *et al.,* 2008).

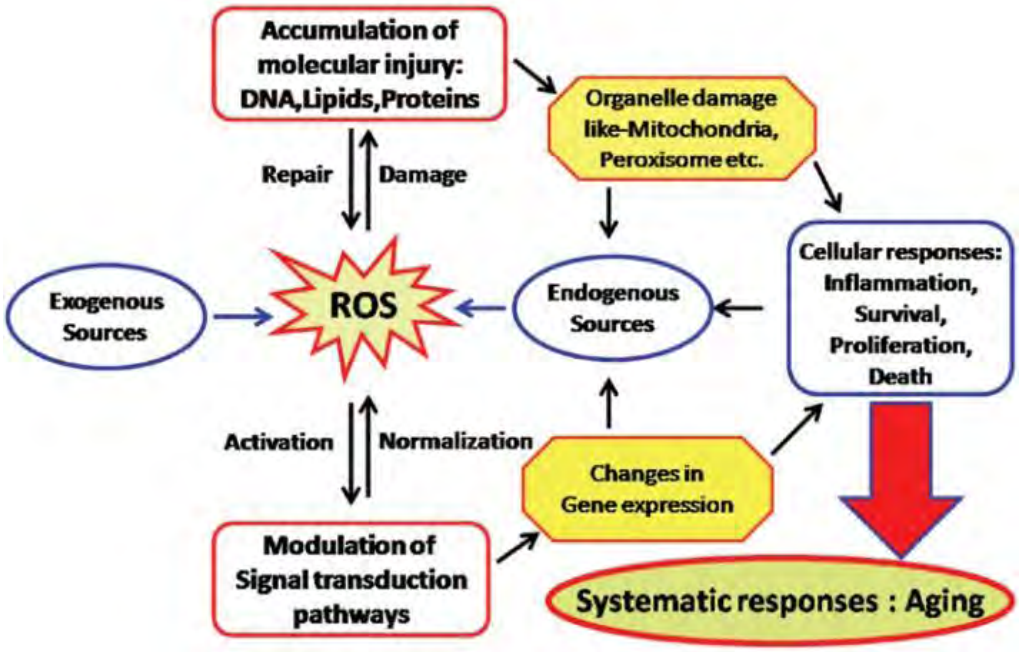


Figure 2.1. Endogenous & exogenous sources resulting in ROS production (Pandey *et al.,* 2010)

2.2.1. Antioxidants and medicinal plants

A prevalent clinical condition that is linked to higher morbidity and death is acute kidney damage (AKI). Reactive oxygen and nitrogen species (ROS) and reactive nitrogen species (RNS) have been shown to increase in oxidative stress (OS)-related AKI whereas endogenous antioxidants have been shown to decrease. Antioxidants obtained from medicinal plants can help treat AKI by lowering LPO and increasing endogenous antioxidant levels and activity. As a result, medicinal herbs are excellent providers of exogenous antioxidants, which may be thought of as crucial treatments to lessen pathogenic changes in oxidative stress-related AKI (Palipoch, 2013).

Historically, medicinal plants have been regarded as a good source for preventing different OS-related illnesses. They are made up of a range of phytochemical constituent molecules, including as carotenoids and phenolic compounds, which have antioxidant capabilities (Awah *et al.,* 2012). Tetraterponoids, or un-oxygenated carotenes, include hydrocarbon carotenoids that are either cyclized, like β -carotene, or linear, like lycopene. Tetraterponoids are classified as either molecules containing oxygen, or xanthophylls, such as lutein and β -cryptoxanthin (Mein *et al.,* 2011). Numerous chronic and degenerative diseases have a much lower risk associated with

high carotenoid intakes (Rao *et al.,* 2007). While flavonoids, tannin, and phenolic acids make up most phenolic chemicals, they are typically found in foods and medicinal plants. Anthocyanidin, anthochlors, auronus, flavonones, dihydroflavone, flavone, flavonols, and isoflavonoid, which likewise exhibit a wide range of antioxidant activities, make up the biggest category of plant phenolics known as flavonoids (Qin *et al.,* 2011).

Tocopherols, lycopenes, flavonoids, nordihydroguaiaretic acid (NDGA), sesamol, gossypol, vitamins, provitamins, and other phytochemicals, as well as enzymes (catalase, glutathione peroxidase, super oxide dismutase), minerals (zinc, selenium), and lecithin, are examples of natural antioxidant molecules that are generally regarded as being safer than synthetic (Thorat *et al.,* 2013). Antioxidants have several physiological effects in the body because they suppress oxidation even at low doses. Additionally, antioxidants serve as free radical scavengers by interacting with reactive radicals and destroying them so that they are no longer as active, hazardous, or long-lived as those that have already been neutralized. Free radicals may be neutralized by antioxidants by receiving or giving electron(s) to eliminate the radical's unpaired condition (Lü *et al.,* 2010). Antioxidants can reduce the number of free radicals in cells by either preventing the activity or expression of enzymes that produce free radicals, such as NAD(P)H oxidase and xanthine oxidase (XO), or by encouraging the activity and expression of enzymes that neutralize free radicals, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) (Shebis *et al.,* 2013: Shih *et al.,* 2007).

2.2.2. Mode of action of tetrachloromethane

Chemical tissue injury inducers like carbon tetrachloride (CCl_4) are extensively utilised in experimental systems. It is thought that the trichloromethyl radical ($^{\bullet}CCl_3$) causes transient tissue problems after the injection of CCl_4 (El-Haskoury *et al.,* 2018). The first step in the production of free radicals caused by CCl_4 is the breaking of the carbon-halogen bond, which is most likely accomplished by CCl_4's one electron reduction with the help of a specific cytochrome P-450. The main early products are chloride ion and the radical trichloromethyl, or $^{\bullet}CCl_3$. By reacting with molecular oxygen, $^{\bullet}CCl_3$ is changed into $^{\bullet}CCl_3O_2$ (Showkat *et al.,* 2010).

The interaction of $^{\bullet}CCl_3O_2$ with the polyunsaturated fatty acids (PUFA) of the membrane lipids starts the oxidation of lipids. Oxidative stress caused by lipid peroxidation results in collection of malondialdehyde (MDA). The defense system contains enzymes that can remove ROS, including superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), and glutathione peroxidase (GPx) (Halliwell *et al.,* 1992). Although the liver is thought to be the main organ affected by CCl_4 poisoning, free radicals can also be produced in other tissues, including the kidneys, heart, and blood (Preethi *et al.,* 2009).

2.3. Kidney

On one side of the spine, near rib cage, are two kidneys (bean shaped), each about the size of a fist. One of the most intricate parenchymatous organs, the human kidney has about one million functioning units called nephrons. A renal glomerulus and a draining tubule make up each nephron. The principal location of urine production is the glomerulus. It contains a three-layered filtration barrier made up of glomerular basement membrane, endothelial cells, and podocytes. A draining tubule, which is made up of several segments with specific roles, including metabolite reabsorption, receives the urine. The kidneys are prone to a variety of issues due to the numerous essential tasks they carry out and the poisons they encounter (Höhne *et al.,* 2018).

2.3.1. Effects of inflammation on kidney

Inflammation and oxidative stress cause harm to the molecular components of the kidney, which increase renal injury. Inflammation and oxidative stress can spread throughout the body and harm tissues far from the original site of injury. Inflammation is essential part of chronic kidney disease (CKD). CKD, a disorder that is progressive and chronic, raises the chance of developing concomitant conditions like type 2 diabetes (Elmarakby *et al.,* 2012) and cardiovascular disease (CVD) (Manning e al., 2005). As CKD inevitably advances, comorbidities and risk factors connected to it (such as hyperglycemia and hypertension) become more difficult to manage, leading to a decline in life expectancy concurrent with a decline in kidney function (Go *et al.,* 2004).

Free radicals interact with molecular components of nephron. The oxidation of amino acids, which results in the loss of crucial functional properties (Avery, 2011), the lipid

peroxidation of cell membranes, which results in decreased membrane viability (Halliwell *et al.,* 1993), and the cleavage and crosslinking of renal DNA, which results in harmful mutations, are just a few examples of the radical-molecule interactions that have been described in several reviews. This type of radical interaction damages the nephron right away and produces secondary radicals with the same destructive potential as the initiating radicals (Turrens, 1997), which sets off a harmful chain reaction that damages the nephron at the cellular and molecular levels and continues to produce radicals.

2.3.2. Inflammatory markers and kidney

Not only is inflammation a pathological aspect of the development of CKD, but it is also one of the classic risk factors for CKD (Miyamoto *et al.,* 2011), along with diabetes (Tsalamandris *et al.,* 2019), high blood pressure (Ku *et al.,* 2019), and dyslipidemia (Arnold *et al.,* 2021). In CKD patients, higher levels of circulating CRP, TNF-α, and IL-6 were linked to a deterioration in renal function as well as an increased risk of morbidity and mortality (Sun *et al.,* 2016).

Leptin, a proinflammatory cytokine, donates to the development of related comorbidities and the course of the disease, mostly through pro-fibrotic and hypersensitive activity. Reduced renal function has been linked to long pentraxin 3 (PTX3) (Valente *et al.,* 2019). In various cohorts of diabetic CKD patients, TNFR1 and TNFR2 were found to be elevated, prior to the development of microalbuminuria and its development to macroalbuminuria (Krolewski *et al.,* 2014). Numerous mechanisms of the inflammatory response have been

uncovered, including phosphoinositide 3-kinase (PI_3K), interleukin-6 (IL-6)/Janus kinase (JAK), and signal transducer and activator of transcription 3 (STAT3) (Yeung *et al.,* 2018). Inflammatory marker groups that are involved in kidney disease are summarized in figure 2.2.

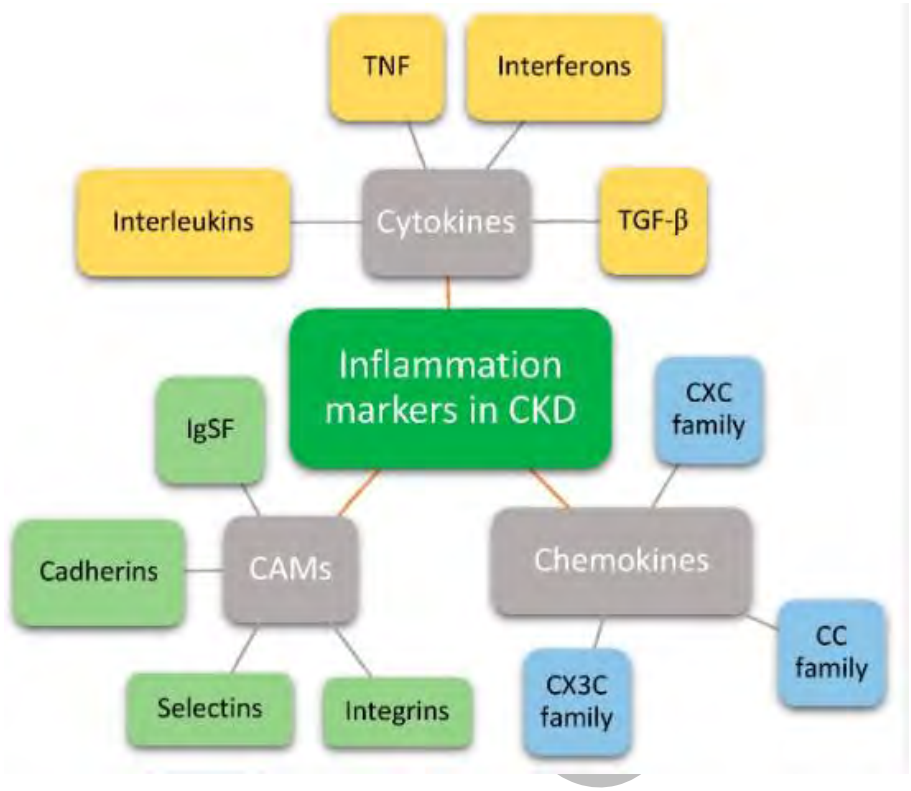


Figure 2.2. Groups of inflammatory markers involved in kidney disease (Petreski *et al.,* 2021)

2.3.3. Nephroprotection by medicinal plants

The maintenance of our endocrine system, erythropoiesis, blood pressure, acid-base balance, etc. is greatly aided by the kidneys. One of the most frequent kidney issues, nephrotoxicity happens when the body is liable to a toxin or medicine (Sundararajan *et al.,* 2014). Numerous medicinal plants showed nephroprotective effects on renal tissues against kidney damage brought on by cytotoxic medicines, chronic stress, sepsis, profenofos, CCl_4, gentamicin, and D-galactosamine (D-GalN) (Radwan *et al.,* 2017).

AKI is a temporary state marked by an abrupt decline in kidney function, a rise in blood urea nitrogen and serum creatinine, and a decreased glomerular filtration rate (GFR) (Marikanty *et al.,* 2016). Nephroprotectors are substances that can reduce the consequences of nephrotoxicity. Due to a variety of intricate chemical compounds, plants offer nephroprotective properties (Chinnappan *et al.,* 2019). The effects of

flavonoids on renal function and the underlying the process which shows that the flavonoids play a crucial role in renal physiology (Vargas *et al.,* 2018). Flavonoids, phenols, glycoproteins, sterols, glycosides, alkaloids, terpenoids, saponins, and phenyl propanoid were found to be the main phytoconstituents, according to phytochemical studies. These substances have notable antioxidant, free radical scavenging, and nephroprotective properties, which are well-documented (Huang, 2000: Nagaraj *et al.,* 2000).

2.3.4. Histopathology of kidney

Histopathology is a field of study wholly reliant on microscopic analysis and interpretation. Correct biopsy technique, appropriate fixation and processing procedures, adequate sectioning, and enough staining are fundamental conditions for arriving at a definitive diagnosis. Finding structural and morphological information about tissue components is crucial for making a firm diagnosis (Chatterjee, 2014).

A very little, thin flap of tissue will be examined under a light microscope for this purpose. After being separated from the animal model, the kidney tissues must be maintained in a 10% formalin solution before being imbedded in paraffin wax to solidify the tissues. Then, using a microtome with a thickness of around 4-5 mm, these fixed tissues are cut into slices. After slicing, the paraffin wax must be eliminated by very gentle heating, cleaning, and allowing the slices to dry out. Later, staining using hematoxylin (a nuclear stain), or eosin (a cytoplasmic stain) stains is necessary.

Materials and Methods

The chemotherapeutic value of *I. cordifolia* is assessed using the *in-vivo* and *in-vitro* assays illustrated below:

3.1. Plant collection

I. cordifolia whole plant was collected from Quaid-i-Azam University Islamabad capital territory during August 2021. This plant was identified from plant taxonomist Dr. Mushtaq Ahmed, faculty of Biological Sciences QAU Islamabad, Pakistan. The Voucher sample of the plant has acquired Accession No.133365 by Herbarium of Pakistan, Quaid-I-Azam University, Islamabad.

3.2. *I. cordifolia* extract preparation

After collection and identification, the plant needed to be cleaned to get rid of any debris or dust that had stuck to it. Then, for about two weeks, it was left to dry out in the shade until completely dehydrated. Electric grinder was used to powder the plant so that it would produce fine powder. Plant powder was run through a 60-mesh topology Willy Mill to create particles of the same size. The finely powdered plant powder was next soaked in methanol for 2-3 weeks to create a methanolic extract of the plants. The isolation or extraction of phytocomponents needed this soaking stage. After two to three weeks, Whatman's filter paper No. 1 was used to filter methanolic-soaked powder to remove plant debris, which was then discarded. The resulting filtrate was permitted to go through distillation using a rotary evaporator to remove the methanol. Semisolid plant extract was produced after the methanol in the plant filtrate was distillated. The remaining methanol in this semisolid mass was placed into petri plates for further evaporation. Scratch it off the plates when it has totally lost all moisture. Consequently obtained *I. cordifolia* methanolic extract was a dried extract.

3.3. Qualitative analysis of Phytochemicals

3.3.1. Phenol

Ellagic acid test:

Few drops of 5 % glacial acetic acid were mixed to 1mL plant sample followed by addition of 5% $NaNO_2$. Muddy brown color formation confirmed presence of phenolic compound.

$FeCl_3$ test:

1mL plant sample mixed with 2mL distilled water followed by addition of a few drops of 10% $FeCl_3$ solution. Blue green color formation confirmed presence of phenols (Harborne, 1998).

3.3.2. Flavonoids

Alkaline reagent base test:

1 mL of 2N NaOH was put in 1mL of plant sample. Yellow color shift gave sign of flavonoids content in sample.

Iron chloride test:

In 1mL plant sample a few drops of $FeCl_3$ solution were added. Blackish red precipitation indicated flavonoids presence (Awoyinka *et al*., 2007).

3.3.3. Coumarins

1mL plant sample mixed with 1mL of 10% NaOH. Yellow color formation indicates presence of coumarins (Harborne, 1998).

3.3.4. Saponins

Olive oil-based emulsion test:

To check for saponins, we would create an emulsion using olive oil. To do this, 5-6 drops of olive oil would be added to 2mL of plant extract. The presence of saponins in the supplied extract was confirmed by seeing if it would produce an emulsion.

Froth formation with Dist. H_2O:

We would add 2 ml d. H_2O to an equivalent amount (2mL) of plant extract and mix it vigorously for a while to check for saponins. If there are saponins, lather-like foam forms.

3.3.5. Tannins

Alkaline reagent test:

2mL NaOH solution added to 2mL of plant sample. Color change from yellow to red show tannins content (Hagerman *et al*., 1978).

$FeCl_3$ test:

2mL of 5% iron chloride was mixed to 1mL of plant sample. Tannins are indicated by dark blue or greenish black color.

3.3.6. Anthocyanin and betacyanin

1mL of plant sample mixed with 1mL of NaOH (2N) followed by heating at 100 ˚C for 5 minutes. Bluish green color indicates anthocyanins while yellow color indicates presence of betacyanin (Mabry *et al*., 1963).

3.3.7. Alkaloids

Hager's Test:

Hager's test, which involved adding 250 mg of picric acid to 28 ml of d.H_2O, 2 ml of HCl (dropwise), and a few drops of Hager's reagent, was used to determine the presence of alkaloids. Yellow precipitates would be a sign of the presence of alkaloids (Mir *et al*., 2013).

Mayer's Test:

The Mayer's reagent (1.28g HgCl2 and 7g KI in 100ml d.H2O) and a few drops of HCl (2ml dropwise) were added to 2ml of plant extract for this test. It would be a sure sign of alkaloid presence if yellow or creamy white ppt. were to appear (Tallent *et al*., 1955).

3.3.8. Terpenoids

To 1mL of plant extract 3mL of chloroform, and a few drops of conc. H_2SO_4 were added. When terpenoids are present, the interface would take on a reddish-brown hue.

3.3.9. Glycosides Test

Concentrated H_2SO_4 test:

1mL of conc. H_2SO_4 was mixed to 1mL of plant sample and left for 2-3 minutes. Radish color precipitation indicate presence of glycosides (Mir *et al*., 2013).

Keller-Killani Test:

1000µl of glacial acetic acid mixed with equal volume of sample and then cooled. After that, 2 drops of $FeCl_3$ were put in it and followed by addition of 2mL of H_2SO_4 along wall of test tube. Glycoside presence is marked by ring formation interaction point of 2 layers (Joshi *et al.,* 2013).

3.3.10. Anthraquinone

- 1mL plant sample mixed with equal volume of benzene followed by addition of 10% ammonium solution. Appearance of red color on addition of ammonium solution indicates presence of anthraquinone.
- In 1mL plan sample few drops of 2% HCl are added. Precipitates of red color sign the occurrence of anthraquinone (Andersen *et al.,* 1991).

3.3.11. Sterols

5ml of chloroform and 2ml of sample were combined for the sterol assessment. 1mL of concentrated H_2SO_4 was then slowly applied along the test tube walls. The presence of sterol was shown by the appearance of a reddish-brown color between the two next layers (Conforti *et al.,* 2008).

3.3.12. Proteins

Xanthoproteic Test:

In this experiment, 2 ml of plant extract was treated with a few drops of concentrated HNO_3, which causes proteins to produce a yellow color (Rasmussen, 1935).

3.4. Quantitative Assays

3.4.1. Total Phenolic Content (TPC)

The total phenolic content was calculated using the spectrophotometric method. 20µL aliquot from stock solution (2 mg/ml dist. H_2O) of plant sample was used, to which 80

µL of the Folin-Ciocalteu reagent was then added and let to stand for 7 minutes. The reaction mixture was then added 80 µL of sodium carbonate (7%) and incubated for 60 min at 23˚C. A microplate reader was used to measure the absorbance at 630 nm. By using comparable operating circumstances, the calibration curve for gallic acid, used as a positive control, was measured. Gallic Acid Equivalent (mg GAE/g DE) was used to express the values as mg per gramme of dry extracts (Tawaha *et al.,* 2007).

3.4.2. Total Flavonoid Content (TFC)

Using spectrophotometry, we would measure the amount of flavonoids in plant extracts based on the development of a yellow-colored complex between flavonoids with aromatic rings that include catechol groups and aluminum chloride. First, stock solutions at a concentration of 1 mg/ml to measure the flavonoid content is prepared. We would add 30 µl each of 10% $AlCl_3$ and 1 M potassium acetate to 70 µl of plant sample followed by incubation at room temperature for half an hour. 415 nm of wavelength was used to measure absorbance. With the aid of the gallic acid standard curve, the flavonoid concentration was assessed (Pękal & Pyrzynska, 2014).

3.5. *In vitro* antioxidant potential assessment

We must investigate *I. cordifolia's* potential as an antioxidant herb. Before determining plant activity in an in-vivo study using rats as a model animal, a few invitro experiments were performed for this aim. One method of learning about a plant's activities is through an *in vitro* investigation. We prepared a stock solution (1000 g/mL) of plant extract to assess the antioxidant activity of *I. cordifolia*, and then we made four serial dilutions of each fraction with concentrations of 500 g/mL, 250 g/mL, 125 g/mL, and 62.5 g/ mL.

3.5.1. Nitric Oxide Scavenging Assay

(Bhaskar & Balakrishnan, 2009) protocol was being followed to observe the nitric oxide scavenging ability of the plant sample. Griess reagent was prepared by mixing equal volume of 1% sulphanilamide in 5% phosphoric acid and 0.1% naphthalenediamine in distilled water. 50µl of plant sample was mixed 450µl of sodium nitroprusside solution prepared in PBS having pH maintained at 7.3. Then it

was incubated for about 3 hours (180 minutes). After incubation, 500µl of freshly prepared Griess reagent was added and was rested side for 30 minutes. Gallic acid was used as a positive control during this assay. Absorbance was measured at 540nm. Nitric oxide scavenging capability was calculated by using the following formula:

$$\text{NO scavenging \%} = [(\text{Abs. of control-Abs. of sample/control})] \times 100$$

3.5.2. Metal chelating assay

(Nehir & Karakaya, 2004) protocol was adopted with slight modifications to measure the iron chelating ability of the plant fractions. 250µl of plant sample was mixed with 50µl of $FeCl_3.2H_2O$ (2.0mM) and 450µl methanol and rested at 10 minutes at room temperature. The reaction will start once 50µl of ferrozine was added to the mixture and incubated at 37°C for 15 minutes. Ethidium bromide (EDTA) was used as a standard during this assay. Absorbance was measured at 562nm. Iron chelating power was calculated by using the formula mentioned below:

$$\text{Chelating activity (\%)} = [(1 - \text{Abs. of sample/Abs. of control}) \times 100]$$

3.6. *In vitro* anti-inflammatory studies

Protein (albumin) denaturation inhibition was used to evaluate the anti-inflammatory properties of IC.

3.6.1. Bovine serum albumin assay

A stock solution containing 100 µg/ml of the standard (diclofenac sodium) and 2000 µg /ml of the plant sample was made in water. The reaction cocktail contained 100µL of sample in the Eppendorf as well as 900 µL of 1% w/v BSA produced in 10X Tris Buffer Saline with a pH adjusted to 5.8 by adding a few drops of 1N HCl. The testing mixtures were heated for the first 30 minutes at 37 °C, then for a further 25-30 minutes at 55 °C. A UV/visible spectrophotometer was used to measure the turbidity at 600 nm after the samples had been cooled (Dharmadeva *et al*., 2018). To calculate the percent protein denaturation inhibition:

$$\%\, inhibition = [(Abs.\ of\ control - Abs.\ of\ sample/control)] \times 100$$

3.6.2. Albumin denaturation inhibiting assay

Numerous inflammatory diseases are brought on by protein breakdown. Results from this assay are assessed spectrophotometrically and are based on the inhibition of protein breakdown brought on by heating. Stock solution of plant sample (1mg/mL) and 250µL egg albumin and 700 µL phosphate buffer saline (pH 6.4) was mixed and firstly incubated for 20 minutes at 37°C. Then second incubation for 5-10 minutes at 70°C followed by cooling at room temperature. Diclofenac sodium was used as standard. Turbidity was measured spectrophotometrically at 660 nm.

$$\%\ inhibition = [(Abs.\ of\ control - Abs.\ of\ sample/control)] \times 100$$

3.7. *In vivo* studies

In-vivo experiments were necessary to clarify the functional properties of the methanolic extract of *I. cordifolia*, including its antioxidant and nephroprotective effects. In this investigation, renal tissues were examined biochemically (including enzymes and biomarkers), histologically, and molecularly.

3.7.1. Study Design

30 Sprague Dawley rats were used in the *in vivo* trial, and at the start of the experiment, their weights ranged from 180 to 230. For any kind of in-vivo investigation, we had to go by all the guidelines recommended by the National Institute of Health (NIH) and the strategic plan put out by the Bioethical Committee of Quaid-i-Azam University, Islamabad. Standard living circumstances for these rats, including a temperature of 25°C, a 12-hour period of darkness and light, and proper dietary care, were provided.

3.7.1.1. Preparation of nephrotoxin

30% CCl4 was produced in olive oil, and rats received the expected volume based on their body weight (bw) through intraperitoneal injection. Carbon tetrachloride-induced friction was lessened by using olive oil.

3.7.1.2. Plant Doses

Water was used to dissolve a predetermined amount of *I. cordifolia* methanolic extract. According to the groups of relevant rats' body mass, the plant dosages (High and Low) were administered.

3.7.1.3. Preparation of reference drug

To compare the potentials of plant extract, silymarin was utilised as a reference medication. Additionally, silymarin was dissolved in water and given to rats in the appropriate manner.

3.7.2. Distribution of rats in groups

Rats were randomly divided into seven groups, each containing three rats. Before the trial began, they were maintained in a fasting state for a whole night. For four weeks on alternate days, CCl_4 was supplied intraperitoneally, whereas IC and silymarin were given orally using pigeon feeding tubes. The grouping and dosage treatment scheme are shown in Table 3.1.

Table 3.1. Distribution of rats in groups

Group number	Treatment
Group I (Control)	No treatment
Group II (CCl_4)	30% CCl_4 in olive oil.
Group III (CCl_4 + Silymarin)	30% CCl_4 + 100 mg/Kg of Silymarin
Group IV (CCl_4 + IC-high dose)	30% CCl_4 + 400 mg/Kg of IC
Group V (CCl_4 + IC-low dose)	30% CCl_4 + 200 mg/Kg of IC
Group VI (IC-High dose)	400 mg/Kg of IC
Group VII (IC-low dose)	200 mg/Kg of IC

Rats were provided their regular diet for 24 hours after the dosage was finished, with no additional treatment. The rats were then dissected to continue the experiment. The ventral side of the body was used for the dissection. Following dissection, the blood from the heart was drawn and put into EDTA containers for serum analysis. The

serum was extracted from each tube after the blood had been centrifuged at 8000 rpm for 30 mins and stored at 4 °C for serum analysis. kidneys were removed, cleaned with ice-cold saline, and kept in liquid nitrogen at -70 °C for enzymatic and molecular analysis. For histological analysis, one kidney was kept in a phosphate-buffered formalin solution.

3.7.3. Body weight and Organ weight

To determine the proportion of altering body weight, the rats were weighed at the beginning of the experiment and again at the conclusion of the plant dose. Separated kidneys were weighed after being washed in saline water during dissection.

3.8. *In Vivo* Assays

With the help of an extract from *I. cordifolia* against CCl_4-induced nephrotoxicity, some experiments were conducted to examine biochemical parameters.

- Serum analysis
- Tissue analysis

3.8.1. Serum Analysis

To evaluate kidney function, serum analysis was done by quantitatively determining serum indicators. For renal physiology, albumin, creatinine, and urea acted as biomarkers.

3.8.1.1. Albumin

A biomarker for kidney function was determined by measuring the serum albumin level. Since the kidneys excrete albumin into the urine, hypoalbuminemia will develop in cases when the kidneys are damaged. The sick state also had an impact on albumin production. The AMP diagnostic kit was used to determine the serum albumin level. The concentration of bromocresol green was determined spectrophotometrically at 625 nm using micro-lab equipment after 1mL of bromocresol green reagent was added to 10µL of serum sample. BSA served as the benchmark.

3.8.1.2. Creatinine

Creatinine is created as a byproduct of the breakdown of creatine phosphate, a muscle protein, and is either digested or excreted by the kidney. When the kidneys are not functioning properly, creatinine cannot be excreted by the kidneys and its amount in the serum is increased. The AMP diagnostic kit was used to quantify creatinine as a biomarker. 100 µL of serum sample was added to 1000µl of reagent, incubated for a predetermined amount of time—the first reading was recorded as the initial value—and after 2 minutes, the second reading was recorded as the final value at 500 nm by micro-lab equipment.

3.8.1.3. Urea

The urea cycle and the metabolic pathway for protein breakdown both resulted in the production of urea that contains nitrogen. About 85% of the urea in the body was converted to urine or eliminated by the kidney. Renal disorders would result in decreased urea clearance, which would raise the quantity of urea in the blood. We would employ AMP diagnostics to measure the serum amount of urea. 10 µL of serum sample was mixed with 800 µL of R1 and 200 µL of R2 from a kit including R1 and R2. The values were taken at 340 nm after 1 and 2 minutes using micro-lab equipment. Urea was used as the standard in this test, and distilled water served as the blank.

3.8.1.4. Total serum protein estimation

By combining 18 µL of serum samples and the reference protein (albumin) with 1000 µL of ready-to-use reagent (370 mmol/L NaOH, 10 mmol/L Na-K tartrate, 3 mmol/L K-I, and 3 mmol/L $CuSO_4$), total serum protein was calculated. The reaction mixtures were incubated at 37 °C for 15 minutes after the addition of the reagent, and the absorbance at 550 nm was measured. For blank distilled water was used. Given formula was used to calculate protein content.

$$Protein\ (mg/dl) = Abs.Sample/Abs.Standard \times n$$

Where: n is concentration of the standard expressed in mg/dl

3.8.2. Tissue Analysis

We had to carry out a number of spectrophotometry-based assays, including CAT, SOD, POD, Protein estimation, Nitrite, TBARs, H_2O_2, and GSH assay, in order to ascertain the activity of antioxidant enzymes, protein content, and oxidative species present in renal tissues under normal or diseased conditions. It was necessary to make renal tissue homogenate to conduct these experiments.

3.8.2.1. Preparation of tissue homogenate

Cut a little piece of kidney and weigh it to roughly 100 mg to prepare tissue homogenate. Then, in the presence of liquid nitrogen, this little fragment of tissue was crushed using a pestle and mortar. For additional homogenization, 1000µl of PO_4^- buffer (0.1 M) containing EDTA (0.001 M) was added to the crushed tissues. After homogenizing the tissue, the homogenate was allowed to go through centrifugation for 25 minutes at 12,000 rpm and 4°C. Later, the supernatant's top layer was removed using a pipette and kept at 4°C for subsequent tests. While the pellet was meant to be thrown away.

3.8.2.2. Catalase Assay (CAT)

The catalase enzyme is well known for its ability to neutralize hydrogen peroxide. It contributes to overcoming oxidative stress since it has the capability to reduce the oxidative species hydrogen peroxide into O_2 and H_2O. Decreased hydrogen peroxide concentration correlates with increased catalase activity because it decreases hydrogen peroxide. We produced a reaction cocktail consisting of 500µL of phosphate buffer (50 mM, pH 6.6), 80 l of H_2O_2 (5.9 mM), and 20µL of tissue homogenate for assessing the catalase activity of the tissue under observation. We were to measure the absorbance of reaction mixture spectrophotometrically at 240 nm, with a 1minute delay between measurements (Weydert *et al.,* 2010).

3.8.2.3. Superoxide Dismutase Assay (SOD)

The catalytic activity of the enzyme superoxide dismutase against superoxide free radicals is well known. The PMS-NADH system generates super oxide radicals. Based on the SOD enzyme's ability to scavenge superoxide free radicals, its activity was examined. To produce the reaction mixture for measuring SOD activity, we

would add 40µL of PMS (188 µM) and 340µL of sodium pyrophosphate buffer to 75µL of tissue homogenate. After that, it was subjected to centrifugation for 20 minutes at 2500 and 10,000 x g. Once centrifugation had been completed, 100µL of NADH was added to start the reaction. After then, set it aside for 25 minutes. The reaction was stopped by adding 500 µL of glacial acetic when the incubation period was complete. Now, at a wavelength of roughly 560nm, we were able to measure its absorbance (Weydert *et al.,* 2010).

3.8.2.4. Peroxidase Assay (POD)

We would take 40 µL of tissue homogenate, 700 µL of PO_4 buffer, 120 µL of hydrogen peroxide, and 60 µL of guaiacol, and add them to it to measure the peroxidase activity. After an interval of 1 minute, we would measure absorbance at 470 nm (Weydert *et al.,* 2010).

3.8.2.5. Nitrite Assay

We would take a tissue sample of around 100 mg, add 100 µL of 0.3 M NaOH, and 100 µL of 5% ZnSO4 to it to estimate the concentration of nitrite. Centrifuge this mixture at 6400xg for 20 minutes. 20-30µL of the supernatant were collected after centrifugation, and the particle was discarded. Its absorbance was measured at 540 nm after we added 1000µl of Griess reagent to it (Beda & Nedospasov, 2005).

3.8.2.6. Hydrogen Peroxide Assay (H_2O_2)

We had to assess the antioxidant activity in this assay against the hydrogen peroxide that was present in the tissue homogenate under evaluation. For this, a reaction mixture containing 400 µL of tissue homogenate, 200 µL of 0.28 mM phenol red, 1.7 units of HRP enzyme, 50 µL of 6.5 mM dextrose, and 150 µL of phosphate buffer was made, and it was then incubated at 37 °C for 60 min. 2 µL of NaOH was added after an hour, and it was centrifuged at 800xg for 5 min. Then, at 610 nm, we had to measure absorbance (Holt *et al.,* 1997).

3.8.2.7. Reduced Glutathione Assay (GSH)

In this test, the amount of GSH in the homogenate of test tissue was measured. When GSH and DTNB interact, colorful complexes are created and GSH is oxidized to GSSG. NADPH and the GR enzyme caused the oxidized form of GSSG to transform

back into the reduced form, GSH. The amount of reduced glutathione GSH would be lowered because of oxidative stress. In this instance, we would mix 100 µL of tissue homogenate with an equal amount of 4% sulfosalicylic acid, and then incubate the mixture at 4 °C for 60 min. After the incubation period, centrifugation was done at a speed of 1200xg for 20 min. at 4 °C. After centrifugation, 10 µL of the upper layer of the supernatant were collected, and 20 µL of 0.1M DTNB and 270 µL of 0.1M phosphate buffer were then added. Then, its absorbance at 412 nm was measured (Mistry *et al.,* 1991).

3.8.2.8. Total Protein Estimation

We weighed 80 mg of tissue, crushed, or homogenized it in phosphate buffer (0.1M), and used the results to determine the amount of total protein in the sample. Then, it was centrifuged at 12,000 rpm for 15 min at 4 °C. A 10-minute incubation period was needed following centrifugation. We removed the top 150 µL of the supernatant and added an equal proportion of FC reagent and alkaline solution. With the use of a vortex, these reagents were completely combined before being incubated once more for 30 minutes. We have to measure its absorbance at 595 nm at this point (Mistry *et al.,* 1991).

3.8.2.9. Lipid peroxidation estimation assay (TBARS)

In this experiment, 30 µL of tissue homogenate was mixed with 3 µL of 0.1 M $FeCl_3$, 30 µL of 0.1 M ascorbic acid, and 70 µL of 0.1 M phosphate buffer, and the mixture was incubated for 60 min at 37 °C on a shaking water bath. The samples were then added 100 µL of TCA (10%) and an equivalent amount of TBA (0.67%), and again they were placed in a water bath for 25 min. at boiling temperature. Place them on ice to chill after removing them from the water bath. After cooling was complete, 10 minutes of 2500xg centrifugation were performed. After that, absorbance was measured at 535 nm (Reilly *et al.,* 1999).

3.9. Histopathological Appraisal of Renal tissues

After being surgically removed from the rat, the kidney was stored in 10% formalin for histological analysis. The kidney tissues were then embedded in paraffin wax and cut into extremely thin pieces using a microtome. Then, ethanol with serially graded

concentrations was used to wash these thin sections (50%, 70%, 90%, 100%). These tissue pieces underwent thorough dehydration following washing. Following eosin and hematoxylin staining, these tissue sections were photographed on slides using a DIALUX 20EB microscope and an automated HDCE 50B system to capture the pictures (Wang *et al.,* 2014).

3.10. Molecular Assessment

Tissues preserved at -70ºC were processed to RNA extraction followed by reverse transcription-polymerase chain reaction (RT-PCR) to examine the efficiency of *I. cordifolia* against CCl_4-induced nephrotoxicity at the molecular level.

3.10.1. RNA Extraction

RNA extraction was necessary in order to examine molecular alterations in kidney tissues under diseased conditions. A triazole reagent-based approach was used for RNA extraction (Rio *et al.,* 2010).

The processes that were taken for RNA extraction are listed here

1. A 100mg-sized portion of liquid nitrogen and -40°C-preserved kidney tissue was cut out. It was put into a cold 1 ml triazole reagent that was used in a homogenizer, and the tissue was homogenized until cell lysis took place.

2. Transferring the homogenized tissue in the triazole reagent from the homogenizer to the pre-chilled Eppendorf and incubating it there for 5 minutes at 25°C followed.

3. 200µl of chloroform was added after 5 minutes to homogenize and slightly shake the mixture by turning Eppendorf upside down for 5 minutes, or around 15 times.

4. After that, it was allowed to centrifuge for 15 minutes at 4°C or 12000xg. There would be two layers after centrifugation. While the lower layer was discarded, the upper layer of supernatant was carefully separated without blending with the other layer and placed into another Eppendorf that had already been refrigerated.

5. Now, 200 µl of isopropanol was gradually added to the layer of separated supernatant, and a small amount of precipitation was seen. I left it aside unmoving for ten minutes.

6. Then, it was centrifuged in a microcentrifuge for 10 minutes at a speed of 12,000xg at a temperature of 4 °C. A clean, white-colored particle will be visible during centrifugation. Supernatant was thrown away in this instance.

7. The resulting pellet was then washed with ethanol at a 75% concentration for 5 minutes at a speed of 7500xg, and the supernatant was discarded. The particle that was created during the initial washing stage was once more exposed to washing, but this time with 100 percent ethanol, then centrifuged for 5 minutes at 7500xg.

8. Following the removal of the supernatant, the pellet was once more centrifuged for 3 minutes at 7500xg to remove any remaining ethanol. Following centrifugation, a properly flipped Eppendorf containing an RNA pellet was tapped on tissue paper.

9. The now-obtained RNA pellet was dissolved in RNAase-free ultrapure distilled water and kept at -80°C temperature for future usage.

3.10.2. RNA Quantification

The RNA was then allowed to undergo quantitative analysis using the Nanodrop technique by adding a small droplet of RNA that had been dissolved in PCR-water and weighed about 2 µl.

3.10.3. cDNA Synthesis

Making cDNA was the next stage in the RT-PCR process because RNA could not be directly used in RT-PCR. Thermo-Fisher Scientific's Revert Aid kit was used to reverse transcribe the extracted RNA into cDNA for further molecular investigation. First, a master mix was made for cDNA synthesis using the ingredients listed below. These reagents were briefly centrifuged for a few seconds and slightly defrosted before use (Meis *et al.,* 2009).

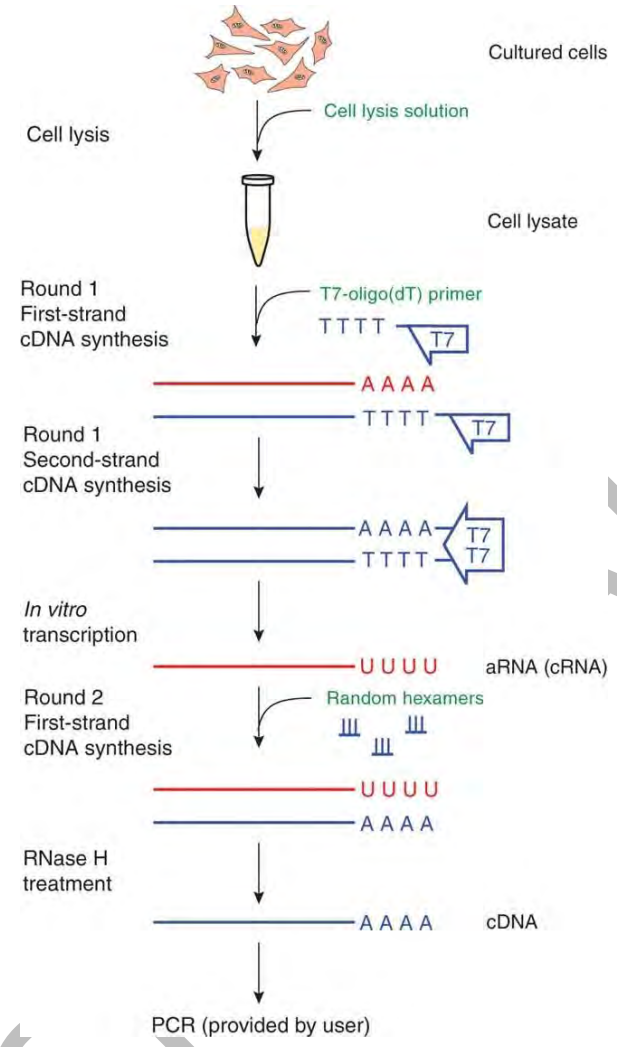


Fig. 3.1: Chemistry of cDNA synthesis

The following substances were utilised to prepare 40 µl of cDNA, as mentioned in [Table 3.2].

Table 3.2. Constituents for cDNA synthesis

Ingredient	Volume (µl)
Template RNA	1
Oligo (dT) 18 primer	0.5
RT Buffer	4
Reverse Transcriptase	1
DEPC water	11
Random Hexamer	0.5
10Mm dNTP Mix	2
Total volume	20

The entire mixture was then placed in a thermocycler for cDNA synthesis after undergoing a brief spin in the centrifuge lasting only a few seconds. After then, the created cDNA was kept for a long time at -80°C.

3.10.4. Real-time polymerase chain reaction (RT-PCR)

The cDNA created using the preceding method was utilised to clarify the changes in target gene expression brought on by nephrotoxicity. For this molecular method, My-Go RT-PCR equipment was used for the RT-PCR assessment that was necessary. The exact target gene in cDNA would be amplified. For performing RT-PCR, the reagents listed below were combined in PCR tubes according to a particular ratio before the tubes were loaded into My-Go PCR. We had to use diluted cDNA and primers rather than concentrated ones when preparing the reaction mixture. For the relative measurement of target gene expression, β -actin was employed as the reference gene.

Table 3.3: Reagents required for RT-PCR reaction

Reagents	**Volume (Per sample)**
cDNA	1 µl
Forward Primer	0.5 µl
Reverse Primer	0.5 µl
PCR water	3 µl
Syber Green Dye	5 µl
Total Volume	10 µl

The RT-PCR was carried out for β -actin, XBPu, XBPs, XBPt, TNF-α, IL-6, TGF-β, CHOP, Bcl-2, and Casp-3. The results of RT-PCR were then quantitatively compared to β -actin, which served as an internal control.

Table3.4. Primers' sequences of investigational genes

Gene		Primer sequences (5′ -3′)
XBPu	Forward	AAAGCGCTGCGGAGGAAA
	Reverse	AGCTGGAGTTTCTGGTTCTCT
XBPs	Forward	TGAGTCCGCAGCAGGTGCA
	Reverse	ACAGGGTCCAACTTGTCCAGAA
XBPt	Forward	CCCTGGTTACTGAAGAGGTC
	Reverse	GTCCAACTTGTCCAGAATGC
IL-6	Forward	GTCAACTCCATCTGCCCTTC
	Reverse	ACTGGTCTGTTGTGGGTGGT
TNF-α	Forward	GCTCCCTCTCATCAGTTCCA
	Reverse	GGTTGTCTTTGAGATCCATGC
TGF-β	Forward	TACCTGAACCCGTGTTGCTCTC
	Reverse	GTTGCTGAGGTATCGCCAGGAA
CHOP	Forward	CCTGTCCTCAGATGATGAAATTG
	Reverse	CTAGGGATGCAGGGTCAAGA
Bcl-2	Forward	TGGATGACTGAGTACCTGAACC
	Reverse	CAGCCAGGAGAAATCAAACAG
Casp-3	Forward	GGAAGCGAATCAATGGACTCTGG
	Reverse	GCATCGACATCTGTACCAGACC
β -actin	Forward	CCTCTATGCCAACACAGAGT
	Reverse	CATCGTACTCCTGCTTGCTG

The temperature profile for RT-PCR also needed to be modified in accordance with the ideal specifications stated in Table 3.5.

Table 3.5: Settings for temperature profile required for RT-PCR

Programs name		Temperature ˚C	Ramp (˚C/s)	Hold (s)
Hold		95	4	600
3-step amplification	**Denaturation**	95	5	15
	Annealing	60	4	30
	Extension	72	5	30
Pre-melt hold		95	5	10
High resolution Melting	**Initial stage**	60	4	60
	Final stage	97	0.05	15

3.11. Quantification of Gene Expression

The relative ΔCT technique was then used to determine the target genes' expression levels. The following formula was used to determine fold changes for the evaluation of gene expression:

$$\Delta CT_{(Treated)} = CT_{(Treated\ gene)} - CT_{(Internal\ control)}$$

$$\Delta CT_{(Reference)} = CT_{(Ref.\ gene)} - CT_{(Internal\ control)}$$

$$\Delta\Delta CT = \Delta CT_{(Treated)} - {}_{\Delta}CT_{(Reference)}$$

$$\text{Fold Change} = (2)^{\wedge -\Delta\Delta CT}$$

3.12. Statistical Analysis

Following *in vivo* tests, the results were statistically analyzed utilizing a variety of programmes and methods. Graph Pad Prism 5 was used to plot the graphs, and ANOVA was also used. Utilizing Statistics 8.1 software, statistics was applied to the data in order to determine the relevance of the derived data. Results were presented as Mean ± S.D. in the documentation.

RESULTS

Numerous scientific studies rely on a variety of biochemical substances with plant origins as their fundamental components. This scientific investigation involved screening for phytochemicals and pharmacological evaluation of *I. cordifolia*.

4.1. *I. cordifolia* yield

We soaked 1.85 kg of finely crushed *I. cordifolia* powder in methanol for two to three weeks to extract plant metabolites. After distillation and drying, the yield of the resulting methanolic extract was 48 g. Due to its propensity to penetrate phytocomponents, methanol was employed to soak plants in order to extract their principal and secondary metabolites. Pure methanol was substantially recovered from plant extract as a result of distillation.

4.2. Phytochemical Investigation

By using various tests designed specifically for phytochemicals, the methanolic fraction of *I. cordifolia* was qualitatively examined for a variety of phytochemicals (phenols, flavonoids, coumarins, saponins, tannins, anthocyanin and betacyanin, glycosides, alkaloids, terpenoids, anthraquinones, sterol, and proteins). Table 4.1 below provides an illustration of these phytochemicals' presence.

Table 4.1. Qualitative evaluation of *I. cordifolia*

Phytochemicals	**ICM**
Phenols	++
Flavonoids	+++
Coumarins	+
Saponins	-
Tannins	-
Anthocyanin	+
Betacyanin	+++
Alkaloids	++
Terpenoids	-
Glycosides	+++
Anthraquinones	-
Sterol	-
Proteins	+++

Phytochemicals in ICM; indicated by (+) component present, (-) component absent, (++) moderate concentration, (+++) maximum concentration

4.2.1. Quantitative assessment of Total Phenolic (TPC) and Total Flavonoid Content (TFC)

The equivalents of standards like mg of gallic acid equivalent/g of dry extract (mg GAE/g dry sample) and mg of catechin equivalent/g of dry extract (mg CE/g dry sample) were calculated via the standard regression lines of gallic acid and catechin in order to determine the TPC and TFC of *I. cordifolia* (Table 4.2).

Table 4.2. The total phenolics and total flavonoids content of *I. cordifolia*

Sample	TPC expressed as mg GAE/g of extract	TFC expressed as mg CE/g of extract
ICM	381.66 ± 8.33	120.0 ± 2.0

TPC and TFC are presented in terms of IC_{50}. Values are expressed in their means along with S.D. (Mean ± S.D.) which are significantly different based on p-value<0.05

4.3. *In-vitro* antioxidant assays

4.3.1. Nitric Oxide Scavenging Assay

I. cordifolia crude extract's half-maximal inhibitory concentration (IC_{50}) for nitric oxide scavenging potency was estimated using ascorbic acid as the reference (Table 4.3).

4.3.2. Metal chelating assay

I. cordifolia crude extract's half-maximal inhibitory concentration (IC_{50}) for iron chelating potential was estimated using Ethylenediamine tetra acetic acid (EDTA) as the reference (Table 4.3).

Fig. 4.1, 4.2 shows visually the percentage of NO scavenging and iron chelating activity that plant components scavenge at various concentrations.

Table 4.3. IC_{50} values of antioxidative activities of ICM

Sample	NO scavenging activity IC_{50} (µg/ml)	Iron chelating activity
ICM	775.78 ± 0.478	741.41 ± 0.54
Ascorbic	423.93 ± 0.699	-
EDTA	-	356.02 ± 0.91

Nitric oxide scavenging and iron chelating activity of methanolic plant fraction in terms of IC_{50} values expressed in their means along with S.D. (Mean ± S.D.) which are significantly different based on p-value<0.05

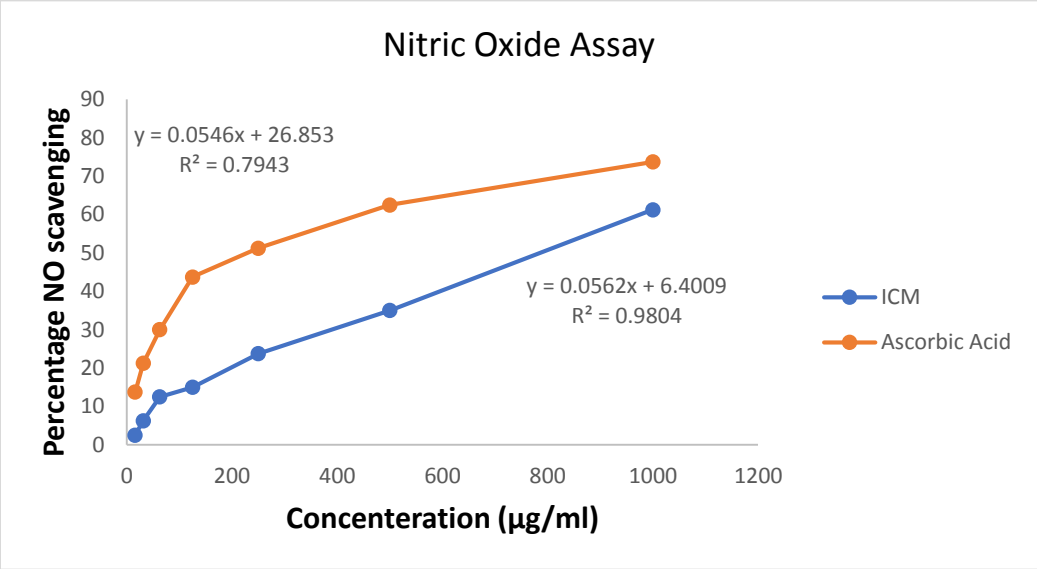


Figure 4.1. Percent Nitric oxide scavenging effect of ICM at different concentrations.

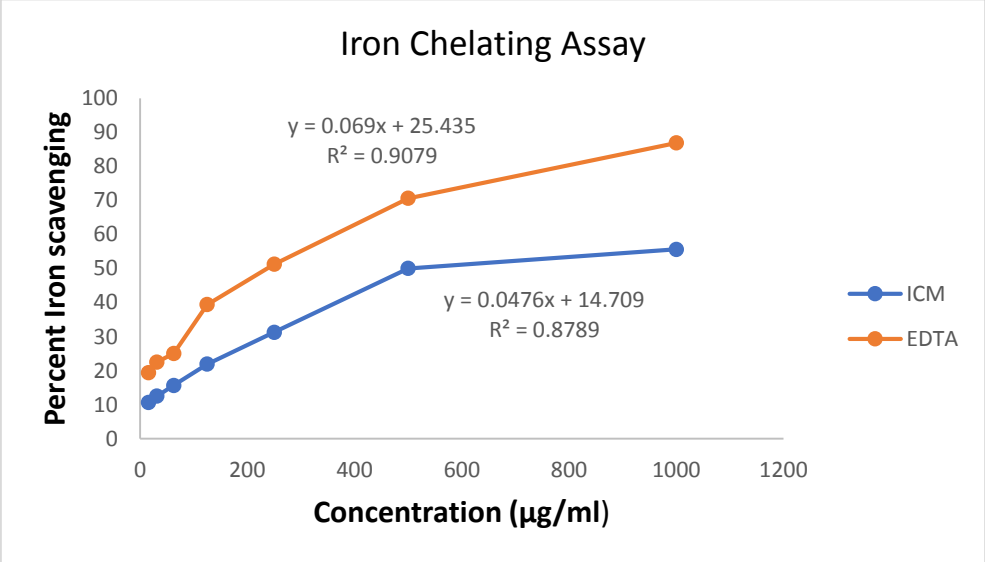


Figure 4.2. Percent Iron chelating activity of ICM at different concentrations

4.4. *In vitro* anti-inflammatory studies

4.4.1. Bovine serum albumin assay

Based on the IC_{50} value, the methanolic plant fraction was determined to be efficient against heat-induced protein denaturation (Table. 4.4). Standard anti-inflammatory drug diclofenac sodium shown a considerable suppression of BSA denaturation. Diclofenac sodium was used as standard. From the absorbance measurements made at various concentrations, Figure 4.3 showed the calculated trend for albumin denaturation inhibition.

4.4.2. Albumin denaturation inhibiting assay

Heat induced albumin denaturation was inhibited by ICM fraction at different concentrations. Diclofenac sodium was used as standard anti-inflammatory drug. Figure 4.4 exhibited the attained trend for albumin denaturation inhibition at different concentrations.

Table 4.4. IC_{50} values of anti-inflammatory activity of ICM

Sample	Bovine serum albumin IC_{50} (µg/ml)	Albumin denaturation
ICM	316.6541 ± 0.917991	428.2296 ± 0.99583
Diclofenac sodium	63.04183 ± 0.839613	242.8821 ± 0.488573

Protein denaturation inhibition activity of methanolic plant fraction in terms of IC_{50} values expressed in their means along with S.D. (Mean ± S.D.) which are significantly different based on p-value<0.05

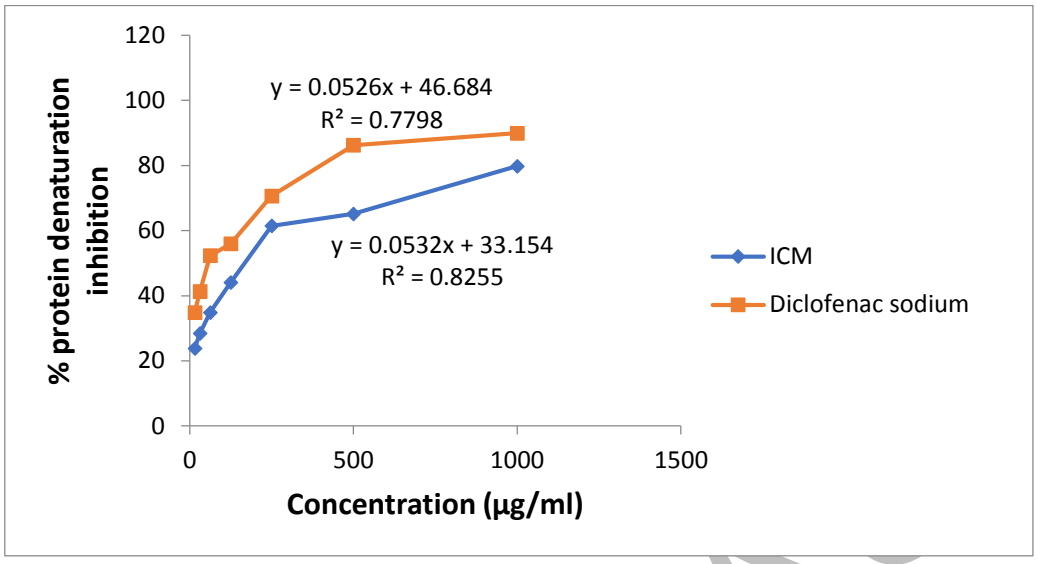


Figure 4.3. % Inhibition of protein denaturation by ICM at different concentrations

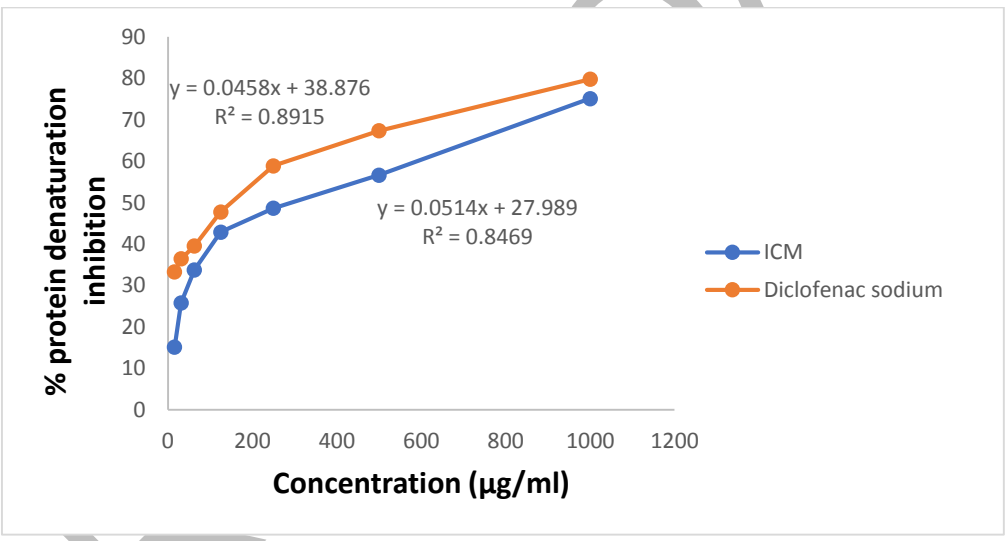


Figure 4.4. % Inhibition of albumin denaturation by ICM at different concentrations

4.5. *In vivo* Assessment

We use the methanolic fraction for further *in vivo* research after determining that the *I. cordifolia* methanolic extract (ICM) has antioxidant activity. It is necessary to quantitatively estimate renal function through the measurement of serum markers for the kidneys (Albumin, Creatinine, and Urea) as well as the activity of antioxidant enzymes (CAT, SOD, POD, Total Protein Content, and GSH) and the level of oxidative species (Nitrite, H_2O_2, and TBARs) in tissues.

4.5.1. Protective effect of ICM on body and kidney weight of rats

When compared to the untreated rat, the CCl_4-treated rat significantly reduced the percent rise in body weight while increasing the absolute and relative weight of the kidney. In a diseased state, the kidney is impacted and experiences morphological changes in addition to physiological ones. These changes include swelling of the kidney tubules and glomerulus, as well as tubule and glomerulus degradation, which reduces the organ weight. Comparing the groups co-administered with ICM to the treated group with CCl_4, it was found that there was a percentage rise in body weight and a considerable repression of the absolute and relative weight of the kidney. The administration of silymarin (100 mg/kg bw), a common nephroprotective drug, to the CCl_4-intoxicated rats caused it to regain its body and organ weight in comparison to the untreated rats. The rats administered with the high (400mg/kg bw) and low (200mg/kg bw) dosages of ICM alone did not see any appreciable difference, though. Based on a p-value of 0.05, the changes in body weight and organ weight are shown in Table 4.5 below.

Table 4.5. Results of ICM different treatments on the rats' % growth in body and organ weight

Treatment groups	Initial body weight (g)	Final body weight (g)	% Increase	Absolute kidney weight (g)	Relative kidney weight (mg/g)
Control	1.34±1.53	180 ± 1.7	25.55 ±1.36^{f}	0.93 ±0.117^{f}	5.16 ± 0.09^{cd}
CCl_4 (1ml/kg)	163 ± 1.53	213 ± 1.68	23.47 ±1.83^{g}	1.68±0.034^{b}	7.88 ± 0.49^{a}
CCl_4 + Silymarin (100mg/kg)	165 ±1.37	245 ± 1.52	32.65 ±2.48^{b}	1.33 ±0.055^{d}	5.42 ± 0.19^{cd}
ICM (400mg/kg)	132 ± 1.12	182 ± 1.62	27.47 ±1.92^{e}	0.95 ± 0.22^{f}	5.21 ± 0.05^{d}
ICM (200mg/kg)	139 ±1.47	204 ± 1.78	31.86 ±1.57^{d}	1.17 ±0.074^{e}	5.73 ± 0.31^{c}
CCl_4 + ICM (400mg/kg)	172 ± 1.09	264 ± 2.23	34.84 ±2.86^{a}	1.93 ± 0.082^{a}	7.31 ± 0.05^{b}
CCl_4 + ICM (200mg/kg)	151 ± 1.53	222 ± 1.49	31.98 ±1.41^{c}	1.64 ± 0.027^{c}	7.38 ± 0.3^{b}

The values are expressed as mean ± SD (n=7). The superscripts (a – f) above each value represent the significant difference ($p<0.05$) among means. CCl_4; tetrachloromethane, ICM; *I. Cordifolia* methanol extract.

4.5.2. Nephroprotective effect of ICM on renal serum markers and protein

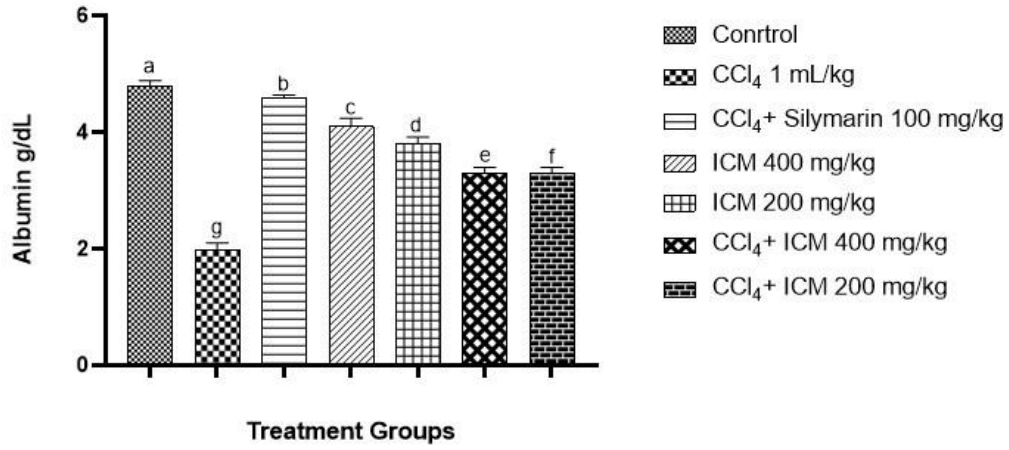
To demonstrate the protecting effect of ICM against nephrotoxicity, levels of serum markers were evaluated. Nephrotoxicity causes the kidney to lose function, making it unable to process and eliminate chemicals, which causes changes in the concentration of various metabolites and other biomolecules. Albumin protein is discharged in urine because it cannot be reabsorbed from renal tubules. Its synthesis may also be impacted, which causes hypo-albunimea, or a decrease in serum albumin levels. Protein breakdown results in the production of urea and creatinine, which are not eliminated by the kidneys and cause an increase in serum levels. Higher albumin and lower levels of urea and creatinine were observed in control groups as well as in groups with oral administration of

standard drug (Silymarin) while reverse patten (lower albumin, higher urea, and creatinine levels) was observed in CCl_4 administered groups. By changing the level of albumin, creatinine, and urea close to control rat levels, high dose (400mg/kg) of ICM has been shown to be more effective than low dose (200mg/kg) in reversing conditions of nephrotoxicity. When compared to the control rats, the rats given CCl_4 had a clearly lower protein level. In comparison to CCl_4-treated rats, the protein content was increased at ICM when CCl_4 was administered in low and high doses. The levels of albumin, urea, creatinine, and protein that differ substantially between distinct groups (n=7) based on p-value<0.05 are listed below in [Table 4.6] and are depicted visually in (Fig. 4.5 and 4.6) for each.

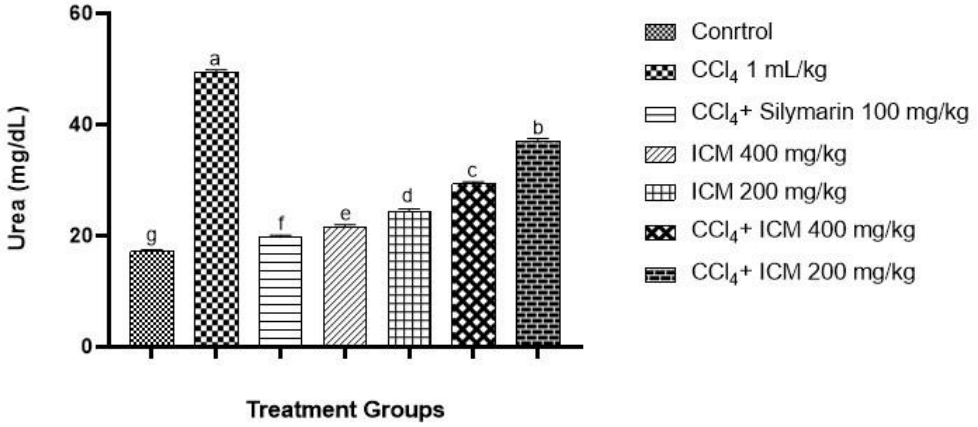
Table 4.6. Effects of ICM treatment on renal serum markers and protein

Groups	Albumin (g/dl)	Urea (mg/dl)	Creatinine (mg/dl)	Total serum protein (mg/dl)
Control	4.78 ± 0.09^{a}	17.13 ± 0.37^{g}	0.33 ± 0.07^{f}	223.47 ± 1.9^{a}
CCl_4 (1ml/kg)	1.97 ± 0.11^{f}	49.46 ± 0.38^{a}	3.02 ± 0.15^{a}	91.57 ± 0.91^{g}
CCl_4 + Sily. (100mg/kg)	4.58 ± 0.05^{b}	19.6 ± 0.43^{f}	0.57 ± 0.11^{f}	180.74 ± 0.75^{c}
ICM (400mg/kg)	4.09 ± 0.13^{c}	21.54 ± 0.40^{e}	0.89 ± 0.16^{e}	187.65 ± 1.9^{b}
ICM (200mg/kg)	3.79 ± 0.11^{d}	24.42 ± 0.42^{d}	1.22 ± 0.18^{d}	169.9 ± 1.89^{d}
CCl_4 + ICM (400mg/kg)	3.31 ± 0.09^{e}	29.29 ± 0.42^{c}	1.52 ± 0.08^{c}	154.78 ± 1.93^{e}
CCl_4 + ICM (200mg/kg)	3.30 ± 0.092^{e}	37.03 ± 0.45^{b}	2.12 ± 0.23^{b}	121.18 ± 1.81^{f}

Nephroprotective effect of ICM on serum marker and protein levels expressed as mean values with standard deviations (Mean S.D.) (n=7) raised to superscript letters as exponent indicating their significance based on p-value <0.05

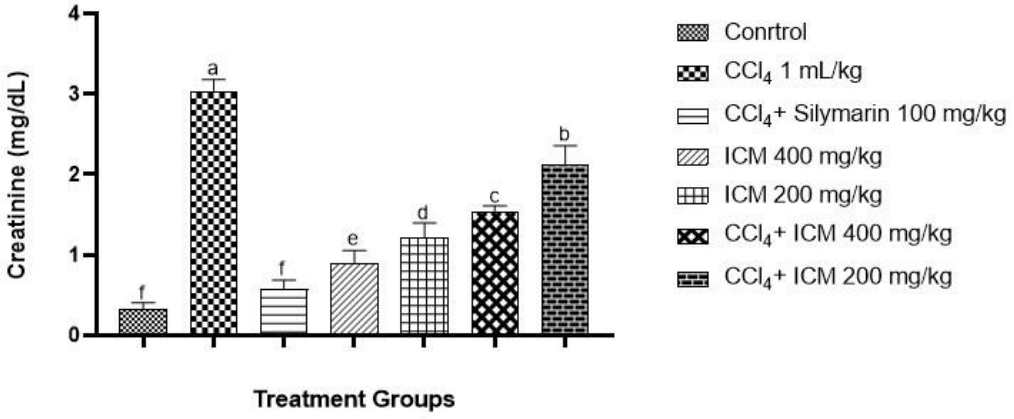


(a)

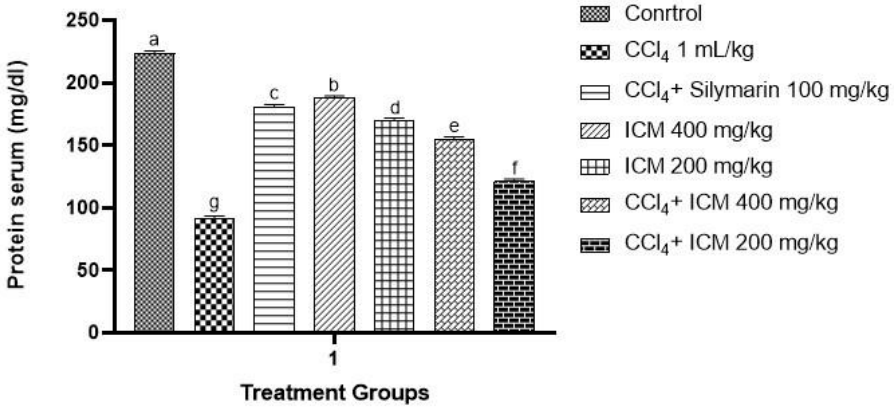


(b)

Figure 4.5. Nephroprotective effects of ICM on albumin (a) and urea (b) levels



(a)



(b)

Figure 4.6. Nephroprotective effects of ICM on creatinine (a) and protein (b) levels

4.5.3. Tissue Analysis

We assessed the quantity of antioxidant enzymes (CAT, SOD, POD, GSH) as well as oxidative free radicals (Nitrite, H_2O_2, and TBAR's) in tissue homogenate to investigate renal functionality.

4.5.3.1. Nephroprotective Effect of ICM on Antioxidant Enzymes

The antioxidant enzymes catalase, superoxide dismutase, and peroxidase are well known for their activity against the oxidative radical's hydrogen peroxide and superoxide in bolstering the antioxidant defense mechanism and reducing oxidative

stress-related kidney damage. The tissue sample from the group that received CCl_4 significantly ($p<0.05$) downregulated the levels of antioxidant enzymes. The level of these antioxidant enzymes is much higher in normal (control) rats and those given a standard medicine orally. In contrast, ICM low and high dose co-administered groups showed higher levels of these antioxidant enzymes. Based on their p-value <0.05, the activity of antioxidant enzymes (CAT, POD, and SOD) in various conditions is shown below in [Table 4.7] and is graphically illustrated in (Fig. 4.7, 4.8, and 4.9) correspondingly.

Table 4.7. Nephroprotective effect of ICM treatment on antioxidant enzymes

Groups	CAT (U/min)	SOD (U/min)	POD (U/min)
Control	19.58 ± 0.93^{a}	8.66 ± 0.14^{a}	3.87 ± 0.78^{a}
CCl_4 (1ml/kg)	5.83 ± 0.77^{e}	3.62 ± 0.03^{g}	1.28 ± 0.03^{g}
CCl_4 + Sily. (100mg/kg)	17.55 ± 0.97^{b}	7.26 ± 0.09^{b}	3.01 ± 0.08^{b}
ICM (400mg/kg)	16.06 ± 0.75^{bc}	6.59 ± 0.53^{c}	2.83 ± 0.06^{c}
ICM (200mg/kg)	14.83 ± 1.16^{c}	6.09 ± 0.29^{d}	2.67 ± 0.14^{d}
CCl_4 + ICM (400mg/kg)	12.75 ± 0.88^{d}	5.38 ± 0.065^{e}	2.38 ± 0.05^{e}
CCl_4 + ICM (200mg/kg)	11.55 ± 0.73^{d}	4.35 ± 0.05^{f}	2.13 ± 0.12^{f}

Values are expressed as Mean ± S.D (n=7) raised to superscripts designating their significance based on p-value<0.05

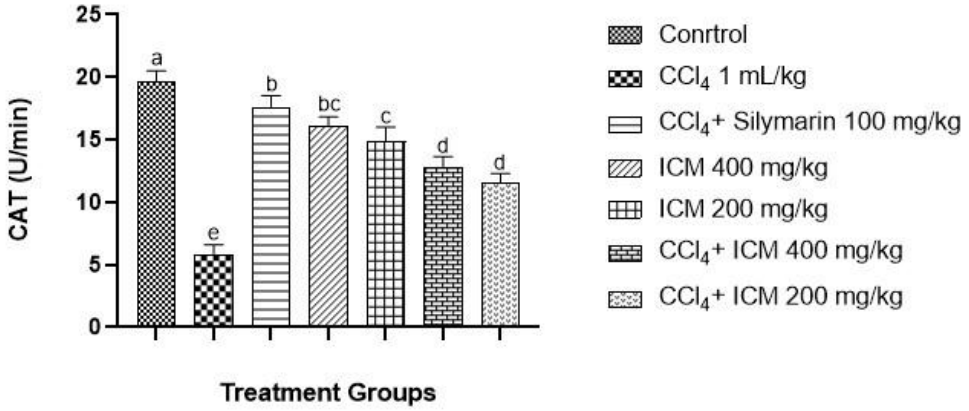


Figure 4.7. Variability in the CAT activity values across several ICM-treated groups. ICM; *I. cordifolia* methanol extract. CCl_4; carbon tetrachloride

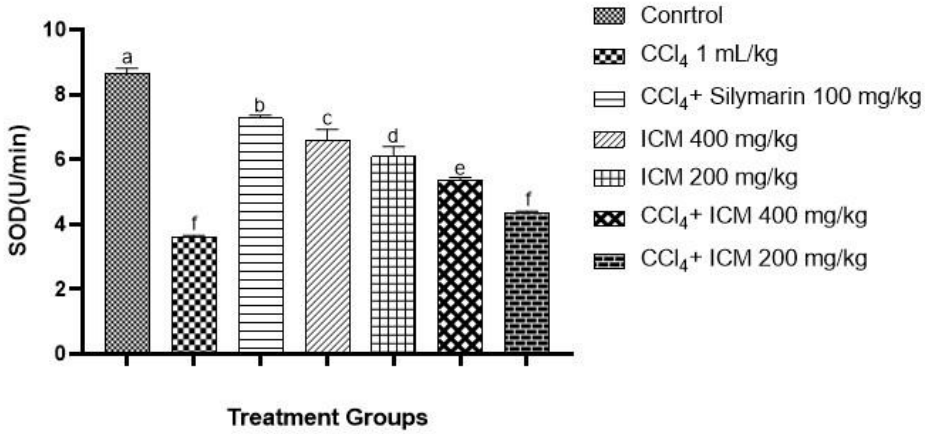


Figure 4.8. Nephroprotective effect of ICM on antioxidant enzyme superoxide dismutase

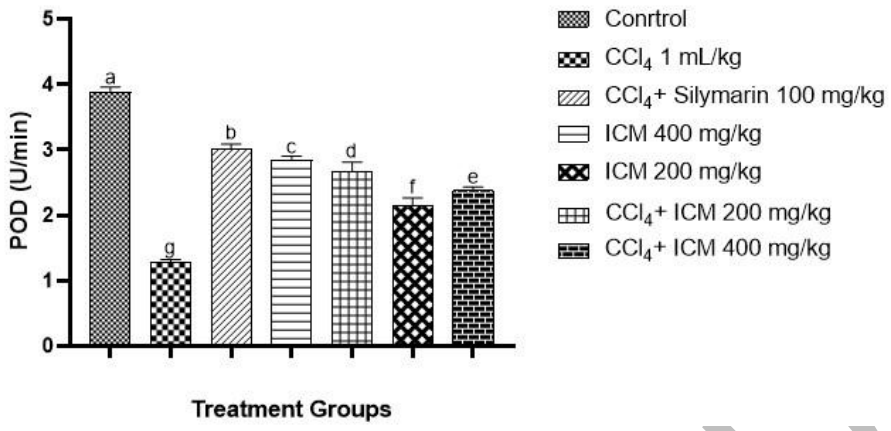


Figure 4.9. Nephroprotective effects of ICM on POD activity

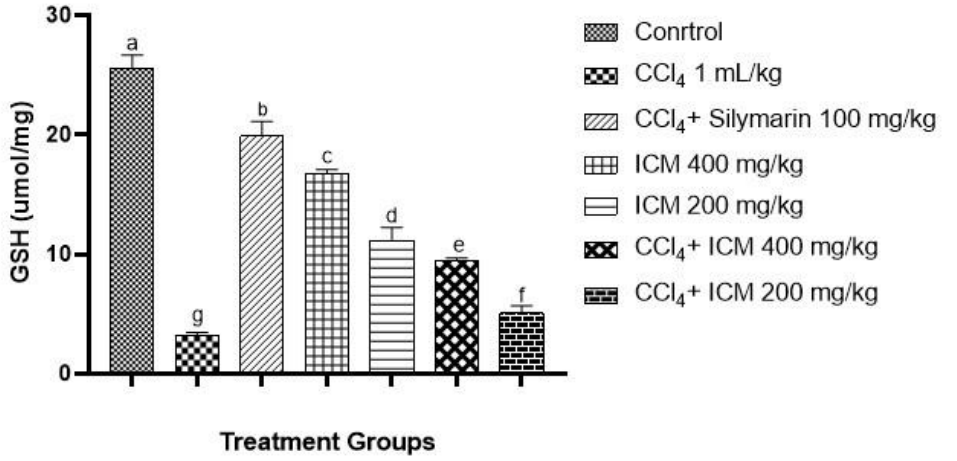
4.5.3.2. GSH and Total Protein Content

Rats given CCl_4 had significantly lower levels of protein and GSH compared to the control rats and rats with standard drugs. Due to the generation of ROS, protein is digested, undergoes greater glomerular filtration, and has a slower rate of reabsorption, which decreases the amount of protein in tissues and increases the excretion of protein in urine. When compared to CCl_4-treated rats, the amount of these antioxidant enzymes was significantly increased in ICM treated with low and high doses. Based on their p-value<0.05, the significant levels of protein and GSH content have been assessed below in [Table 4.8] and are graphically displayed in (Fig. 4.10).

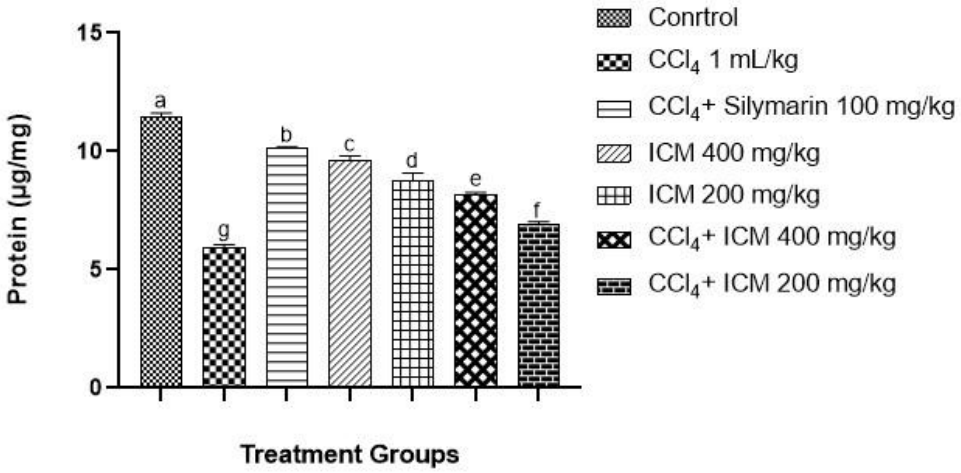
Table 4.8. Nephroprotective effect of ICM treatment on GSH and protein content

Groups	GSH content (µmol/mg)	Total Protein (µmol/mg)
Control	25.59 ± 1.04^{a}	11.43 ± 0.18^{a}
CCl_4 (1ml/kg)	3.22 ± 0.22^{g}	5.96 ± 0.10^{g}
CCl_4 + Sily. (100mg/kg)	19.88 ± 1.21^{b}	10.15 ± 0.02^{b}
ICM (400mg/kg)	16.75 ± 0.33^{c}	9.61 ± 0.20^{c}
ICM (200mg/kg)	11.12 ± 1.12^{d}	8.73 ± 0.35^{d}
CCl_4 + ICM (400mg/kg)	9.51 ± 0.14^{e}	8.16 ± 0.09^{e}
CCl_4 + ICM (200mg/kg)	5.08 ± 0.62^{f}	6.93 ± 0.08^{f}

Values are expressed as Mean ± S.D (n=7) raised to superscripts designating their significance based on p-value<0.05



(a)



(b)

Figure 4.10. Nephroprotective effects of ICM on GSH (a) and protein content(b)

4.5.3.3. Protective role of ICM on H_2O_2, Nitrite, and TBAR's content

Due to the oxidation of membrane lipids, proteins, and DNA damage, CCl_4 treatment considerably ($p< 0.05$) increased the quantity of oxidative species (lipid peroxides, nitrite ions, and hydrogen peroxide). In rats given a high dose of ICM (400 mg/kg), the amount of these species is noticeably reduced (Figures 4.13, 4.12, and 4.13). Different levels of these species in different groups are illustrated in Table 4.9.

Table 4.9. Nephroprotective effect of ICM on levels of oxidative species

Groups	H_2O_2 (µg/µL)	Nitrite (nM/min/mg)	TBAR's (nM/min/mg)
Control	3.29 ± 0.08^{f}	7.10 ± 1.93^{e}	73.77 ± 3.1^{f}
CCl_4 (1ml/kg)	7.41 ± 0.21^{a}	13.49 ± 0.23^{a}	183.31 ± 5.0^{a}
CCl_4 + Sily. (100mg/kg)	$3,48 \pm 0.13^{ef}$	8.15 ± 0.15^{de}	77.50 ± 2.8^{ef}
ICM (400mg/kg)	3.59 ± 0.11^{e}	8.61 ± 0.36^{de}	81.66 ± 2.9^{e}
ICM (200mg/kg)	4.24 ± 0.17^{d}	$9.54 \pm 1.^{05cd}$	98.36 ± 4.0^{d}
CCl_4 + ICM (400mg/kg)	4.99 ± 0.09^{c}	10.32 ± 0.24^{bc}	119.69 ± 1.7^{c}
CCl_4 + ICM (200mg/kg)	5.45 ± 0.12^{b}	11.84 ± 0.25^{b}	132.05 ± 2.7^{b}

Values are expressed as Mean ± S.D (n=7) raised to superscripts designating their significance based on p-value<0.05

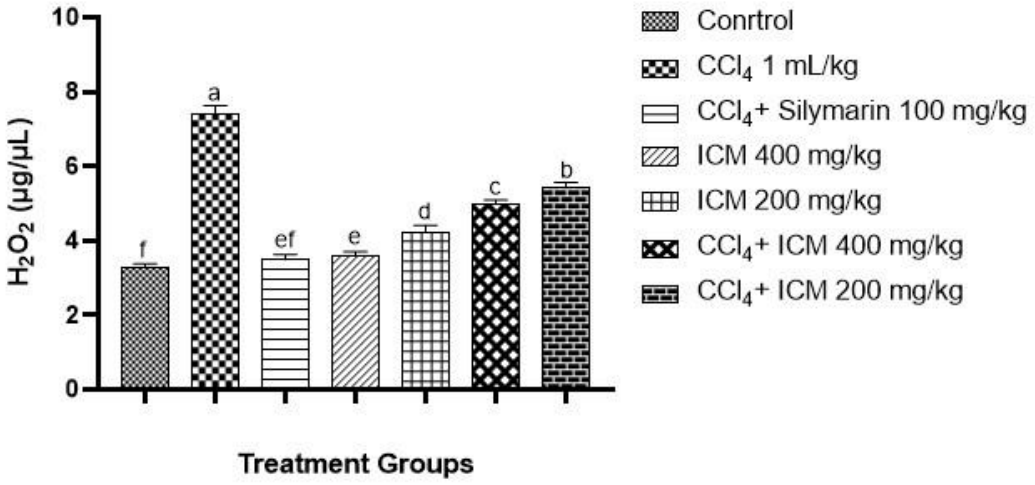


Figure 4.11. Effect of different ICM treatments on H_2O_2 content

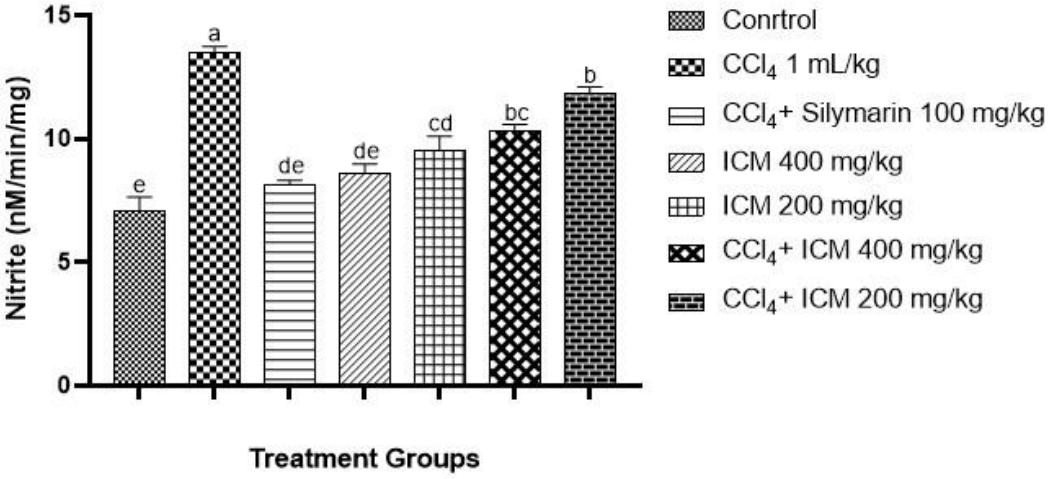


Figure 4.12. Effect of different ICM treatments on Nitrite concentration

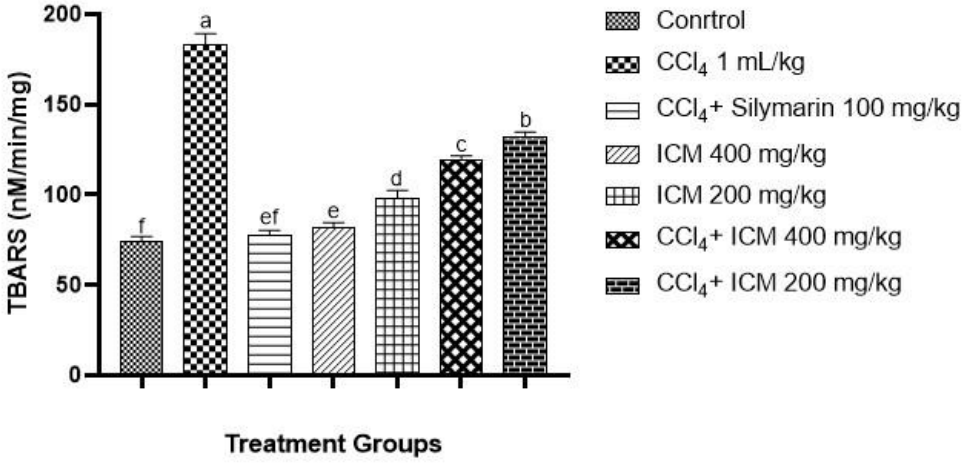


Figure 4.13. Effect of different ICM treatments on TBAR's content

4.6. Molecular Analysis

By harming the glomerulus and renal tubular cells, nephrotoxicity causes kidney injury. Apoptosis, cellular uptake and efflux, vascular injury, oxidative and endoplasmic reticulum stress, and inflammation are all caused by altered molecular pathways that arise as a result of renal cell damage. Multiple molecular processes that are altered as renal cells deteriorate cause the up- or down-regulation of certain genes. Increased levels of tumor necrosis factor alpha (TNF- α) and reactive oxygen species (ROS) are produced because of nephrotoxicity, which also promotes oxidative stress,

vascular damage, and apoptotic pathways. Numerous mechanisms, including the TGF- β, EMT, ERK, and other pathways, contribute to nephrotoxicity.

RT-PCR analysis was used to track how the nephrotoxicity caused by CCl_4 affected the regulation of several genes. The ER stress proteins XBPu, XBPs, and XBPt were shown to have significantly ($p<0.05$) increased mRNA expression in CCl_4-intoxicated rats compared to the control group as shown in Fig 4.14, 4.15, and Fig 4.16. The silymarin and ICM groups both downregulated the expression of these genes and normalized the fold change versus the group that had received CCl4 treatment at various rates. Non-significant fold change was shown by ICM high and low dose treatment alone.

The fold change of the anti-apoptotic marker (Bcl-2) was significantly suppressed in the CCl_4-mediated nephrotoxic rats, whereas the opposite was shown in the case of the apoptotic mediators (Casp-3 and CHOP) in comparison to the control group shown below in Fig 4.17, Fig 4.18, and Fig 4.19. In the groups receiving silymarin and ICM low and high doses together, a variety of fold change tendencies were seen. However, the ICM administration alone did not show any appreciable differences from the control group.

Significantly ($p<0.05$) higher pro-inflammatory cytokines (IL-6 and TNF-α) and anti-inflammatory cytokine (TGF-β) mRNA expression was seen in the CCl4 treated group (Fig 4.20, Fig 4.21, Fig 4.22). The fold change of these markers was noticeably improved in the standard (silymarin) and ICM high and low dose treated groups when compared to the control group while using CCl_4. The expression of these genes was not significantly altered by plant dosage treatments alone.

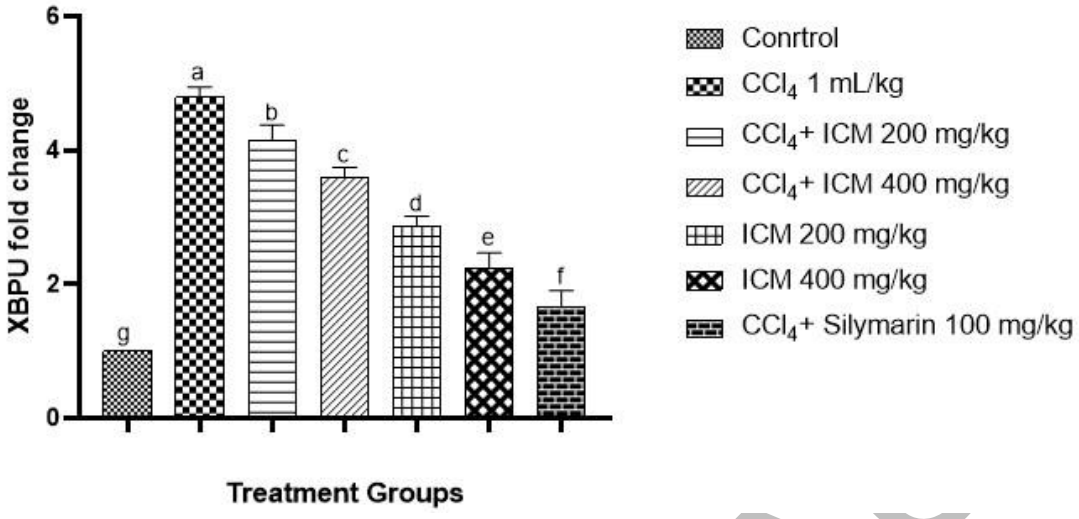


Figure 4.14. Effect of ICM on XBPu (ER stress maker), evaluated as fold change

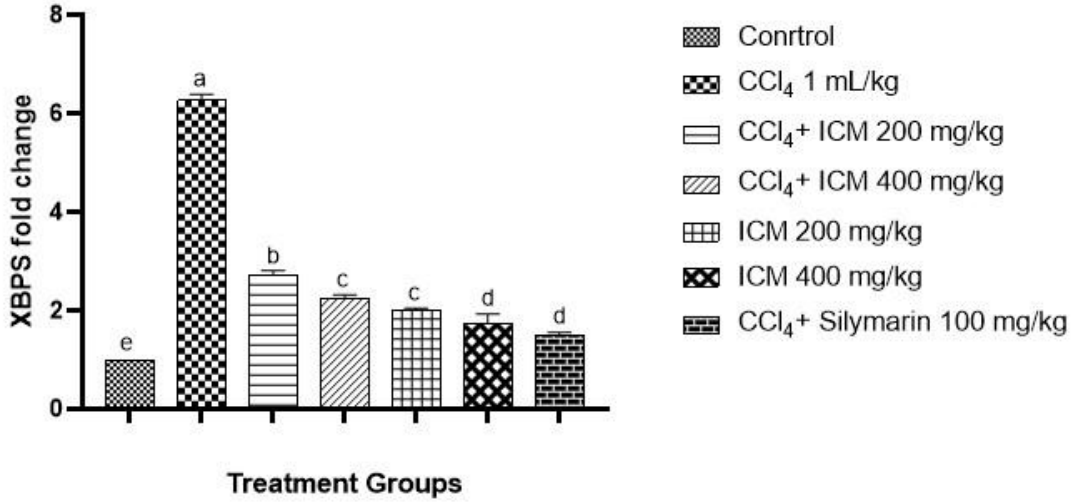


Figure 4.15. Effect of ICM on XBPs (ER stress maker), evaluated as fold change

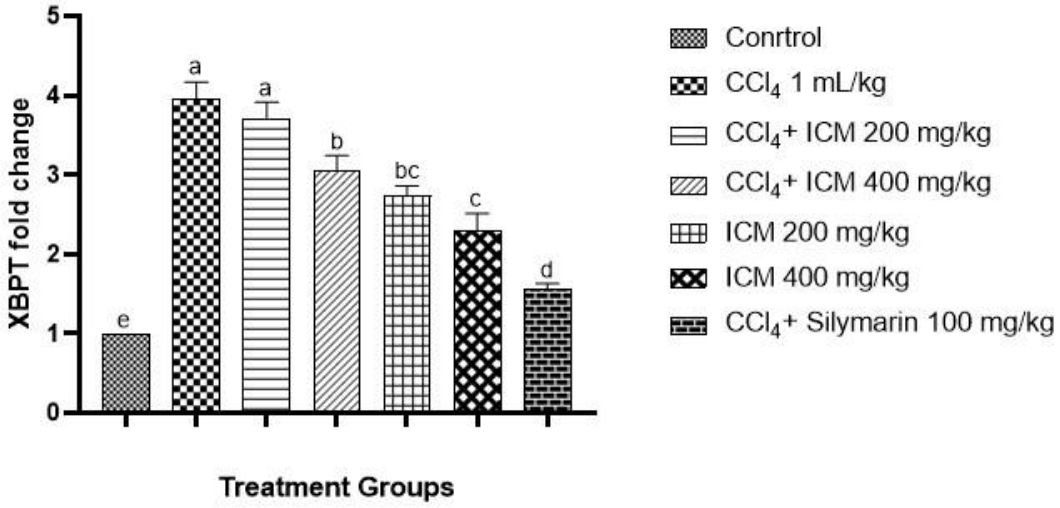


Figure 4.16. Effect of ICM on XBPt (ER stress maker), evaluated as fold change

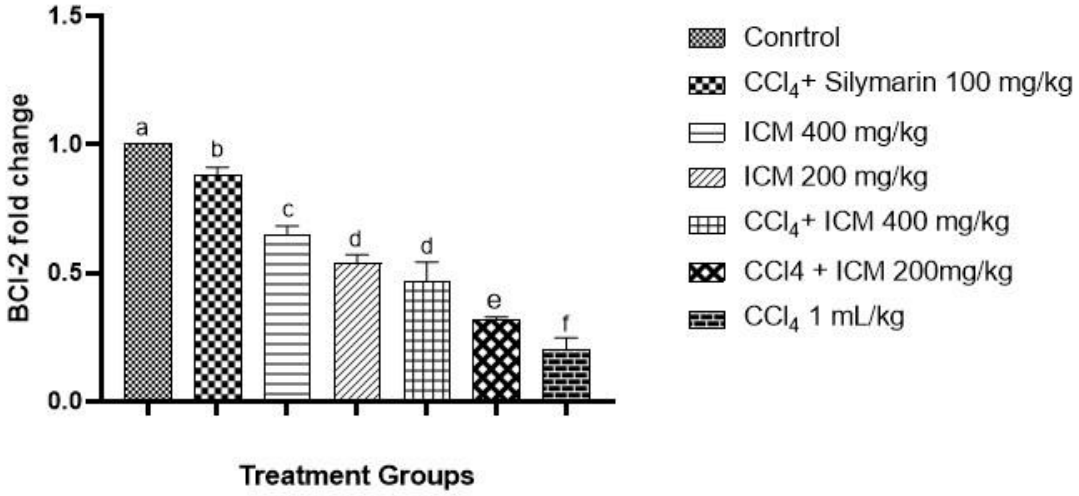


Figure 4.17. Effect of ICM on Bcl-2 (anti apoptotic maker), evaluated as fold change

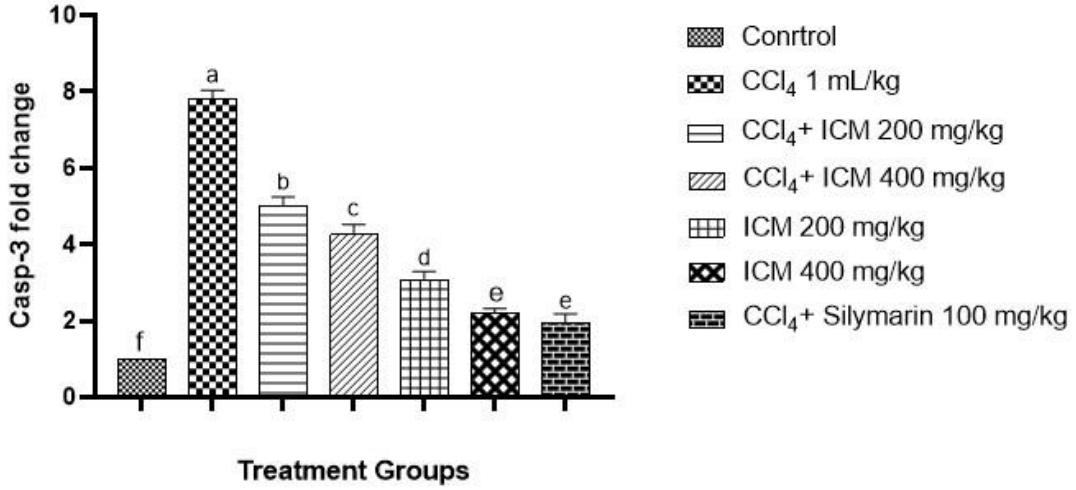


Figure 4.18. Effect of ICM on Casp-3 (apoptotic maker), evaluated as fold change

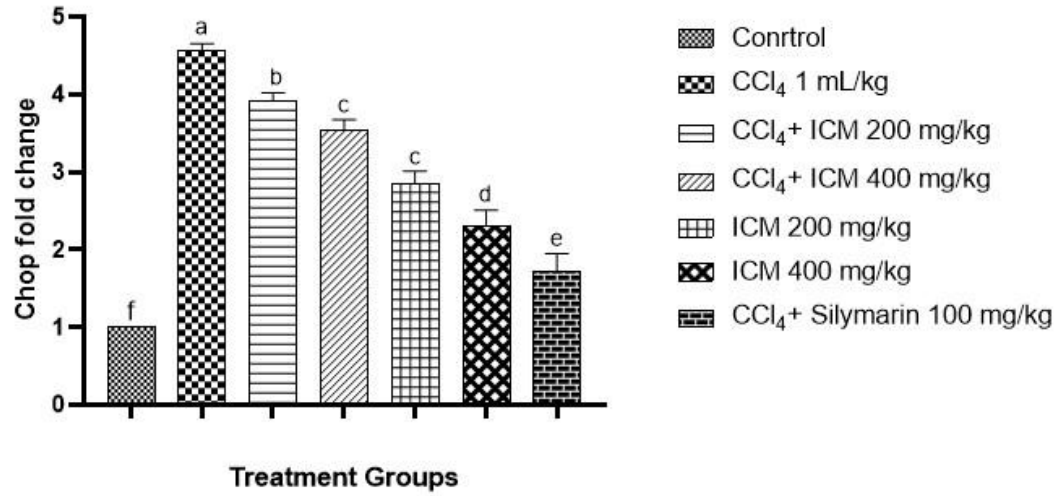


Figure 4.19. Effect of ICM on Chop (apoptotic maker), evaluated as fold change

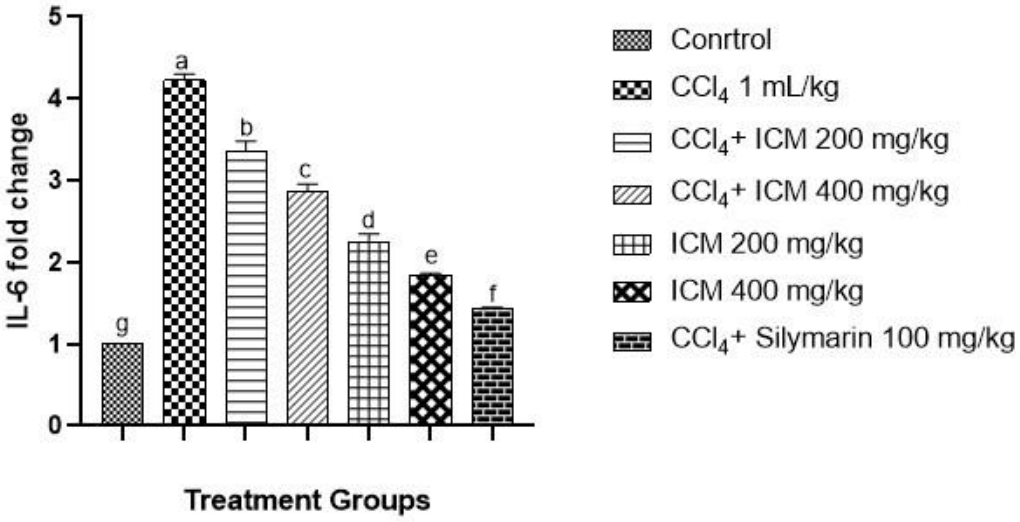


Figure 4.20. Effect of ICM on IL-6 (pro-inflammatory maker), evaluated as fold change

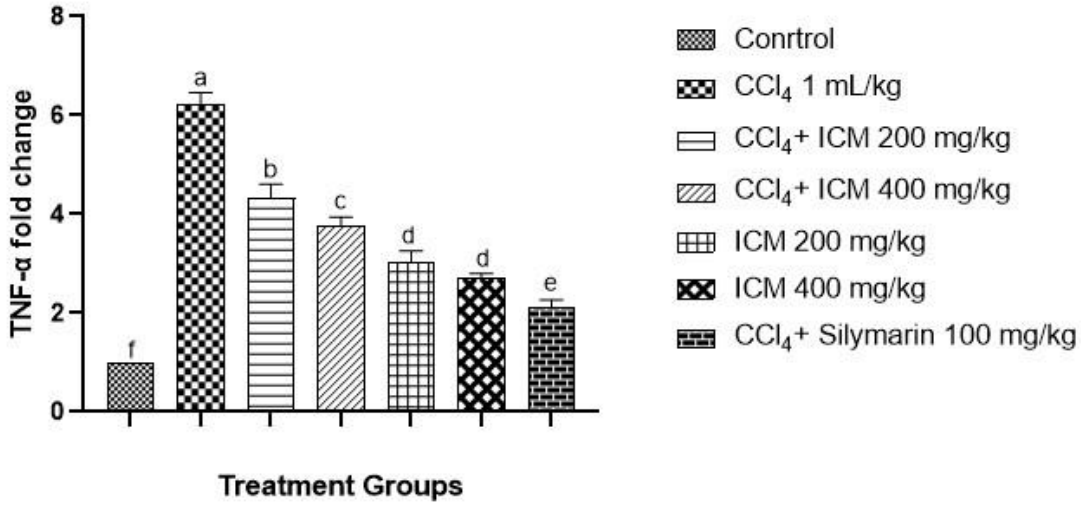


Figure 4.21. Effect of ICM on TNF- α (pro-inflammatory maker), evaluated as fold change

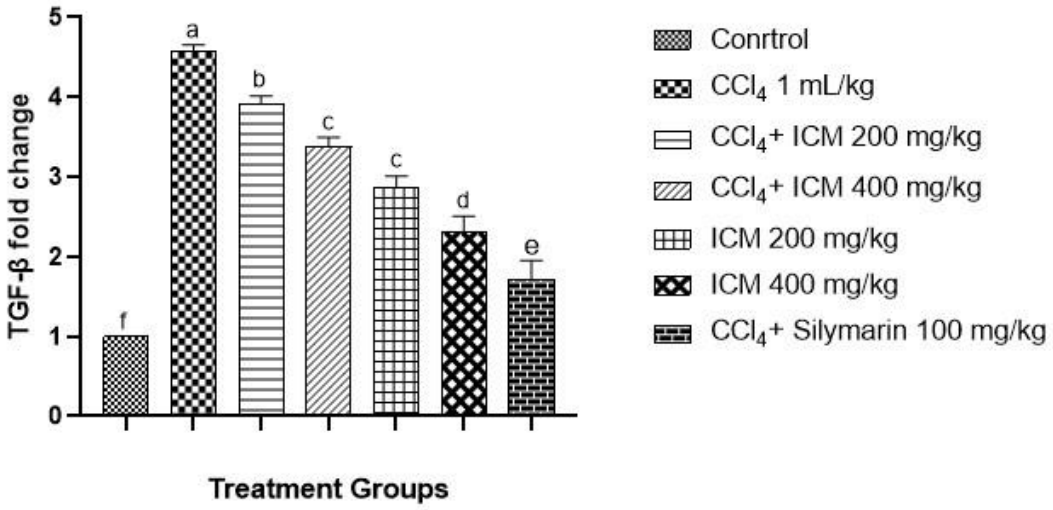


Figure 4.22. Effect of ICM on TGF-β (anti-inflammatory maker), evaluated as fold change

4.7. Histopathological Analyses of kidney

Histology of renal tissues has been done to observe morphological changes occurring in kidney nephron. By looking at slides under a light microscope, we can see that cellular enlargement, widening of the bowman's capsule, hydropic degeneration of the proximal convoluted tubules, and the deterioration of glomerular capillaries as well as tubular cells are signs of nephrotoxicity. Control rat's groups and rats given a standard medication show nephrons with normal morphological characteristics. The normal architecture of the nephron cannot be restored by a low dose of ICM. However, it has been observed that a high dose of ICM can mitigate the negative effects of toxicity on the structure of nephrons. Fig. 4.23 describes the impact of plant doses in contrast to normal rats and rats receiving regular medication.

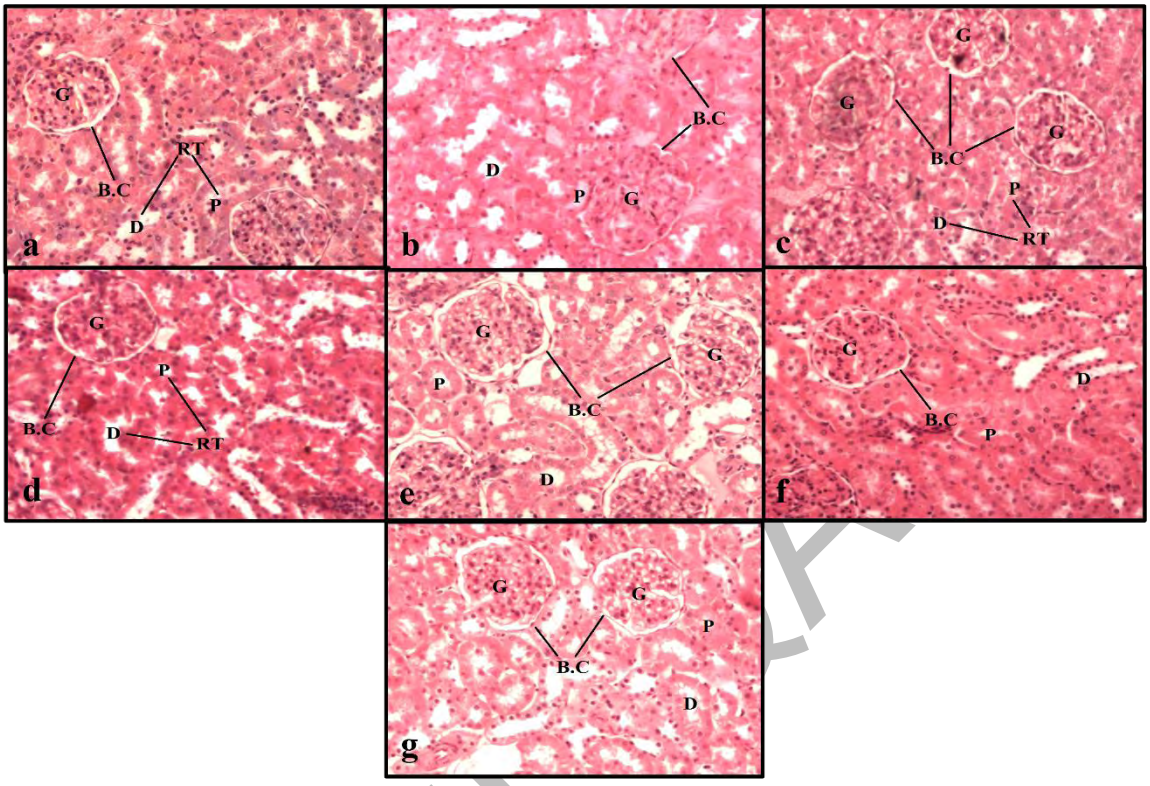


Fig 4.23. Nephroprotective effect of ICM on CCl_4 induced nephrotoxicity. (a) Control group showing normal glomeruli (G) surrounded by Bowman's capsule (BC) and normal renal tubules; (b) CCl_4 group (1 ml/kg b.w.) showing hypercellularity of glomeruli and widening of the Bowman's space. Some renal tubules exhibited vacuolar degeneration and their lumens were filled with cellular debris, glomerular and tubular necrosis; normal histological appearance was observed in (c) CCl_4 + silymarin, (d) ICM (400 mg/kg b.w.), and (e) ICM (200 mg/kg b.w.); groups treated with (f) CCl_4+ ICM (400 mg/kg) and (g) CCl_4+ ICM (200 mg/kg) prevented CCl_4 induced damage in glomeruli distal tubules and proximal tubules. ICM: *Indigofera cordifolia*; CCl_4: carbon tetrachloride; b.w.: body weight.

Discussion

An increase in oxidative stress and inflammation are symptoms of chronic renal disease. Increased inflammation and oxidative stress may cause further kidney injury as well as initial or additional damage to distal tissues, leading to the onset or advancement of concurrent illnesses. In light of this, it is clear that lowering oxidative stress and inflammation is essential since the damage they cause is cyclical and potentially systemic, harming both local tissue and tissues distant from the original injury (Tucker *et al.,* 2015). Kidney failure is defined as having a GFR of less than 15 mL/min per 1.73 m^2 or needing dialysis or a transplant. An increased risk of cardiovascular disease, acute renal injury, infection, cognitive decline, and physical function impairment are all side effects of low GFR (Hailpern *et al.,* 2007). When cells are not able to sufficiently eliminate the excess of free radicals produced, oxidative stress can result. In other words, oxidative stress is caused by an imbalance between the production of ROS and RNS and their neutralization (Pham-Huy *et al.,* 2008). Intoxication with CCl_4 produces free radicals in a variety of tissues, including the blood, liver, kidneys, heart, lungs, testicles, and brain (Khan *et al.,* 2009). The cytochrome P450-mediated transfer of a single electron to the C-Cl bond is the first step in the tissue damage caused by CCl_4. This process results in a radical anion as a temporary intermediary that removes chlorine to generate the trichloromethyl radical ($^{\bullet}CCl_3$) and chloride (Halliwell and Gutteridge, 1984). The $^{\bullet}CCl_3$ is then transformed into the even more dangerous trichloromethyl peroxyl radical ($^{\bullet}OOCCl_3$) in the presence of oxygen, which starts the process of lipid peroxidation and generates membrane-damaging compounds such MDA (Halliwell, 2007). These free radicals then go after the polyunsaturated fatty acids in membrane lipids, starting a chain reaction that degrades membrane structure and interferes with protein synthesis and cell energy processes (Adewole *et al.,* 2007). Through their different pharmacological effects, medicinal plants have anti-nephrotoxic properties. Examples of plants with nephroprotective characteristics include *Nigella sativa, Ginkgo biloba, Trianthema portulacastrum, Zingiber officinale, Ficus carica, Moringa oleifera, Costus afer, Cissampelos pareira, Tephrosia purpurea, and Olea europaea* (Rad *et al.,* 2017).

I. cordifolia is used to treat kidney disorders to some extent in Pakistan and other places where it is grown. *I. cordifolia* whole plant extract is employed in studies to assist its well-established nephroprotective benefits. Numerous phytochemicals, including as alkaloids, betacyanin, flavonoids, coumarins, quinones, tannins, glycosides, and many others, are found in varying concentrations. Flavonoids and glycosides were the phytochemicals with the highest amounts. We carried out various *in-vitro* biological experiments to evaluate the antioxidant and anti-inflammatory effects of plant extracts with maximal activity. Following the completion of antioxidant testing, it was shown that ICM possessed the antioxidant activity against NO free radicals and OH ions. Griess reagent is employed in the NO scavenging assay to produce NO-, which in turn induces the creation of nitrite or nitrate ions. Based on ICM's ability to scavenge them, their production is prevented by reducing the amount of oxygen accessible (Jagetia *et al.,* 2004). ICM has much IC_{50} (775.78 ± 0.478) values and robust NO scavenging activity. The H_2O_2-ascorbate Iron-EDTA system in the OH- ion scavenging test produces OH ions, which are known to degrade nucleic acids. It is a highly harmful radical that damages cellular membranes, reducing the strength of the cell. By scavenging these ions, plant antioxidant activity contributes to cellular integrity maintenance (Valentao *et al.,* 2002). According to the results of this test, ICM has a lower IC_{50} (741.41 ± 0.54) value against these ions, indicating a high antioxidant capability. The egg albumin assay demonstrated the anti-inflammatory properties of the plant extract ICM, as indicated by a lower IC_{50} value (316.65 ± 0.917). We first demonstrated the antioxidant and anti-inflammatory properties of ICM, and then use *in-vivo* tests to demonstrate its nephroprotective abilities. After approximately 21 days of treatment with plant dosage and conventional Silymarin, we examined ICM's dose-dependent impact on the level of renal serum indicators, tissue antioxidant enzymes, and the expression of certain genes in addition to its potential nephroprotective effects. Nephrotoxicity was caused by the chemical CCl_4. The harmful chemical substance CCl_4 speeds up lipid peroxidation. From CCl_4, the radicals trichloromethyl ($•CCl_3$) and peroxytrichloromethyl ($•OOCCl_3$) are created. Free radicals and polyunsaturated fatty acids (PUFAs) combine to generate lipid peroxides, which are highly reactive, interfere with enzyme activity, and ultimately lead to damage or necrosis (Adewole *et*

al., 2007). Histological analysis revealed that CCl_4 markedly increased the quantity of inflammatory cell infiltration and fibrosis in kidney tissues, albeit this was significantly reduced in the silymarin groups. According to our research, silymarin can prevent oxidative kidney damage by boosting antioxidant enzyme activity and reducing lipid peroxidation. The body weight study of nephrotoxic rats showed that the CCl_4-induced rats had a lower body weight than the control group. The body's weight decreased as a result of CCl_4's induction of free radical damage, which also caused oxidative damage to kidney tissues (Johnston and Kroening, 1998). Rats given ICM high dose 400/mg and standard silymarin significantly reduced kidney and body weight by repairing tissue damage, reducing oxidative stress, and regaining normal morphological properties. At the conclusion of dosing and dissection, ICM low dose 200/mg does not show a significant difference in kidney and body weight.

By assessing the amount of serum indicators Albumin, Urea, and Creatinine that indicate renal functionality, we must establish nephroprotective efficacy. The CCl_4 metabolites induced renal cell inflammation, which raised the levels of urea and creatinine. The kidney structures get deformed as a result of CCl_4's nephrotoxicity, which causes dramatic alterations in blood indicators. Due to excessive albumin excretion and obstruction of urea and creatinine excretion in urine, the level of serum indicators is altered as a result of nephron dysfunction in such a way that albumin serum level continues to decrease while that of urea and creatinine increases. By turning around the typical levels of urea, creatinine, and albumin, the silymarin-treated rats were observed to be restoring the kidney's normal functionality. When we examined that the high dose ICM 400/mg treated rat group was seen restoring normal nephron functionality, the ICM demonstrated its nephroprotective properties. In contrast to low dosages, high doses of ICM have been shown to be successful in restoring the levels of the serum indicators albumin, urea, and creatinine (3.31 ± 0.09 g/dl, 29.29 ± 0.42 mg/dl, 1.52 ± 0.08 mg/dl), which indicate improvements in renal function. The ICM is effective at raising levels of enzymes that fight inflammation and free radicals, which lowers levels of oxidative stress. Catalase, peroxidase, and superoxide dismutase levels were shown to be lower in CCl_4-treated rats (5.83 ± 0.77 U/min, 1.28 ± 0.03 U/min, 3.62 ± 0.03 U/min), although oxidative species (hydrogen peroxide, nitrites, and TBARs) levels were higher (7.41 ± 0.21 µg/µL, 13.49 ± 0.23

nM/min/mg, 183.31 ± 5.0 nM/min/mg). In nephrotoxic conditions, oxidative stress also causes a decrease in protein content (5.96 ± 0.10 µmol/mg). The amount of GSH declines in renal tissues as well (3.22 ± 0.22 µmol/mg) because the glutathione reductase enzyme, which may change the oxidized form of GSH, or GSSG, back to its reduced form, loses function. Since GSSG cannot be converted back into GSH, its level decreases (Khan *et al.,* 2010). As compared to plant ICM low dose 200mg/kg, ICM high dose 400mg/kg significantly increased CAT, SOD POD, GSH, and protein levels (16.06 ± 0.75, 6.59 ± 0.53, 2.83 ± 0.06 U/min, 16.75 ± 0.33, 9.61 ± 0.20 µmol/mg) while decreasing nitrite, TBAR'S, and H_2O_2 (8.61 ± 0.36, 81.66 ± 2.9 nM/min/mg, 3.59 ± 0.11 µg/µL). By calculating the expression of specific genes expressed in tissues following oral administration of ICM for around 3 weeks, the nephroprotective effect of ICM on kidney is considered on a molecular level.

By calculating the expression of specific genes expressed in tissues following oral administration of ICM for around 3 weeks on alternative days, the nephroprotective effect of ICM on kidney is taken into account on a molecular level. By producing ROS, CCl_4 causes kidney damage, which then triggers the EMT pathway. High ROS levels trigger PI3k/Akt activation, which in turn prevents GSK3- β from functioning. Because GSK3-β is inhibited, β-catenin escapes degradation, enters the nucleus, and triggers transcription of genes implicated in EMT (Lu *et al.,* 2019). Oxidative injury caused by CCl_4 increased expression of transforming growth factor (TGF- β), tumor necrosis factor alpha (TNF-α).

CCl_4-intoxicated rats showed elevated mRNA expression of ER stress markers (XBPs, XBPu, and XBPt), apoptotic mediators (Casp-3 and CHOP), pro-inflammatory cytokines (IL-6 and TNF- α) and anti-inflammatory cytokine (TGF-β) as compared to normal rat groups and rats administered with standard drug. In the CCl_4-mediated nephrotoxic rats, the fold change of the anti-apoptotic marker (Bcl-2) was considerably decreased. When compared to the control group, the fold change of these markers was considerably better in the standard (silymarin) and ICM high and low dose treatment groups when injecting CCl_4.

To observe the changes in renal structure brought on by CCl_4 nephrotoxicity and ICM dosages' restorative effects, histopathological examinations were conducted. It shows

tube damage, glomerular breakdown, and corpuscular changes. Loss of tubular epithelial cell brush boundary membranes, tubular collapse, cellular necrosis, and interstitial fibrosis are the characteristics of tubular injury. The kidney histology of the ICM high dose group of rats was observed returning to normal, demonstrating the therapeutic potential of ICM on nephropathy.

Conclusion

The methanolic extract of *I. cordifolia* (ICM) has been shown in this study to have considerable levels of egg denaturation inhibitory activity against proteins, supporting its anti-inflammatory and antioxidative potential against ions and oxidative free radicals. By replenishing serum indicators, antioxidant enzymes, and gene expression in renal tissues by neutralizing generated oxidative stress, oral dosing of ICM to CCl_4-induced nephrotoxicity in rats exerts nephroprotective effect.

Future Perspective

We believe that this investigation has shed light on the therapeutic potential of *I. cordifolia* against the kidney-damaging effects of CCl_4. It is also necessary to chemically characterize the phytoconstituents that give *I. cordifolia* plants their nephroprotective effects. Additionally, research is required to determine whether the plant has the ability to treat a number of diseases and whether it can harm other organs. ICM has phytoconstituents that have therapeutic value, and their derivatives can be generated to treat a variety of pathogenic disorders.

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