CARDIOPROTECTIVE EFFECT OF *FICUS HISPIDA* LINN. LEAF EXTRACT ON CYCLOPHOSPHAMIDE PROVOKED OXIDATIVE MYOCARDIAL INJURY IN A RAT MODEL

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"He who has the mind to acknowledge
Is sure to gain knowledge"

- H.A. Williams.

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LIST OF ABBREVIATIONS

1.	ALT	Alanine amino transferase
2.	AST	Aspartate amino transferase
3.	CAT	Catalase
4.	CDNB	1-chloro-2,4-dinitro benzene
5.	CP	Cyclophosphamide
6.	CPK	Creatine phosphokinase
7.	$CuSO_4$	Copper sulphate
8.	DTNB	5,5-dithiobis (2 nitrobenzoic acid)
9.	DNA	Deoxy ribonucleic acid
10.	DNPH	2,4-dinitrophenyl hydrazine
11.	DOX	Doxorubicin
12.	EDTA	Ethylene diamine tetra acetic acid
13.	FH	*Ficus hispida*
14.	GSH	Reduced glutathione
15.	GST	Glutathion-S-transferase
16.	GR	Glutathione reductase
17.	HDL	High Density Lipoprotein
18.	H_2O_2	Hydrogen peroxide
19.	H_2O	Water
20.	H_2SO_4	Sulphuric acid
21.	IU	International unit
22.	LDH	Lactate dehydrogenase

23.	LPO	Lipid per oxidation
24.	MDA	Malondialdehyde
25.	MIC	Minimum Inhibitory concentration
26.	NAD+	Nicotinamide adenine dinucleotide
27.	NADPH	Nicotinamide adenine-dinucleotide phosphate
28.	NaOH	Sodium hydroxide
29.	$OH^{\bullet}$	Hydroxy radical
30.	OD	Optical density
31.	O_2	Oxygen molecule
32.	RBC	Red blood cell
33.	ROS	Reactive oxygen species
34.	RNS	Reactive nitrogen species
35.	SOR	Superoxide reductase
36.	SDS	Sodium dodesyle sulphate
37.	SOD	Superoxide dismutase
38.	TCA	Trichloro acetic acid
39.	TBARS	Thiobarbituric acid
40.	TBA	Thiobarbituric acid
41.	UV	Ultraviolet spectroscopy

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INTRODUCTION

Heart – Structure[1]

The heart is a hollow, four-chambered (two atria; two ventricles) muscular organ that is specialized for pumping blood through the vessels of the body. It is located in the mediastinum where it is surrounded by a tough fibrous membrane called the pericardium. The parietal pericardium is a loose sac composed of an outer fibrous layer that protects the heart and an inner serous layer that secretes pericardial fluid. The visceral pericardium is a serous membrane that makes up the outer layer of the wall of the heart (the epicardium). The space between the parietal pericardium and the visceral pericardium is called the pericardial cavity. Pericardial fluid is found within this cavity and functions to lubricate the surface of the heart.

Layers of the heart

The heart is composed of three layers from superficial to deep:

- Epicardium : Outer serous layer.
- Myocardium : Middle muscular layer; thickest layer.
- Endocardium : Inner epithelial layer.

Electrical conduction system of the heart[2]

Inherent firing rate is the rate at which the SA node or another pacemaker site normally generates electrical impulses.

A. SA Node - Sinoatrial node

1. Dominant or primary pacemaker of the heart.
2. Inherent rate 60 – 100 beats per minute.
3. Located in the wall of the right atrium, near the inlet of the superior vena cava.

4. Once an impulse is initiated, it usually follows a specific path through the heart, and usually does not flow backward

B. Intra-atrial tracts - Bachmann's bundle

As the electrical impulse leaves the SA node, it is conducted through the left atria by way of the Bachmann's bundles, through the right atria, via the atrial tracts.

C. AV Junction - Made up of the AV node and the bundle of His.

1. AV node

a) Is responsible for delaying the impulses that reach it.

b) Located in the lower right atrium near the interatrial septum.

c) Waits for the completion of atrial emptying and ventricular filling, to allow the cardiac muscle to stretch to it's fullest for *peak cardiac output*.

d) The nodal tissue itself has no pacemaker cells, the tissue surrounding it (called the junctional tissue) contains pacemaker cells that can fire at an inherent rate of 40 – 60 beats per minute.

2. Bundle of His

a) Resumes rapid conduction of the impulses through the ventricles.

b) Makes up the distal part of the AV junction then extends into the ventricles next to the interventricular septum.

c) Divides into the Right and Left bundle branches.

3. Purkinje Fibers

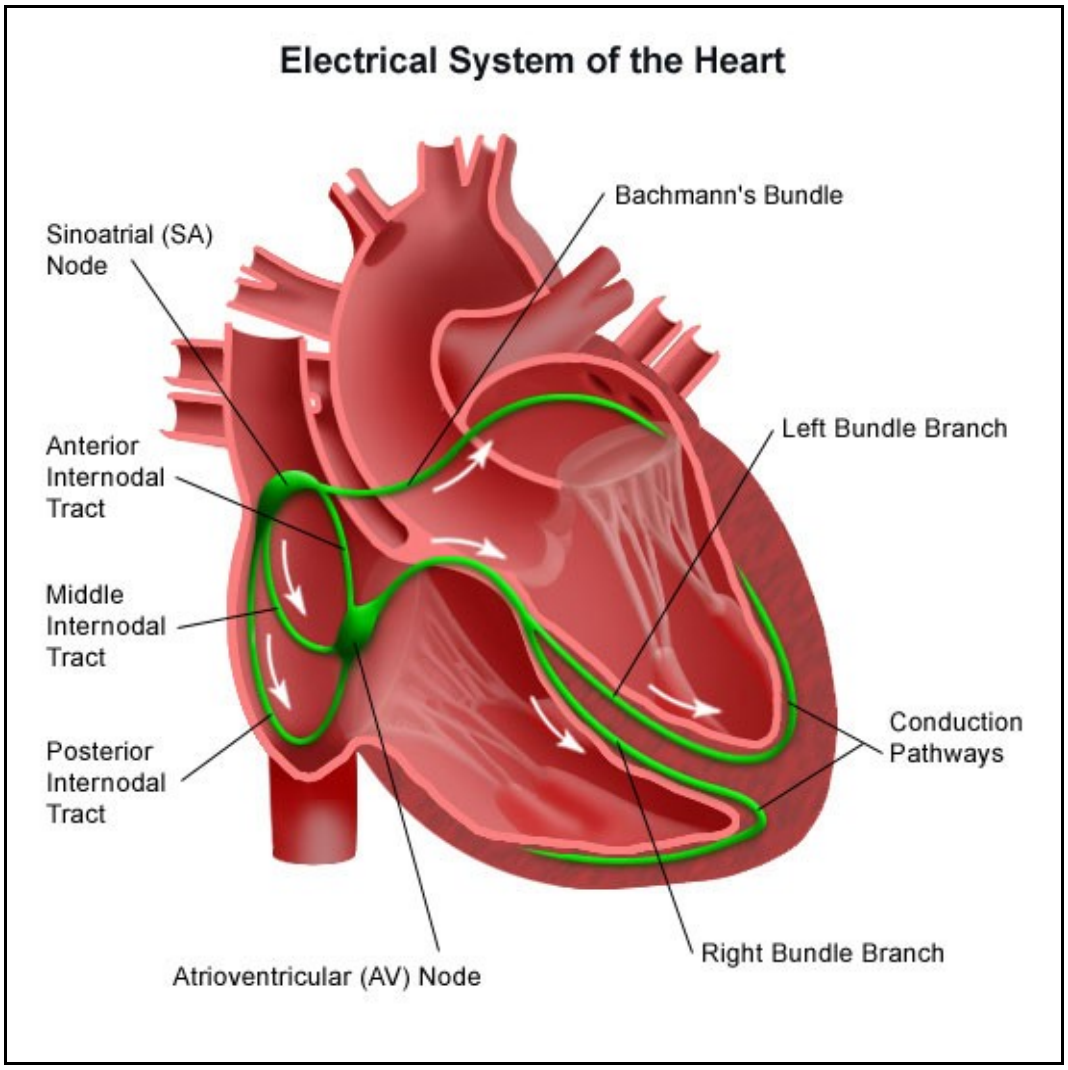
a) Conduct impulses rapidly through the muscle to assist in depolarization and contraction.

b) Can also serve as a pacemaker, discharges at an inherent rate of 20 – 40 beats per minute or even more slowly.

c) Are not usually activated as a pacemaker unless conduction through the bundle of His becomes blocked or a higher pacemaker such as the SA node or AV junction do not generate an impulse.

d) Extends form the bundle branches into the endocardium and deep into the myocardial tissue.

Figure 1: Electrical conduction system of the heart



Death by Medicine

Many new pharmaceutical agents have been withdrawn from the market or severely restricted to specific indications because of unexpected adverse events, including fatalities. Cardiac, hepatic, and hematological abnormalities are the major causes of withdrawal of drugs or restriction in their labeling. A short review of medical peer-review journals and government health statistics shows that modern medicine imposes more harm than benefits. Of note, a group of researchers meticulously reviewed the statistical evidence and their findings are absolutely shocking. These researchers have authored a paper titled **"Death by Medicine"** that presents compelling evidence that today's medical system

frequently causes more harm than good[3]. This fully referenced report shows that the deaths caused by conventional medicine were chiefly due to the heart disease. Hence, recent regulatory developments are thrusting cardiac safety to the forefront of clinical development.

Cardiotoxic Drugs

The major culprits for cardiotoxicity include:

- Cytostatics (anti-neoplastic agents)
- Immunomodulators
- Calcium channel blocking agents
- Antidepressants
- Non-steroidal anti-inflammatory drugs
- Anti-arrhythmics
- β-adrenergic blockers
- Anesthetics.

Cytostatics at the front[4]

Since cancer is a leading cause of mortality worldwide, the number of therapeutic modalities available for the treatment of neoplastic processes has increased. This has resulted in a large number of patients being exposed to a wide variety of cancer therapy. Historically, it has been well recognized that antineoplastic agents may have adverse effects on multiple organs and normal tissues. The most commonly associated toxicities occur in tissues composed of rapidly dividing cells (e.g. hematopoietic cells) and may spontaneously reverse with minimal long-term toxicity. However, the myocardium consists of cells that have limited regenerative capability, which may render the heart susceptible to permanent or transient adverse effects from chemotherapeutic agents. Such toxicity encompasses a heterogeneous group of disorders, ranging from relatively benign arrhythmias to potentially lethal conditions such as myocardial ischemia/infarction and cardiomyopathy. In some instances, the pathogenesis of these toxic effects has been elucidated, whereas in others the precise etiology

remains unknown. In this context, an ample literature data suggest that “oxidative stress” plays a pivotal role in the cardiotoxicity of cytotoxic drugs[5].

Oxidative Stress

Oxidative stress, implicated in the pathogenesis of a wide variety of clinical disorders, refers to the cytological consequence of a mismatch between the production of free radicals and the ability of the cell to defend against them. Oxidative stress can thus occur when the generation of free radicals increases or the capacity to scavenge free radicals and repair of oxidatively modified macromolecules decreases, or both[6]. This imbalance leads to the accumulation of oxidatively modified molecules, referred to as reactive oxygen species (ROS).

Types of Reactive Species

- **Reactive Oxygen Species (ROS)**
  - Oxygen-centered radicals
    - Superoxide anion ($\cdot O_2–$)
    - Hydroxyl radical ($\cdot OH$)
    - Alkoxyl radical ($RO\cdot$)
    - Peroxyl radical ($ROO\cdot$)
  - Oxygen-centered non-radicals
    - Hydrogen peroxide (H_2O_2)
    - Singlet oxygen ($^1O_2$)
- **Reactive Nitrogen species (RNS)**
    - Nitric oxide ($NO\cdot$)
    - Nitric dioxide ($NO_2\cdot$)
    - Peroxynitrite ($OONO^–$).

The presence of free radicals in biological materials was discovered 50 years ago[7]. Soon thereafter, Harman described free radicals as a Pandora’s Box of evils that may account for gross cellular damage, mutagenesis, cancer, and, last but not least, the degenerative process of biological aging.

Mitochondria and ROS[8]

Aerobic energy metabolism is dependent on oxidative phosphorylation, a process by which the oxidoreduction energy of mitochondrial electron transport (via a multicomponent NADH dehydrogenase enzymatic complex) is converted to the high-energy phosphate bond of ATP. O_2 serves as the final electron acceptor for cytochrome-c oxidase, the terminal enzymatic component of this mitochondrial enzymatic complex that catalyzes the four-electron reduction of O_2 to H_2O. Partially reduced and highly reactive metabolites of O_2 may be formed during these (and other) electron transfer reactions. These O_2 metabolites include superoxide anion ($\cdot O_2–$) and hydrogen peroxide (H_2O_2), formed by one- and two-electron reductions of O_2, respectively. In the presence of transition metal ions, the even more reactive hydroxyl radical (·OH) can be formed. These partially reduced metabolites of O_2 are often referred to as “reactive oxygen species” (ROS) due to their higher reactivities relative to molecular O_2. ROS from mitochondria and other cellular sources have been traditionally regarded as toxic by-products of metabolism with the potential to cause damage to lipids, proteins, and DNA.

Oxygen Paradox

Under physiological conditions, approximately 1–3% of the molecular oxygen (dioxygen; O_2) consumed by the body is converted into superoxide and other ROS. Thus, although molecular oxygen is absolutely essential for aerobic life, it can be toxic under certain conditions. This ‘double-edged sword’ aspect of oxygen may be regulating longevity and the phenomenon has been termed as “oxygen paradox”[9].

ROS in Cell Signaling

Accumulating evidence suggests that ROS are not only injurious by-products of cellular metabolism but also essential participants in cell signaling and regulation[10,11]. Although this role for ROS is a relatively novel concept in vertebrates, there is strong evidence of a physiological role for ROS in several

non-mammalian systems. The apparent paradox in the roles of ROS as essential biomolecules in the regulation of cellular functions and as toxic by-products of metabolism may be, at least in part, related to differences in the concentrations of ROS produced.

Analogy of ROS and Nitric Oxide[8]

The paradoxical behavior of ROS is analogous to the effects of nitric oxide (NO·), which has both regulatory functions and cytotoxic effects depending on the enzymatic source and relative amount of NO· generated. NO· functions as a signaling molecule mediating vasodilation when produced in low concentrations by the constitutive isoform of nitric oxide synthase (NOS) in vascular endothelial cells and as a source of highly toxic oxidants utilized for microbicidal killing when produced in high concentrations by inducible NOS (iNOS) in macrophages. Indeed, all phagocytic cells have a well-characterized $\cdot O_2$—-generating plasma membrane oxidase capable of producing the large amounts of ROS required for its function in host defense.

NO is synthesized enzymatically from L-arginine by NO synthase (NOS).

$$\text{L-arginine} + O_2 + \text{NADPH} \rightarrow \text{L-citrulline} + \text{NO} + \text{NADP}^+$$

Lipid peroxidation (LPO)

ROS exert their cytotoxic effect by peroxidation of membrane phospholipids leading to change in permeability and loss of membrane integrity. Lipid oxidation is a free-radical chain reaction, and reactive oxygen species can accelerate lipid oxidation. Cell membranes are phospholipid bilayers with extrinsic proteins and are the direct target of lipid oxidation. Malondialdehyde (MDA), one of the lipid oxidation products, can react with the free amino group of proteins, phospholipid, and nucleic acids leading to structural modification, which induce dysfunction of immune systems. A high level of lipid peroxidation products can be detected in cell degradation after cell injury or disease.

ROS and Apoptosis[12]

1. Induction and execution of apoptosis

Apoptosis is a special form of programmed cell death that plays an indispensable role in the development and homeostasis of multicellular organisms. The potential for mitochondrial ROS to mediate cell signaling has gained significant attention in recent years, particularly with regard to the regulation of apoptosis. An increase in cellular ROS production is often observed in apoptotic processes triggered by various stimuli. Also, high ROS concentrations induce apoptotic cell death in various cell types, suggesting that ROS contribute to cell death whenever they are generated in the context of the apoptotic process. Exposure of T-lymphocytes to relatively moderate concentrations of hydrogen peroxide was found to induce a CD95-independent apoptotic process that requires mitochondrial ROS production and the activation of NF-kB. This underscores the need to transfer lymphocytes to more reducing conditions for the development of immunological effector functions.

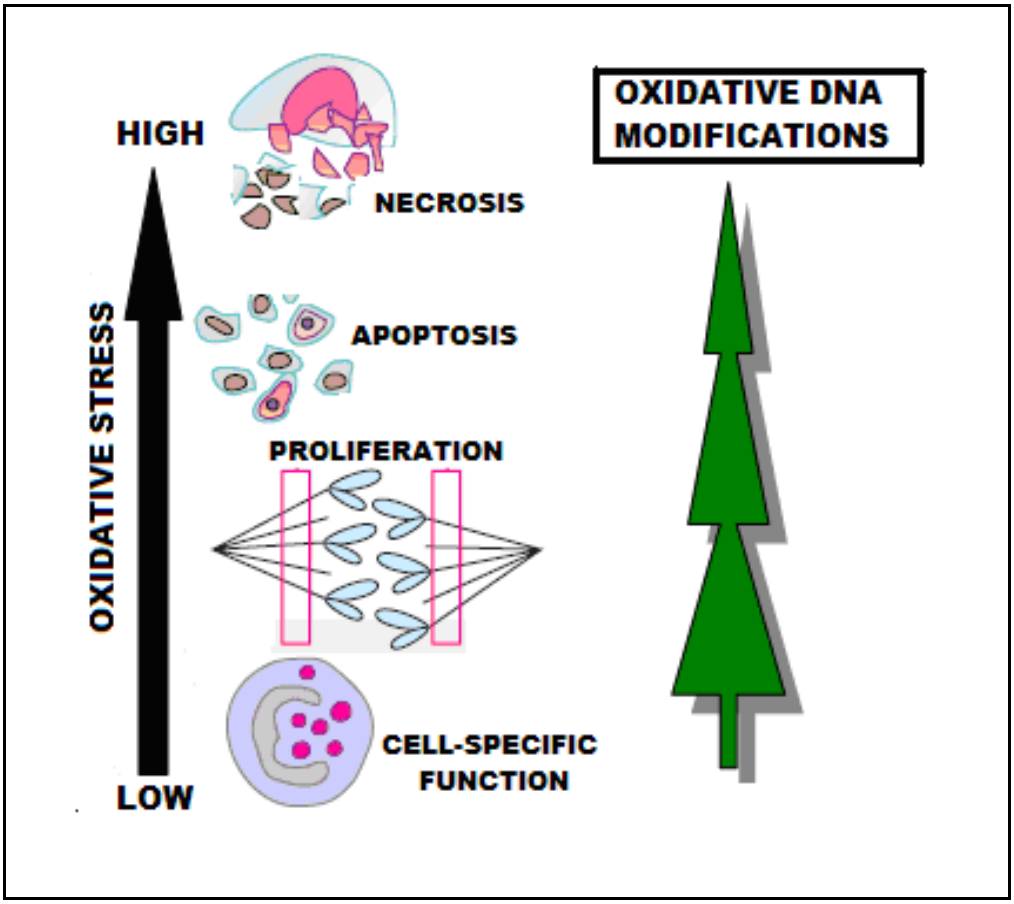
2. NO-dependent apoptosis

NO-dependent apoptosis has been observed in several experimental models and certain clinical pathologies. Induction of apoptosis by NO is associated with a decrease in the concentration of cardiolipin, decreased activity of the mitochondrial electron transport chain, and release of mitochondrial cytochrome *c* into the cytosol. However, some cell types, such as endothelial cells from the microvasculature, are extremely resistant to the induction of apoptosis by NO, and low concentrations of NO provide protection from apoptotic cell death in various cell types by inhibiting certain caspases. High intracellular glutathione levels are associated with increased resistance to NO-mediated apoptosis.

3. Induction of cell death by TNF-α

TNF- α induces cell death in many types of tumor cells and has been used in model systems for studies of the molecular mechanisms of cell death. In transformed cell lines TNF-α induces endogenous ROS production by mitochondria. Whether and how these ROS contribute to the induction of cell death depends on the signaling and execution pathways that are activated. In leukocytes and fibroblasts, TNF- α induces the release of superoxide by the activation of membrane-bound NADPH oxidases. This process induces proliferation or cell death depending on the condition of the ROS-producing cell. There is evidence to suggest that tumor necrosis factor (TNF)-α and interleukin (IL)-1-induced generation of mitochondrial ROS has been implicated in apoptotic cell death. It has also been suggested that the mitochondria may function as an "O_2 sensor" to mediate hypoxia-induced gene transcription.

Figure 2: Cellular response to oxidative stress. Different levels of oxidative stress cause different outcomes in cells.



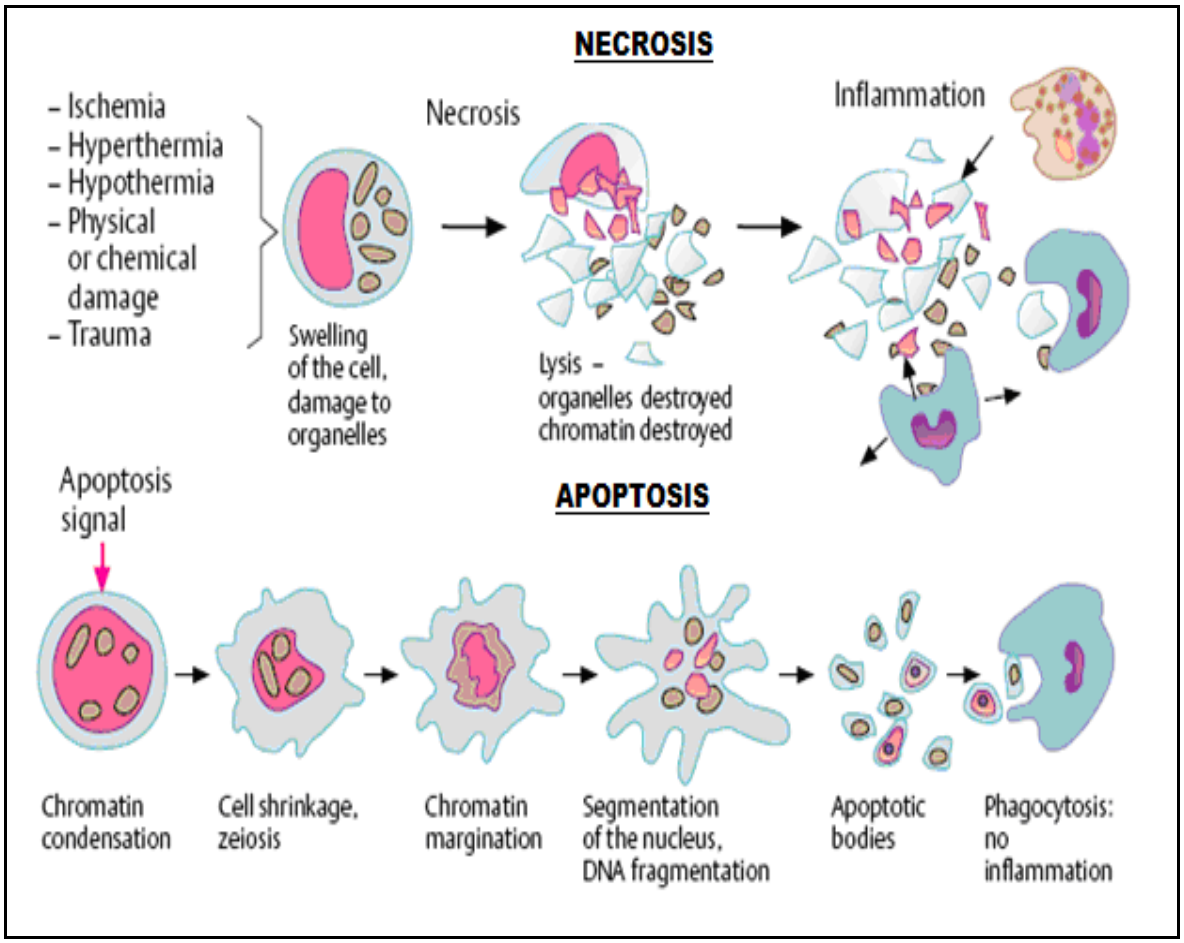
Apoptosis and Necrosis[13]

Cells must constantly die to ensure the normal development of an organism and to maintain the balance between the generation and loss of cells (*homeostasis*). This activity is regulated by programmed cell death, a type of “cell suicide”, the morphological correlative of which is apoptosis. Apoptosis plays an important role for the correct function of the immune system.

Apoptosis is a very subtle process. The first signs of apoptosis are chromatin condensation and shrinkage of the cell. The cell membrane that develops small bulges in a process known as zeiosis (*blebbing*). The cell that begins to expel its contents into vesicles, some of which contain parts of the fragmented and condensed (pyknotic) cell nucleus. The vesicles are ingested and degraded by macrophages. Since the cytoplasmic enzymes and toxic metabolites are always surrounded by membranes, no inflammatory reaction occurs.

Cell necrosis, on the hand, occurs as the result of severe injuries due, for example, to burns, oxygen deficiency (e.g., heart attack), and trauma. In necrosis, the cell membrane loses its integrity. As a result, the cell starts to swell, and the contents, together with a number of toxic substances are released into the surrounding tissue, thus inducing an inflammatory reaction.

Figure 3: Differences between Necrosis and Apoptosis



The potential role of NO in cardiac pathology

Fogli *et al*.[14] proposed the plausible link between elevated NO level and the heart pathology. Increased iNOS expression has been reported in human cardiac disease including dilated cardiomyopathy, heart failure, and contractile dysfunction of the left ventricle after heart transplantation. It is conceivable that the balance between the physiologic and pathologic production of NO may be critical in regulating cardiac function and preserving cardiomyocyte integrity whereas the pathological switch may be expected when there is an abnormal level of NO production, and/or when there is an excess of superoxide anion. For example, excessive production of superoxide in reperfused myocardium can inactivate NO, and this may contribute to the coronary vasoconstriction. On the other hand, excess superoxide reacts with NO enhancing the production of peroxynitrite, a potent oxidant that is able to oxidize lipids, nitrate tyrosine residues on proteins, and decompose to toxic products including the highly reactive hydroxyl radicals. Thus, the abundant synthesis of NO and/or $O_2^{\cdot-}$ generates a free radical cascade that, in turn, may disrupt key cellular targets in the heart and represent a cause of cardiomyopathy. In this regard, evidence is available demonstrating a significant contribution of increased RNS/ROS production and protein nitration in the progression of cardiovascular disease. Finally, the relevance of mitochondria in the damaging effect of NO further support the role of ONOO– in NO-mediated apoptotic damage and cardiotoxicity[15-17]. iNOS up-regulation by cytokines increases apoptosis in cultured myocytes by a process that is independent of guanylate cyclase/cGMP pathway and associated with the shift in the cellular balance toward proapoptotic Bak vs. antiapoptotic Bcl-xL[18,19]. Thus, the available data demonstrate the multiplicity of NO molecular targets and point to the important role of the disarray of NO homeostasis in cardiac cell injury in various heart diseases.

Cellular antioxidant defensive system[20]

To protect against the potentially damaging effects of ROS, mammalian cells encompass an impressive gamut of antioxidant defensive system which includes:

- ❖ Superoxide dismutases (SOD)
- ❖ Catalase (CAT)
- ❖ Glutathione peroxidase (GPx)
- ❖ Glutathione-S-transferase (GST)
- ❖ Glutathione disulphide reductase (GR)
- ❖ Glutathione (GSH)

Superoxide dismutases (SOD)

Superoxide dismutases (SOD) were the first genuine ROS-metabolizing enzymes discovered. In eukaryotic cells, $O_2\cdot^-$ can be metabolized to hydrogen peroxide by two metal-containing SOD isoenzymes:

♥ A tetrameric Mn-SOD present in mitochondria and

♥ A dimeric Cu/Zn-SOD present in the cytosol.

Catalases (CAT)

Antioxidative role of catalase is to lower the risk of hydroxyl radical formation from H_2O_2 via the Fenton-reaction catalyzed by Cu or Fe ions.

Glutathione peroxidases (GPx):

There are at least four different GPx in mammals (GPx1–4), all of them containing selenocysteine. All glutathione peroxidases may catalyze the reduction of H_2O_2 using glutathione as substrate. They can also reduce other peroxides (e.g., lipid peroxides in cell membranes) to alcohols.

Other Glutathione-related systems

Hydrogen peroxide is reduced by glutathione peroxidases (GPx) by oxidation of two molecules of glutathione (GSH) forming glutathione disulfide (GSSG) that subsequently can be reduced by glutathione reductase (GR) under consumption of NADPH. Glutathione also reduces glutaredoxins (Grx) that in their turn reduce various substrates. Specific for glutaredoxins is the reduction of glutathione mixed disulfides such as glutathionylated proteins. Glutathione S-transferases (GST) catalyze the conjugation of glutathione with other molecules, thereby functioning as an intermediate step in the detoxification of miscellaneous toxic substances.

Figure 4: Schematic summary of the major glutathione-associated antioxidant systems

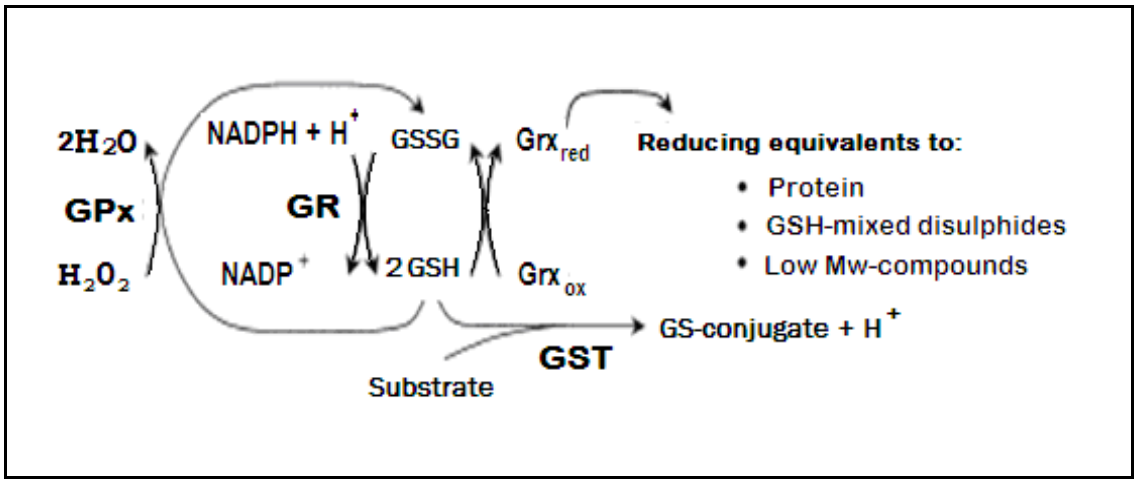
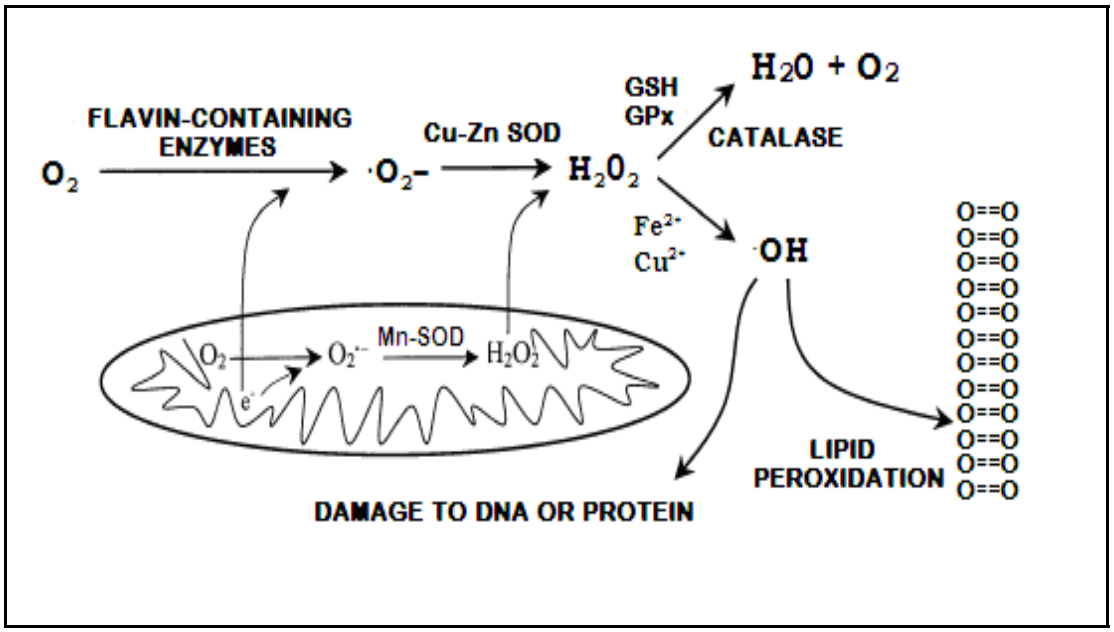


Figure 5: Simplified non-stoichiometric scheme of oxidative and antioxidative systems in cells



Superoxide is produced in significant amounts intracellularly, both in the cytosol via flavin-containing enzymes, and in mitochondria, mainly due to escape of electrons from the respiratory chain. Two molecules of superoxide rapidly dismutate, either spontaneously or via superoxide dismutases to dioxygen and hydrogen peroxide, the latter permitting flux of ROS between cellular compartments. Hydrogen peroxide can be enzymatically metabolized to dioxygen and water by a number of different enzyme systems or converted to the hydroxyl radical, which is extremely reactive, via a chemical reaction catalyzed by transition metals.

Antineoplastic agents

The anticancer drugs either kill cancer cells or modify their growth. A tumor (neoplasm) consists of cells that proliferate independently of the body's inherent "building plan." A malignant tumor (cancer) is present when the tumor tissue destructively invades healthy surrounding tissue or when dislodged tumor cells form secondary tumors (metastases) in other organs[21].

Cancer chemotherapeutic modalities[22]

A. Curative therapy

In curative therapy, the malignant cells are completely removed. This is now possible in:

- Acute leukemia
- Retinoblastoma
- Lymphosarcoma
- Testicular teratoma
- Hodgkin's disease
- Mycosis fungoides

B. Palliative therapy

When curing is not possible, attempts can be made to slow tumor growth and thereby prolong the patient's life or improve quality of life, which is termed as "palliative therapy". Chemotherapy is faced with the problem that the malignant cells are endogenous and are not endowed with special metabolic properties.

C. Adjuvant therapy

Drugs are used to mop up any residual malignant cells after surgery or radiotherapy. This is routinely employed now.

Classification of the Anticancer Drugs[23]

I. Alkylating agents

A. Nitrogen mustards

1. Cyclophosphamide
2. Chlorambucil
3. Melphalan
4. Ifosfamide

B. Alkyl sulfonates

1. Busulfan

C. Nitrosoureas

1. Carmustine
2. Streptozocin

D. Ethylenimines

1. Thiotepa

E. Triazenes

1. Dacarbazine

II. Antimetabolites

A. Folate antagonist

1. Methotrexate

B. Purine analogues

1. Thioguanine
2. Mercaptopurine

C. Pyrimidine analogues

1. Cytarabine
2. Fluorouracil

III. Antibiotics

A. Anthracyclines

1. Doxorubicin hydrochloride (*Adriamycin*)
2. Daunorubicin

IV. Plant-derived products

- *A. Vinca alkaloids*
    1. Vincristine
    2. Vinblastine
- *B. Epipodophyllotoxins*
    1. Etoposide
    2. Teniposide

V. Enzymes

- *A. L-Asparaginase*

VI. Hormonal agents

- *A. Glucocorticoids*
- *B. Estrogens, antiestrogens*
    1. Tamoxifen citrate
- *C. Androgens, antiandrogens*
- *D. Progestins*
- *E. Luteinizing hormone–releasing hormone (LH-RH) antagonists*
- *F. Octreotide acetate*

VII. Miscellaneous agents

- *A. Hydroxyurea*
- *B. Procarbazine*
- *C. Cisplatin*
- *D. Carboplatin*

VIII. Monoclonal antibodies

IX. Immunomodulating agents

- *A. Levamisole*
- *B. Interferons*
- *C. Interleukins*

General toxicological properties of anticancer drugs

Most of the drugs used in cancer treatment have a therapeutic index that approaches unity, exerting toxic effects on both normal and tumor tissues even at optimal dosages. This lack of selective toxicity is the major limiting factor in the chemotherapy of cancer. Rapidly proliferating normal tissues, such as bone marrow, gastrointestinal tract, and hair follicles, are the major sites of acute toxicity of these agents. In addition, acute toxicities like cardiotoxicity, urothelial toxicity, and even chronic and cumulative toxicities may occur. The most commonly encountered toxicities of antineoplastic agents are described in the following section:

- Bone marrow toxicity
- Gastrointestinal tract toxicity
- Hair follicle toxicity
- Cardiotoxicity.

Bone marrow toxicity

Chemotherapy may result in the destruction of actively proliferating hematopoietic precursor cells. White blood cell and platelet counts may in turn be decreased, resulting in an increased incidence of life-threatening infections and hemorrhage. The risk of serious infections has been shown to increase greatly when the peripheral blood granulocyte count falls below 1000 cells/mm^3. A chronic bone marrow toxicity or hypoplastic state may develop after long-term treatment with nitrosoureas, other alkylating agents, and mitomycin C. Thus, patients frequently will require a progressive reduction in the dosages of *myelosuppressive* drugs when they are undergoing long-term therapy, since such treatment may result in chronic pancytopenia.

Gastrointestinal tract toxicity

The nausea and vomiting frequently observed after anticancer drug administration are actually thought to be caused by a stimulation of the vomiting center or chemoreceptor trigger zone in the central nervous system (CNS) rather than by a direct gastrointestinal effect. These symptoms are ameliorated by treatment with phenothiazines and other centrally acting antiemetics. Commonly, nausea begins 4 to 6 hours after treatment and lasts 1 or 2 days. Although this symptom is distressing to patients, it is rarely severe enough to require cessation of therapy. Anorexia and alterations in taste perception also may be associated with chemotherapy.

The serotonin antagonist ondansetron has proved effective in the prevention of nausea and vomiting due to chemotherapy. Damage to the normally proliferating mucosa of the gastrointestinal tract may produce stomatitis, dysphagia, and diarrhea several days after treatment. Oral ulcerations, esophagitis, and proctitis may cause pain and bleeding.

Hair follicle toxicity

Most anticancer drugs damage hair follicles and produce partial or complete alopecia. Patients should be warned of this reaction, especially if paclitaxel, cyclophosphamide, doxorubicin, vincristine, methotrexate, or dactinomycin is used. Hair usually regrows normally after completion of chemotherapy.

Cardiotoxicity[24]

Cardiotoxicity occurs during therapy with several cytotoxic drugs and may be the dose limiting factor in cancer treatment and hence tumour response. Furthermore, cardiotoxicity can also be responsible for long term side effects and may cause severe morbidity in surviving cancer patients, which may be relevant especially in pediatric oncology. Cardiotoxicity from cytotoxic treatment is known to have a high prevalence. Cardiotoxicity includes a wide range of cardiac effects from small changes in blood pressure and arrhythmias to cardiomyopathy. In

literature different mechanisms of chemotherapy induced cardiotoxicity are postulated including cellular damage due to the formation of free oxygen radicals and the induction of immunogenic reactions with the presence of antigen presenting cells in the heart. Moreover, the influence of the cytotoxic agent on certain phospholipids, especially cardiolipin, may also explain the development of cardiotoxicity. The anthracyclines, such as doxorubicin and epirubicin, are potent cytotoxic drugs but their clinical use is often limited by their cardiotoxic side effects. Other cytotoxic drugs that have reported cardiotoxicity include **cyclophosphamide**, 5-fluorouracil, capecitabine, mitoxantrone, cisplatin, the taxoids paclitaxel and docetaxel and newer drugs such as the monoclonal antibody trastuzumab.

Plants and human health

For centuries people have used plants for healing. Plant products – as parts of foods or botanical potions and powders – have been used with varying success to cure and prevent diseases throughout history. Written records about medicinal plants date back at least 5000 years to the Sumerians[25], and archeological records suggest even earlier use of medicinal plants.

The US Federal Food and Drug Administration (FDA) recently published guidance for standardized multifunctional and multicomponent plant extracts, referred to as botanical drugs, thus making it possible to market these products under the New Drug Application (NDA) Approval Process[26]. In response to the public demand for trustworthy and effective alternatives to new chemical entity (NCE) pharmaceuticals, the agency proposed abbreviated preclinical and clinical testing protocols for botanical drugs derived from plants with a safe history of human use. This has enabled US industrial and academic scientists to become involved in botanical drug R&D efforts. Botanical drugs are fully accepted and widely prescribed in China, Japan, India and other Asian and African countries. In addition, some countries in Europe, such as Germany, allow physicians prescribe botanical drugs.

Botanical dietary supplements

Botanical dietary supplements – also called botanical nutraceuticals or herbals – can be best defined as plant-derived materials with medical benefits aimed at disease prevention or treatment that go beyond satisfying basic nutritional requirements.

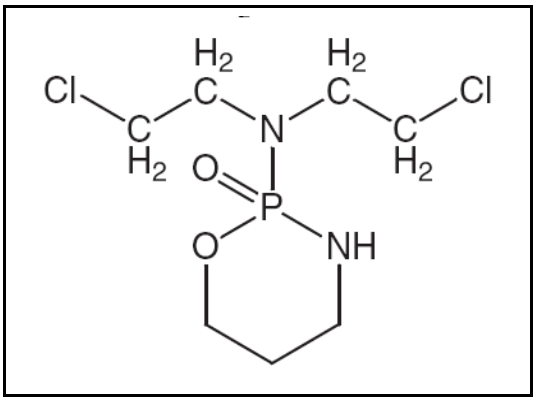
Common botanical dietary supplements sold in the USA and their therapeutic indications[27]

Echinacea purpurea L.angustifolia, DC. and *pallida* Nutt.	- Respiratory infections, Immunostimulant.
Panax ginseng, L.A. Mey (ginseng)	- Fatigue and stress, high cholesterol, diabetes, gastro-intestinal disorders.
Serenoa repens (W. Bartam) Small (saw palmetto)	- Benign prostatic hyperplasia (BPH), inflamations, impotence.
Ginkgo biloba L.	- Dementia, cognitive decline, mental fatigue.
Hypericum perforatum L. (St John's wort)	- Mild and moderate depression, Epilepsy.
Valeriana officinalis L. (valerian)	- Sleep improvements, anxiety, Hypertension.
Allium sativum L. (garlic)	- Cancer, high cholesterol, diabetes, arteriosclerosis, hypertension,
(contd.)	
Allium sativum L. (garlic)	respiratory infections.
Hydrastis canadensis L. (goldenseal)	- Diarrhea, respiratory and Gastrointestinal infections, Constipation.
Matricaria chamomilla L. (German chamomile)	- Intestinal disorders, wound healing, inflammations, anxiety.
Silybum marianum gaertn. (milk thistle)	- Liver disorders, lactation problems.

Trigonella foenum-graecum L. (fenugreek)	- Diabetes, loss of appetite, skin Inflammation.
Tanacetum –parthenium Schultz-Bip. (feverfew)	- Migraines, inflammation.
Ephedra sinica Stapf. (Ephedra, Ma Huang)	- Stimulant, obesity, asthma, congestion, fluid retention.
Cimicifuga racemosa Nutt. (black cohosh)	- Premenstrual symptoms, dysmenorrhea, menopausal symptoms.

DRUG PROFILE

Cyclophosphamide



Properties[28,29]

- Cyclophosphamide is an alkylating agent and a derivative of nitrogen mustard.
- It is an odorless, fine white crystalline powder.
- Molecular weight is 261.1 and melting point is 49.5°C to 53°C.

- ❖ It is soluble in water and ethanol; slightly soluble in benzene, ethylene glycol, carbon tetrachloride, and dioxane; and sparingly soluble in diethyl ether and acetone. Its log octanol-water partition coefficient is 0.63.
- ❖ It reacts with strong oxidizing agents.
- ❖ It is sensitive to moisture and light, and is hydrolyzed in aqueous solutions above 30°C.

Indications

- ► Cyclophosphamide is used as a drug to treat cancer and other medical conditions. In chemotherapy, it may be used alone, but more frequently is used concurrently or sequentially with other anticancer drugs.
- ► It is used to treat malignant lymphoma, multiple myeloma (bone-marrow cancer), leukemia, breast and ovarian cancer, neuroblastoma (childhood nerve-cell cancer), retinoblastoma (childhood cancer of the retina), and mycosis fungoides (lymphoma of the skin)[30-32].
- ► Cyclophosphamide also is used as an immunosuppressive agent following organ transplants
- ► It is used to treat autoimmune disorders such as rheumatoid arthritis, Wegener's granulomatosis (an inflammation of the blood vessels), and nephrotic syndrome (a kidney disorder) in children[33].

Proprietary names[34]

- ❖ Ledoxan® - Dabur Pharmaceuticals Ltd.
- ❖ Cyphos® - Intas Pharmaceuticals Pvt. Ltd.
- ❖ Eldamide® - Elder Pharmaceuticals Pvt. Ltd.
- ❖ Neophos® - Cipla Ltd.
- ❖ Oncophos® - Cadila Pharmaceuticals Ltd.
- ❖ Cydoxan® - Alkem Laboratories Ltd.

Preparations[34]

- Cyclophosphamide is available in the strength of 50-mg tablet preparations.
- In a crystalline hydrate form for injection in strengths of 100 to 2,000 mg.

Dosage[34]

- Doses used in medical treatment depend on the patient and the specific disease.
- Cyclophosphamide may be given orally (in 25- or 50-mg tablet form) or by i.v. injection (100-mg, 200-mg, 500-mg, 1-g, or 2-g vials).
- The initial treatment for cancer patients with no hematologic deficiency may be 40 to 50 mg/kg of body weight in divided i.v. doses over two to five days, 10 to 15 mg/kg every seven to ten days, or 3 to 5 mg twice a week.
- The adult dosage for tablets typically is 1 to 5 mg/kg of body weight per day for both initial and maintenance treatment.
- For nonmalignant diseases, an oral dose of 2.5 to 3 mg/kg per day is administered for 60 to 90 days[32].

Contraindications

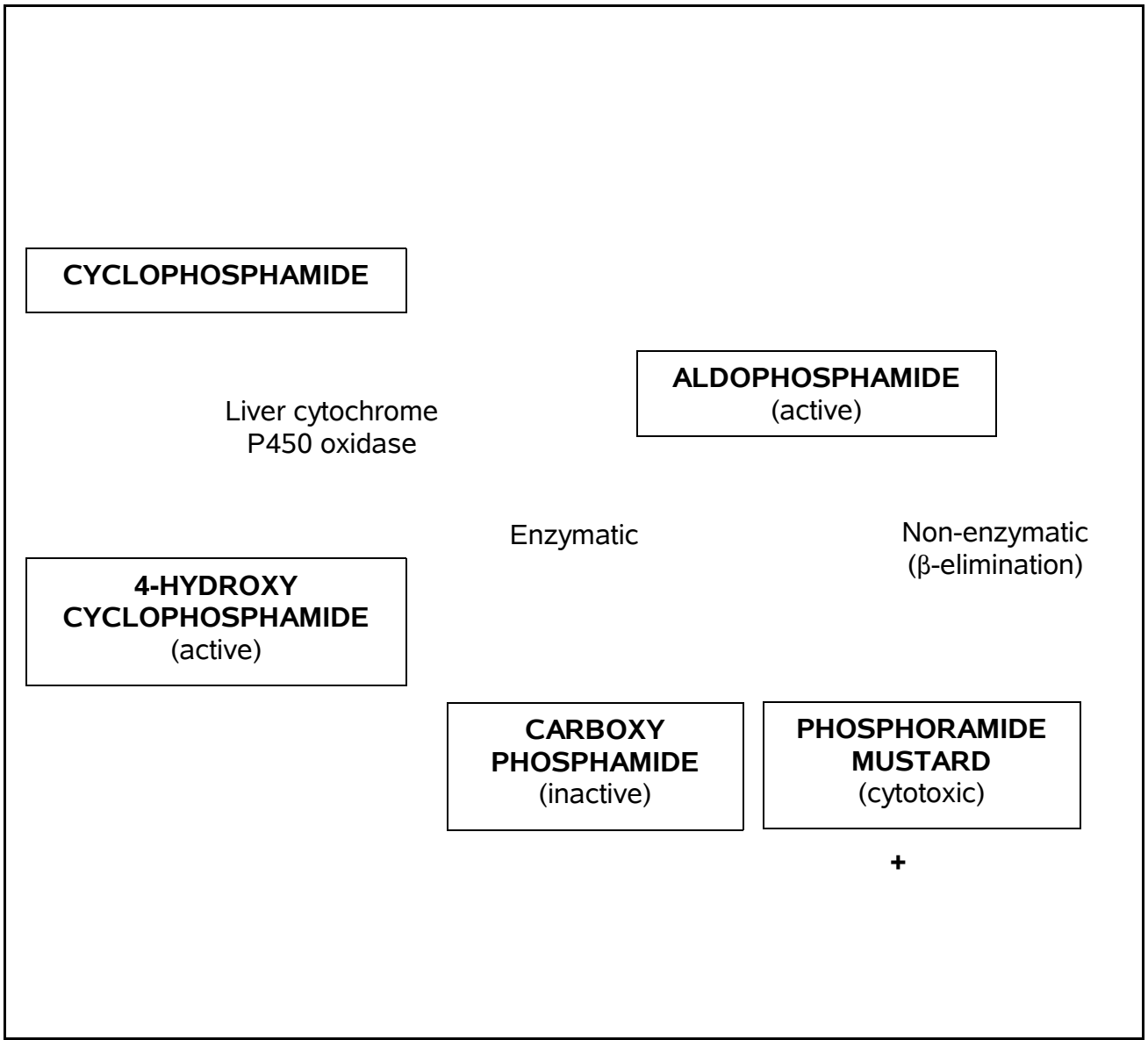
- Bladder haemorrhage
- Pregnancy
- Lactation.

Precautions

- Diabetics
- Liver, heart or kidney impairment
- Geriatrics
- Pediatrics.

Adverse effects

- Alopecia
- Myelosuppression
- GI Toxicity
- Cardiotoxicity
- Haemorrhagic cystitis
- Pulmonary toxicity
- Infertility
- Carcinogenesis



+

ACROLEIN
(cytotoxic)

4-KETO CYCLOPHOSPHAMIDE
(inactive)

PLANT PROFILE

Taxonomic classification

Plant name	**:** *Ficus hispida* Linn.
Division	**:** Magnoliophyta
Class	**:** Magnoliopsida
Subclass	**:** Rosidae
Order	**:** Rosales
Family	**:** Moraceae (Mulberry)
Genus	**:** *Ficus*
Species	**:** *hispida*

Vernacular names

Bengali - Dumoor

English - Rough-leaved fig

Hindi - Gobla

Tamil - Peyatti

Telugu - Bhramhamedi.

Distribution

Ficus hispida Linn. Is widely distributed throughout India, Sri Lanka, Myanmar, sothern regions of the People's republic of China, New Guinea, Thailand, Vietnam, Nepal and Queensland in Australia.

Habitat

F. hispida is a shrub or moderate sized tree that grows in secondary forests, open lands, and riverbanks, at an altitude of 450-1200 m.

Description[36]

Coarsely hairy; dioecious. Stipules usually 4 and decussate on leafless fruiting branchlets, ovate-lanceolate. Leaves opposite; petiole with short thick hairs; leaf blade ovate, oblong, or obovate-oblong, thickly papery, abaxially with coarse gray hairs, adaxially rough and with short thick hairs, margin entire or bluntly toothed, apex acute to mucronate.

Uses in Indigenous medicine[37,38]

- Different plant parts are used in skin diseases, leucoderma, and jaundice and as anti-poisonous.
- Leaves are used as an anti-diarrhoeal, antitussive, antipyretic, astringent, anti-inflammatory, haemostatic, anti-ulcer, vulnerary and in treatment of liver disorders.
- Bark as emetic, anti-diarrhoeal and in the treatment of diabetes mellitus.
- Fruits as tonic, lactogogue, cooling, astringent and in hepatic obstruction.

Phytoconstituents

- The presence of phenanthroindolizidine alkaloids, *n*-alkanes, coumarins, triterpenoids, flavonoids, oxyterpene, tannins, and saponins was documented.
- The leaves are reported to contain *O*-methyltylophorinidine[39], oleanolic acid, hispidin, bergapten, psoralene, β-amyrin, and β-sitosterol[40].
- The bark was reported to contain lupeol acetate, 10-ketotetracosyl arachidate[41], β-amyrin and triacontanol acetate[42].

LITERATURE REVIEW

Cyclophosphamide

Santos *et al.*[43] reported the first human fatality of cyclophosphamide (CP) cardiotoxicity as a complication of bone marrow transplantation. High-dose cyclophosphamide was introduced as a mainstay of numerous preparative regimens for haemopoietic stem-cell transplantation and its potential to cause myocardial damage was soon recognized.

De Souza *et al.*[44] demonstrated that high-dose cyclophosphamide (HDCY) therapy could cause multiple organ toxicities including cardiac, hepatic and pulmonary damage. Clinical trials performed by their team infer that pretreatment with antioxidants like amifostine might confer protection against high-dose cyclophosphamide therapy could cause multiple organ toxicities. With amifostine, side effects were mild and easily controlled. They concluded that amifostine protection should be useful in HDCY to protect normal tissues, with acceptable side effects.

Gharib and Burnett[45] stated that cardiotoxicity is a potential side effect of few chemotherapeutic agents. The anthracycline class of cytotoxic antibiotics is the most famous, but other chemotherapeutic agents can also cause serious cardiotoxicity and are not so well recognised. Examples include cyclophosphamide, ifosfamide, mitomycin and fluorouracil. High therapeutic doses of cyclophosphamide could cause a lethal cardiotoxicity that presents a combination of symptoms and signs of myo-pericarditis which could lead to fatal complications such as congestive heart failure (CHF), arrhythmias and cardiac tamponade.

Lindley *et al*.[46] proposed that CP itself is a prodrug and it is bioactivated by hepatic cytochrome P450 enzymes via the predominant pathway, 4-hydroxylation, resulting in the formation of 4-hydroxycyclophosphamide (HCY), the major active circulating metabolite that is converted intracellularly to its tautomer aldophosphamide which is further metabolised to the DNA cross-linking agent phosphoramide mustard (PM) and a toxic-byproduct, acrolein.

Yousefipour *et al*.[47] identified that acrolein, an environmental pollutant and a lipid peroxidation product is implicated in vascular pathogenesis. This study investigated the effects of acrolein on vascular function to understand the underlying mechanism of acrolein-induced vascular responses. Urine and blood samples were collected. Changes in systolic blood pressure (SBP) and responses to acetylcholine and phenylephrine were determined. Acrolein (4 mg/kg, 7 days) significantly increased SBP by 25%, phenylephrine vasoconstriction by 2-fold, but decreased urinary excretion of nitrite by 25%. Acrolein inhibited generation of cyclic guanosine 3'5'-monophosphate (cGMP) by 98%, and did not alter expression of nitric oxide synthase (eNOS). Acrolein increased the generation of lipid hydroperoxide in plasma and aortic tissue by 21% and 124% respectively, increased glutathione-S-transferase (GST) and glutathione peroxidase (GSH-Px) activities. Acrolein up-regulated the expression of GST by 2 fold. These data suggest that induced SBP and altered vasoconstriction/vasodilatation in acrolein treated rats may be due to reduced availability of NO via increased free radical generation and reduced antioxidant defense.

Lee *et al*.[48] postulated that "**oxidative stress"** might play a pivotal role in cyclophosphamide-induced cardiotoxicity. Lee and his colleagues also suggested that acute decompensating cardiomyopathy induced by cyclophosphamide is usually irreversible. To investigate the clinical course and the outcome of therapy, 13 patients (1.7%) with grade III acute cardiomyopathy and hypotension who were treated with ablative transplant regimens between January 1980 and September 1995 were analyzed by their team. Eight of nine patients died of acute fatal restrictive cardiomyopathy with unresponsive hypotension (ARCH), whereas three of four patients who survived the initial episode died of subacute congestive heart failure (SCHF). Acute fatal restrictive cardiomyopathy was characterized with extreme sensitivity to volume overload, myocardial edema and a rapidly fatal course. It was associated with progressive, unresponsive hypotension, reduced left ventricular stroke work index and markedly reduced

systemic and pulmonary vascular resistance indices. Of 10 patients who received conventional therapy, nine died and one sustained chronic CHF. One of three patients with ARCH on antioxidant therapy of ascorbic acid and theophylline survived the episode. The data suggests peripheral vascular collapse may also be responsible for fatal ARCH.

Sies[6] defined "oxidative stress" as an imbalance between oxidants and antioxidants in favour of the oxidants, potentially leading to damage. Oxidants are formed as a normal product of aerobic metabolism but can be produced at elevated rates under pathophysiological conditions. Antioxidant defense involves several strategies, both enzymatic and non-enzymatic. In the lipid phase, tocopherols and carotenes as well as oxy-carotenoids are of' interest, as are vitamin A and ubiquinols. In the aqueous phase, there are ascorbate, glutathione and other compounds. In addition to the cytosol, the nuclear and mitochondrial matrices and extracellular fluids are protected. Overall, these low molecular mass antioxidant molecules add significantly to the defense provided by the enzymes superoxide dismutase, catalase and glutathione peroxidases.

Ficus hispida

Mandal *et al.*[49] evaluated the methanol extract of the leaves of ***Ficus hispida*** Linn. (Moraceae) for hepatoprotective activity in rats by inducing acute liver damage by paracetamol (750 mg/kg, p.o.). The extract at an oral dose of 400 mg/kg exhibited a significant protective effect by lowering the serum levels of transaminase (SGOT and SGPT), bilirubin and alkaline phosphatase (ALP). These biochemical observations were supplemented by histopathological examination of liver sections. The activity of extract was also comparable to that of Liv-52 a known hepatoprotective formulation.

Khan *et al.*[40] isolated oleanolic acid from the leaves of ***Ficus hispida*** and he had cited that ***Ficus hispida*** leaves contain bergapten, β-amyrin and β-sitosterol.

Huong and Trang[50] extracted six alkaloids in the total alkaloid residue from the leaves of ***Ficus hispida*** L. One of them was elucidated as hispidin by spectrocopic method which has demonstrated cytotoxicity toward human hepatic cancer cell line Hep-2 and human heart cancer line RD.

Senthil *et al.*[51] investigated the protective effect of oleanolic acid (OA) against isoproterenol-induced myocardial ischemia in rat myocardium. Wistar strain rats were pretreated with OA (20, 40, and 60 mg/kg, s.c) for 7 days and then intoxicated with isoproterenol (ISO, 85 mg/kg, sc for 2 consecutive days). Heart were excised from the experimental animals and assessed for the activities of marker enzymes, the levels of lipid peroxide products [thiobarbituric acid reactive substances (TBARS), lipid hydroperoxides (HP) and conjugated dienes (CD)], myeloperoxidase (MPO), lipid profiles, and membrane-bound ATPase enzymes. Troponin T and I were estimated in plasma. Leakage of cardiac markers, elevated lipid peroxidation with increased lipid profiles and decreased activities of membrane-bound ATPase enzymes were confirmed the severe myocardial damage occurring as a consequence of isoproterenol-induced ischemia, and they also showed the significant improvement effected by oleanolic acid pretreatment. These findings provided evidence that oleanolic acid was found to be protecting rat myocardium against ischemic insult and the protective effect could attribute to its anti-oxidative, anti-hyperlipedemic, and anti-arrhythmic properties as well as its membrane-stabilizing action.

Park *et al.*[52] in the course of screening for reactive oxygen species scavengers from natural products, isolated an antioxidant from the mycelial culture broth of Phellinus linteus and identified as hispidin. The hispidin content was reached its maximum level at 12 days after onset of inoculation. About 2.5 mg/mL of hispidin was produced by P. linteus in a yeast-malt medium. Hispidin inhibited 22.6 and 56.8% of the super oxide anion radical, 79.4 and 95.3% of the

hydroxyl radical, and 28.1 and 85.5% of the DPPH radical at 0.1 and 1.0 mM, respectively. The positive control alpha-tocopherol scavenged 25.6 and 60.3%, 74.6 and 96.3%, and 32.7 and 77.5% of each radical, respectively, at the same concentrations.

Peraza-Sánchez[39] isolated a new norisoprenoid, ficustriol (1), and the known phenanthroindolizidine alkaloid O-methyltylophorinidine (2), from a $CHCl_3$ extract of the leaves and twigs of Ficus hispida. O-Methyltylophorinidine showed potent cytotoxic activity when tested against a small panel of human cancer cells, while ficustriol was inactive.

Yang *et al.*[53] examined the molecular mechanisms for the anti-inflammatory activity of phenanthroindolizidine alkaloids were in an in vitro system mimicking acute inflammation by studying the suppression of lipopolysaccharide (LPS)/interferon-gamma (IFNgamma)-induced nitric oxide production in RAW264.7 cells. Two of the phenanthroindolizidine alkaloids, NSTP0G01 (tylophorine) and NSTP0G07 (ficuseptine-A), exhibited potent suppression of nitric oxide production and did not show significant cytotoxicity to the LPS/IFNgamma-stimulated RAW264.7 cells, in contrast to their respective cytotoxic effects on cancer cells. Tylophorine was studied further to investigate the responsible mechanisms and it was found that NSTP0G01 exerts its anti-inflammatory effects by inhibiting expression of the proinflammatory factors and related signaling pathways.

Liu[54] reviewed the pharmacology of oleanolic acid and cited that oleanolic acid has protective effect against cyclophosphamide-induced toxicities.

OBJECTIVE AND SCOPE OF RESEARCH

The most imperative obstacle in cancer chemotherapy is the non-specific cytotoxic action on both the tumor cells and normal healthy cells.[55] Cyclophosphamide (CP), an oxazophosphorine-alkylating agent, is extensively used as an antineoplastic and immunosuppressant drug. Despite its wide spectrum of clinical uses, CP is known to cause multiple organ toxicity.[44] High therapeutic doses of cyclophosphamide could cause a lethal cardiotoxicity that presents a combination of symptoms and signs of myo-pericarditis which could lead to fatal complications such as congestive heart failure (CHF), arrhythmias and cardiac tamponade.[45]

The prime factor for therapeutic and toxic effects of cyclophosphamide is the requirement of bioactivation by hepatic microsomal cytochrome P450 mixed functional oxidase system, to phosphoramide mustard and acrolein, which are the active cytotoxic metabolites.[46,56] Bioconversion of CP to these metabolites leads to the formation of high levels of reactive oxygen species (ROS), which culminate in oxidative stress.[57] An ample literature implicate that oxidative stress plays a key role in the pathogenesis of CP induced cardiotoxicity.[5,48]

Cytoprotectants like amifostine, mesna and dexrazoxane were used to manage the toxic effects of cancer chemotherapy, but these agents are not approved for wide clinical use due to lack of efficacy, gastrointestinal side effects, hypotension, hypersensitivity reactions, anxiety, urinary retention and myelosuppression.[58-60] Limitations to such conventional treatment have spurred the development of new treatment modalities.

Plants are arguably poised for a comeback as sources of human health products, mainly due to their enormous propensity to synthesize complex mixtures of structurally diverse compounds, which could provide a safer and more holistic approach to disease treatment and prevention.[27] Plant extracts and natural compounds have also shown protective effect on CP-induced toxicity.[61-65]

The genus *Ficus* constitutes an important group of trees, not only of their immense medicinal value but also of their growth habits and religious significance. The genus *Ficus* is an exceptionally large pantropical genus with over 700 species and belongs to the family Moraceae. Almost all parts of this plant are used as a folklore remedy for the treatment of various ailments by Indian traditional healers but the leaves are of particular interest from a medicinal point of view, as an anti-diarrhoeal,[38] hepatoprotective,[49] anti-inflammatory,[66] antitussive, antipyretic, astringent, vulnerary, haemostatic and anti-ulcer drug, among other parts.[67,68]

Ficus hispida leaves have been reported to contain hispidin, oleanolic acid, bergapten, psoralen, β-amyrin and β-sitosterol[40,50] and the bark comprises lupeol acetate, β-sitosterol and β-amyrin acetate.[41,69] An ample literature suggests that these compounds exhibit significant antioxidant and/or cardioprotective properties.[70-74]

In this light, I hypothesized that *F. hispida* could be evaluated for its cardioprotective effect. The claim that the cardioprotective activity of *F. hispida* resides in the leaves is speculative and has not yet been documented. The present study was designed to investigate the cardioprotective activity of the methanolic leaf extract of ***Ficus hispida*** on cyclophosphamide induced oxidative cardiac injury in rats.

DESIGN OF WORK

CHAPTER 1: Collection and processing of plant

- Collection and authentication of plant
- Drying and pulverization
- Preparation of extract

CHAPTER 2: Phytochemical investigation

- Preliminary phytochemical screening

CHAPTER 3: *In vivo* animal studies

- Purchase of drugs and chemicals
- Procurement of animals
- Treatment regimen
- Biochemical investigation
    - Assay of enzymatic indices
    - Assay of tissue antioxidants
    - Assay of lipid peroxidation
    - Assay of protein content
    - Assay of TNF-α
    - Assay of nitric oxide.
- Histopathological investigation
- Statistical analysis.

EXPERIMENTAL PROTOCOLS

CHAPTER 1: COLLECTION AND PROCESSING OF PLANT

I. Collection and authentication of plant

The leaves of *Ficus hispida* Linn. (Moraceae) were collected during the month of February 2007 from the herbal garden of Anna Siddha Hospital and Research Centre, Chennai, India. A voucher specimen (PARC/2007/Vel's/28) was deposited in the Plant Anatomy Research Centre, Pharmacognosy Institute, Chennai, India and was authenticated by Dr.Jayaraman.

II. Drying and pulverization

The leaves were dried under shade and pulverized in a mechanical grinder and stored in a closed container for further use.

III. Preparation of extract

The powdered leaves were defatted with petroleum ether (B.P. 60–80°C) and then extracted with methanol in a Soxhlet extractor. On evaporation of methanol from the methanol extract *in vacuo*, a greenish coloured residue was obtained (yield 4.7% (w/w) with respect to the dry starting material) and was stored in a desiccator and used for further studies.

CHAPTER 2: PHYTOCHEMICAL INVESTIGATION

The extract obtained from the powdered leaves of *Ficus hispida* was subjected to qualitative tests for the identification of various phytoconstituents[75-78].

I. Test for alkaloids

1. **Mayer's test**: To the 1ml of extract, 1 ml of Mayer's reagent (Potassium mercuric iodide solution) was added. Appearance of yellowish-whitish precipitate indicates the presence of alkaloids.
2. **Dragendorff's test**: To the 1ml of extract, 1 ml of Dragendorff's reagent (potassium bismuth iodide solution) was added. An orange-red precipitate indicates the presence of alkaloids.
3. **Hager's test**: To 1 ml extract, 3ml of Hager's reagent (saturated aqueous solution of picric acid) was added; yellow coloured precipitate indicates the presence of alkaloids.
4. **Wagner's test**: To the 1 ml of extract 2 ml of Wagner's reagent (iodine in potassium iodide) was added, formation of reddish brown precipitate indicates the presence of alkaloids.

II. Test for triterpenoids

1. **Noller's test:** Two or three granules of tin metal were added to 2 ml thionyl chloride solution. Then 1ml of the extract was added into test tube and warmed, the formation of pink colour indicates the presence of triterpenoids.

III. Test for flavonoids

1. **Shinoda's Test:** The alcoholic extract was treated with magnesium foil and concentrated HCl. Appearance of orange red colour indicates the presence of flavonols.
2. The drug in alcoholic and aqueous solution with few ml of ammonia was seen under U.V. and visible light; formation of fluorescence indicates the presence of flavonoids.

3. Little quantity of extract was treated with amyl alcohol, sodium acetate and ferric chloride. A yellow colour solution formed, disappears on addition of an acid indicates the presence of flavonoids.
4. The extract was treated with sodium hydroxide; formation of yellow colour indicates the presence of flavones.
5. The extract was treated with concentrated H_2SO_4, formation of yellow or orange colour indicates the presence of flavones.
6. The alcoholic and aqueous extract was treated with 10% sodium chloride; formation of yellow colour indicates the presence of coumarins.

IV. Test for steroids

1. **Liebermann-Burchard test:** The extract was dissolved in a few drops of chloroform, 10 drops of acetic anhydride, 3ml of glacial acetic acid were added, warmed and cooled under the tap and drops of concentrated sulphuric acid were added along the sides of the test tube. Appearance of bluish-green colour indicates the presence of sterols.
2. **Salkowski test:** The extract was dissolve in chloroform and equal volume of conc. H_2SO_4 was added. Formation of bluish-red to cherry colour in chloroform layer and green fluorescence in the acid layer represents the steroidal components in the tested extract.

V. Test for tannins and phenolics

1. **Lead acetate test:** To the filtrate, basic lead acetate solution (10%) was added. Formation of white precipitate indicates the presence of tannins.
2. **Ferric chloride test:** To 1ml of filtrate, ferric chloride solution was added, formation of a deep blue or greenish black colour shows the presence of tannins.
3. The filtrate was treated with potassium ferric cyanide and ammonia solution. A deep red colour indicates the presence of tannins.

4. To the test extract, strong potassium dichromate solution was added. A yellow colour precipitate indicates the presence of tannins and phenolics.

VI. Test for Saponins:

1 ml of the extract was diluted separately with distilled water to 20 ml and was shaken in a graduated cylinder for 15 minutes lengthwise. A one cm layer of foam indicates the presence of saponins.

VII. Test for carbohydrates

1. **Molisch's test:** To 2ml of the extract, 1 ml of α-napthol solution was added; concentrated sulphuric acid was added through the side of the test tube. Purple or reddish violet colour at the junction of the two liquids reveals the presence of carbohydrates.
2. **Fehling's test:** To 1ml of the extract, add equal quantities of Fehling's solution A and B, upon heating formation of a brick red precipitate indicates the presence of sugars.
3. **Benedict's test:** To 5 ml of Benedict's reagent, add 1ml of extract solution and boil for 2 minutes and cool. Formation of red precipitate shows the presence of sugars.

VIII. Test for proteins and amino acids

1. **Biuret test:** 2 drops of 1% $CuSO_4$ solution was added to 1 ml of 40% sodium hydroxide solution till the appearance of blue colour, and then 1 ml of the extract was also added. Formation of pinkish or purple violet colour indicates the presence of proteins.
2. **Ninhydrin test:** Two drops of freshly prepared 0.2% ninhydrin reagent (0.1% solution in n-butanol) were added to small quantity of the extract solution and heated. Development of blue colour reveals the presence of proteins, peptides or amino acids.
3. **Xanthoprotein test:** To 1ml of the extract, 1ml of concentrated nitric acid was added. A white precipitate appeared, it was boiled and cooled. Then 20% of sodium hydroxide or ammonia was added. Orange colour indicates the presence of aromatic amino acids.
4. **Millon's test:** 1ml of test solution was acidifed with sulphuric acid and Millon's reagent was added and this solution was boiled. A yellow precipitate was formed which indicates the presence of proteins.

CHAPTER 3: *IN VIVO* ANIMAL STUDIES

I. Purchase of drugs and chemicals

Cyclophosphamide (Ledoxan®) was purchased from Dabur Pharma Limited, New Delhi, India. All other chemicals and solvents used were of the highest purity and analytical grade.

II. Procurement of animals

The study was conducted on male Wistar rats (150±10 g). Animals were obtained from the Animal House, Vel's College of Pharmacy, The Tamilnadu Dr. M.G.R. Medical University, Chennai, India. Animals were fed with commercially available standard rat pelleted feed (M/s Pranav Agro Industries Ltd., India) under the trade name Amrut rat/mice feed and water was provided *ad libitum*. The animals were deprived of food for 24 h before experimentation but allowed free access to tap water. The rats were housed under conditions of controlled temperature (25±2 °C) and were acclimatized to 12-h light: 12-h dark cycles. Experimental animals were used after obtaining prior permission and handled according to the University and institutional legislation as regulated by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India.

III. Treatment regimen

The experimental animals were randomized into four groups of six rats each as follows:

Group I : Control rats received normal saline (1 mL/kg body weight), orally for 10 days.

Group II : Rats were injected intraperitoneally with a single dose of CP (200 mg/kg body weight) dissolved in saline, on the first day of the experimental period.

Group III: Rats received FH extract by oral gavage (400 mg/kg body

weight for 10 days).

Group IV: Rats were administered CP as in Group II, immediately followed by supplementation with FH extract (400 mg/kg body weight) by oral gavage for 10 consecutive days.

After the 10 days experimental period (i.e., on the 11th day), all the animals were anesthetized and decapitated. Heart tissues were immediately excised and rinsed in ice cold physiological saline. The tissues were homogenized in 0.01 M Tris-HCl buffer (pH 7.4) and aliquots of this homogenate were used for the assays. Blood was collected and serum was separated for analysis of biochemical parameters.

IV. BIOCHEMICAL INVESTIGATION

A. Assay of enzymatic indices

1. Creatine phosphokinase (CPK, creatine kinase, E.C. 2.7.3.2)

This enzyme catalyzes the reaction:

Creatine + ATP → Creatine phosphate + ADP

The enzyme activity was assayed by the method of Okinaka *et al.*[79]

Reagents

1. Substrate: Creatine solution
2. ATP solution
3. Cysteine hydrochloride solution
4. 10% Trichloro-acetic acid
5. Ammonium molybdate solution
6. 1-amino-2-naphthol-6-sulphonic acid (ANSA).

Procedure

The reaction mixture comprised of 0.1 ml of plasma/tissue homogenate, 0.1 ml of substrate, 0.1 ml of ATP solution and 0.1 ml of cysteine-hydrochloride solution. The final volume was made up to 2.0 ml with distilled water and incubated at 37.8^{O}C for 30 min. The reaction was arrested by the addition of 1.0 ml of 10% TCA (trichloroacetic acid) and the contents were subjected to centrifugation. To 0.1 ml of the supernatant, 4.3 ml distilled water and 1.0 ml ammonium molybdate were added and incubated at room temperature for 10 minutes. 0.4 ml of ANSA was added and the colour developed was read at 640 nm after 20 min. The activity of the enzyme was expressed as µmoles of phosphorus liberated/min/mg protein.

2. Lactate dehydrogenase (LDH, E.C. 1.1.1.27)

This enzyme catalyzes the reaction:

Lactate + NAD+ → Pyruvate + NADH

The enzyme activity was assayed by the method of King.[80]

Reagents

1. 0.1 M glycine buffer: 7.5 g of glycine and 5.85 mg of sodium chloride were dissolved in 1 litre of distilled water; pH 10.
2. Substrate: 2.76 g of lithium lactate was dissolved in glycine buffer *in situ* and the volume was made up to 100 ml.
3. 0.4 N sodium hydroxide.
4. NAD^{+} solution.
5. 2,4-dinitro phenyl hydrazine (DNPH): 0.2% in 1 N HCl, prepared by dissolving 200 mg of DNPH in 1 litre of 1 N HCl.
6. Standard pyruvate solution: 11 mg of sodium pyruvate was dissolved in 100 ml of phosphate buffer. This contained 1 µmole of pyruvate/ml.

Procedure

About 1.0 ml of the buffered substrate, 0.1 ml of plasma/tissue homogenate was added and the tubes were incubated at 37.8ºC for 15 min. After adding 0.2 ml of NAD^+ solution, the incubation was continued for another 15 min. The reaction was arrested by adding 0.1 ml of DNPH (2, 4-dinitrophenyl hydrazine), and the tubes were incubated for a further period of 15 min at 37.8°C after which 7.0 ml of 0.4N NaOH was added and the colour developed was measured at 420 nm. Suitable aliquots of the standards were also analyzed by the same procedure. The activity of the enzyme was expressed as µmoles of pyruvate liberated/min/mg protein.

3. Aspartate aminotransferase (AST, glutamic-oxaloacetic transaminase, E.C. 2.6.1.1)

This enzyme catalyzes the reaction:

L-aspartate + 2-oxoglutarate → oxaloacetate + L-gluatamate

The enzyme activity was assayed by the method of King.[81]

Reagents

1. Substrate: 1.33 g of L-aspartic acid and 15 mg of 2-oxo glutaric acid were dissolved in 20.5 ml of buffer and 1 N sodium hydroxide to adjust the pH to 7.4 and made up to 100 ml with the phosphate buffer.
2. Sodium hydroxide: (0.1 N).
3. 2,4-dinitro phenyl hydrazine (DNPH): 0.2% in 1 N HCl.
4. Standard pyruvate solution: 11 mg of sodium pyruvate was dissolved in 100 ml of phosphate buffer. This contained 1 µmole of pyruvate/ml.

Procedure

1 ml of buffered substrate was incubated at 37˚C for 10 minutes. Then 0.2 ml of plasma/tissue homogenate was added in the test tubes and incubated at 37˚C for 1 hour. The reaction was arrested by adding 1.0 ml of DNPH reagent and tubes were kept at room temperature for 20 minutes. Then 10 ml of 0.4N sodium hydroxide solution was added. A set of pyruvic acid was also treated in a similar manner for the standard. The colour developed was read at 520 nm against the reagent blank. The activity of the enzyme was expressed as µmoles of pyruvate liberated/min/mg protein.

4. Alanine aminotransferase (ALT, glutamic-pyruvic transaminase, E.C. 2.6.1.2)

This enzyme catalyzes the reaction:

L-alanine + 2-oxoglutarate → Pyruvate + L-gluatamate

The enzyme activity was assayed by the method of King.[81]

Reagents

1. Phosphate buffer: 0.1 M; pH 7.4.
2. Substrate: 1.78 g of DL-alanine and 30 mg of 2-oxo glutaric acid were dissolved in 20 ml of buffer. About 0.5 ml of 1 N sodium hydroxide was added and made up to 100 ml with buffer.
3. Sodium hydroxide: (0.1 N).
4. 2,4-dinitro phenyl hydrazine (DNPH): 0.2% in 1 N HCl.
5. Standard pyruvate solution: 11 mg of sodium pyruvate was dissolved in 100 ml of phosphate buffer. This contained 1 µmole of pyruvate/ml.

Procedure

1 ml of buffered substrate was incubated at 37˚C for 10 minutes. Then 0.2 ml of plasma/tissue homogenate was added in the test tubes and incubated at 37˚C for 30 minutes. The reaction was arrested by adding 1.0 ml of DNPH reagent and tubes were kept at room temperature for 20 minutes. Then 10 ml of 0.4N sodium hydroxide solution was added. A set of pyruvic acid was also treated in a similar manner for the standard. The colour developed was read at 520 nm against the reagent blank. The activity of the enzyme was expressed as µmoles of pyruvate liberated/min/mg protein.

B. Assay of Tissue Antioxidants

1. Superoxide dismutase (SOD, E.C. 1.15.1.1)

This enzyme catalyzes the dismutation of superoxide anion ($O_2^{-\cdot}$) to hydrogen peroxide and molecular oxygen in the following manner:

$$H_20 + 2O_2^{-\cdot} + 2H^+ \rightarrow 2H_2O_2 + O_2$$

The enzyme activity was assayed by the method of Misra and Fridovich.[82]

Reagents

1. Carbonate-bicarbonate buffer: 0.1 M; pH 10.2.
2. EDTA solution: 0.6 mM.
3. Epinephrine: 1.8 mM (prepared *in situ*).
4. Absolute ethanol.
5. Chloroform.

Procedure

0.1 ml of tissue homogenate was added to the tubes containing 0.75 ml ethanol and 0.15 ml chloroform (chilled in ice) and centrifuged. To 0.5 ml of supernatant, added 0.5 ml of 0.6 mM EDTA solution and 1 ml of 0.1 M carbonate-bicarbonate (pH 10.2) buffer. The reaction was initiated by the addition of 0.5 ml of 1.8 mM epinephrine (freshly prepared) and the increase in absorbance at 480 nm was measured. One unit of the SOD activity was the amount of protein required to give 50% inhibition of epinephrine autoxidation.

2. Catalase (CAT, E.C. 1.11.1.6)

This enzyme catalyzes conversion of hydrogen peroxide into water and molecular oxygen.

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

The enzyme activity was assayed by the method of Sinha.[83]

Reagents

1. Dichromate-acetic acid reagent: 5% potassium dichromate in water was mixed with glacial acetic acid in the ratio of 1:3 (v/v).
2. Phosphate buffer: 0.01 M; pH 7.0.
3. Hydrogen peroxide: 0.2M.

Procedure

0.1 ml of the tissue homogenate was added to the reaction mixture containing 1ml of 0.01 M phosphate buffer (pH 7.0) pre-warmed to 37°C, 0.4 ml of distilled water and the mixture was incubated at 37°C. The reaction was initiated by the addition of 0.5 ml of 0.2 M hydrogen peroxide and the reaction mixture was incubated at 37°C for one minute. The reaction was terminated by the addition of 2 ml of dichromate-acetic acid reagent after 15, 30, 45, and 60 seconds. Standard hydrogen peroxide in the range of 4-20 µmoles were taken

and treated in the same manner. All the tubes were heated in a boiling water bath for 10 minutes, cooled and and the green colour that developed was read at 590 nm against blank containing all components except the enzyme. Catalase activity was expressed in terms of µmoles of H_2O_2 consumed/min/mg protein.

3. Glutathione peroxidase (GPx, E.C. 1.11.1.9)

This enzyme catalyzes the reduction of H_2O_2 using glutathione as substrate.

$$2GSH + H_2O_2 \rightarrow GSSG + 2H_2O$$

The enzyme activity was assayed by the method of Rotruck *et al*.[84]

Reagents

1. Sodium phosphate buffer: 0.32 M; pH 7.0.
2. Ethylene diamine tetra-acetate (EDTA): 0.8 mM.
3. Sodium azide: 10 mM.
4. Reduced glutathione: 4mM.
5. Hydrogen peroxide: 2.5 mM.
6. Trichloro acetic acid (TCA): 10%
7. Disodium hydrogen phosphate: 0.3M.
8. 5,5'-dithiobis (2-nitro benzoic acid) (DTNB): 0.04%; 40 mg of DTNB in 1% sodium citrate.
9. Standard reduced glutathione: 10 mM.

Procedure

The assay mixture containing 0.5 ml sodium phosphate buffer, 0.1 ml of 10mM sodium azide, 0.2 ml of 4 mM reduced glutathione, 0.1 ml of 2.5 mMH2O2, and 0.5 ml of 1:10 cell extract was taken and the total volume was made up to 2.0 ml with distilled water. The tubes were incubated at 37 8C for 3 min and the reaction was terminated by the addition of 0.5 ml of 10% trichloroacetic acid (TCA). To determine the residual glutathione content, the supernatant was removed after centrifugation, and to this 4.0 ml disodium

hydrogen phosphate (0.3 M) solution and 1 ml dithio-bis-nitrobenzoic acid (DTNB) reagent were added. The colour that developed was read at 412 nm against a reagent blank containing only phosphate solution and DTNB reagent in a spectrophotometer. Suitable aliquots of the standard were also treated similarly. The enzyme activity is expressed in terms of µg of GSH utilized/min/mg protein.

4. Glutathione-*S*-transferase (GST, E.C. 2.5.1.18)

This detoxifying enzyme catalyzes the conjugation of xenobiotics/ electrophiles (E) with the reduced glutathione (GSH), which acts as an electrophile.

$$E + GSH \rightarrow EH + GSSG$$

The enzyme activity was assayed by the method of Habig *et al*.[85]

Reagents

1. Phosphate buffer: 0.3 M; pH 6.5.
2. 1-chloro-2, 4-dinitrobenzene (CDNB) in 95% ethanol: 30 mM.
3. Reduced glutathione: 30 mM.
4. Double distilled water.

Procedure

The reaction mixture contained 1.0 ml of 0.3 M phosphate buffer, 0.1 ml of 30 mM 1-chloro-2, 4-dinitrobenzene (CDNB) in 95% ethanol and 1.7 ml of double distilled water. After pre-incubating the reaction mixture at 37°C for 5 min, the reaction was started by the addition of 0.1 ml of tissue homogenate and 0.1 ml of 30 mM glutathione as substrate. Then, the total reaction mixture (3 ml) was mixed well and the absorbance was read at 340 nm for 3 min at an interval of 30 sec. Reaction mixture without the enzyme was used as blank. The activity of GST is expressed as µmoles of GSH-CDNB conjugate formed/min/mg protein.

5. Glutathione reductase (GR, E.C. 1.6.4.2)

This enzyme catalyzes the conversion of oxidized glutathione (GSSG) to reduced glutathione (GSH) by the oxidation of NADH to NAD^+.

$$GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+$$

The enzyme activity was assayed by the method of Staal *et al*.[86]

Reagents

1. Sodium phosphate buffer: pH 7.4.
2. EDTA: 25 mM.
3. Oxidized glutathione (GSSG): 12.5 mM.
4. NADPH: 3 mM.

Procedure

To the assay mixture containing 0.2 ml tissue homogenate, 1.5 ml sodium phosphate buffer, 0.5 ml of 25 mM EDTA, 0.2 ml of 12.5 mM oxidized glutathione, and 0.1 ml of 3 mM NADPH were added and immediately read at 340 nm against blank containing all the components except the enzyme for 3 min at 30 sec. interval. The activity of GR is expressed as µmoles of NADPH oxidized/min per mg protein.

6. Reduced glutathione: (GSH)

The total reduced glutathione was determined according to the method of Ellman.[87]

Reagents

1. Phosphate buffer: 0.2 M; pH 8.0.
2. DTNB [5, 5 dithiobis (2-nitrobenzoic acid)] reagent: 0.6 mM.
3. Trichloro-acetic acid (TCA): 5%.

Procedure

0.1 ml of tissue homogenate was precipitated with 5% TCA (trichloroacetic acid). The contents were mixed well for complete precipitation of proteins and centrifuged. To 0.1 ml of supernatant, 2.0 ml of 0.6 mM DTNB [5, 5 dithiobis (2-

nitrobenzoic acid)] reagent and 0.2 M phosphate buffer (pH 8.0) were added to make up to a final volume of 4.0 ml. The absorbance was read at 412 nm against a blank containing TCA (trichloroacetic acid) instead of sample. A series of standards treated in a similar way also run to determine the glutathione content. The amount of glutathione was expressed as nmoles/g heart tissue.

C. Assay of lipid peroxidation

Lipid peroxidation (LPO) was assayed by the method of Ohkawa *et al*.[88] in which the malondialdehyde (MDA) released served as the index of LPO.

Reagents

1. Standard: 1, 1, 3, 3-tetra ethoxypropane (TEP).
2. Sodium dodecyl sulphate: 8.1%
3. Acetic acid: 20%
4. Thiobarbituric acid (TBA): 0.8%
5. n-butanol: pyridine mixture (15:1 v/v).

Procedure

To 0.2 ml of tissue homogenate, 0.2 ml of 8.1% SDS, 1.5 ml of 20% acetic acid (pH 3.5) and 1.5 ml of 0.8% TBA were added. The mixture was made up to 4.0 ml with water and then heated in a water bath at 95.8ºC for 60 min using glass ball as a condenser. After cooling, 1.0 ml of water and 5 ml of n-butanol: pyridine (15:1 v/v) mixture were added and shaken vigorously. After centrifugation at 4000 rpm for 10 min, the organic layer was taken and its absorbance was measured at 532 nm. The level of lipid peroxides was expressed as nmoles of MDA formed/mg of protein.

D. Assay of protein content

The protein content of heart tissue was estimated by the method of Lowry *et al*.[89]

Reagents

1. Solution A: 2% sodium carbonate in 0.1 N sodium hydroxide solution.
2. Solution B: 0.5% copper sulphate in 1% sodium or potassium tartrate. 50 ml of solution A was mixed with 1 ml of solution B *in situ*.
3. Folin's phenol reagent: Commercial sample.
4. Standard solution: 100 mg of bovine serum albumin (BSA) was dissolved in 100 ml of distilled water with the aid of a small amount of alkali. This stock solution was diluted 1 in 10 (*in situ*) to give a working standard containing 100 µg/ml.

Procedure

To 0.1 ml of tissue homogenate, 0.9 ml of distilled water and 5 ml of alkaline copper reagent were added and mixed well and allowed to stand at room temperature for 10 minutes. Standard solution containing bovine serum albumin (BSA) in the range of 10-100 µg/ml and the blank containing 1.0 ml of water were treated similarly. 0.5 ml of Folin's reagent was added and the blue colour developed was read at 640 nm.

The protein level was expressed as mg/g of tissue.

E. Assay of tumor necrosis factor (TNF)-α

TNF-α was assayed by using the commercially available TNF-α assay kit.

E. Assay of nitric oxide (NO)

NO was assayed by using the commercially available NO assay kit.

V. HISTOPATHOLOGICAL INVESTIGATION[90,91]

Reagents

1. Formal saline: 10% formalin in normal saline.
2. Ethanol/isopropanol.
3. Xylene.
4. Paraffin wax.
5. Hematoxylin and eosin (H & E) stain.
6. Mordant: Potassium aluminium sulphate (Potash alum).

Procedure

Tissue biopsies from the heart were fixed in 10% formal saline, subjected to dehydration with increasing concentrations of ethanol and then embedded with paraffin wax. Following dehydration and embedding, histological sections were cut (5-7 µm) with rotary microtome and the paraffin was washed off with three xylene baths, followed by three isopropanol baths, and rehydration. Finally, the section were stained with hematoxylin and eosin (H & E) and examined microscopically.

Summary and explanation

- The purpose of fixation is to prevent substantially any physicochemical alteration of the tissue and to maintain it in the initial state in which it was collected.
- The purpose of dehydration is to replace the water present in the tissues by a compound which is itself substituted- possibly with the use of solvents--by the infiltration/embedding compound.
- The purpose of the infiltration/embedding is to solidify the tissue to make it possible to make the slices as thin as possible, thus enabling a more extensive examination.
- Rehydration restores the tissue more or less to its initial condition, permitting the desired analysis.

- Hematoxylin (a natural dye), stains the nuclei with blue/black, whilst eosin (a xanthene dye) stains the cytoplasm and the connective tissue with varying shades of pink.
- The addition of a mordant to an appropriate dye solution results in a very sudden, dramatic change in colour, which occurs due to the chelation between the mordant (potash alum) and the dye (hematoxylin).

Principle

Hematoxylin, a basic bye is oxidized to hematein with a mordant, a metallic ion such as the salts of aluminium. The positively charged aluminium-hematein complex combines with the negatively charged phosphate groups of the nucleic acids (DNA and RNA) forming blue/purple colour, which is characteristic of hematoxylin stains. Eosin is an acidic dye, which is considered to have a selective affinity for the basic parts of the cell, i.e., the cytoplasm. Thus, the hematoxylin and eosin (H & E) stain is used to demonstrate different structures of the tissue.

VI. STATISTICAL ANALYSIS

The results were expressed as mean ± standard deviation (S.D.) for six animals in each group. Differences between groups were assessed by one-way analysis of variance (ANOVA) using the SPSS 13.0 software package for Windows. Post hoc testing was performed for inter-group comparisons using the least significance difference (LSD) test. $P < 0.05$ has been considered as statistically significant.

RESULTS

Effect of *Ficus hispida* on the levels of diagnostic marker enzymes

In the present study, intraperitoneal administration of a single dose of CP (200 mg/kg body weight) induced severe biochemical changes as well as oxidative damage in cardiac tissue. There was a significant ($P < 0.05$) rise in the levels of diagnostic marker enzymes (CPK, LDH, AST and ALT) in the serum of Group II CP administered rats as compared to that of Group I control rats (Table 1). The administration of *Ficus hispida* leaf extract to Group IV animals restored the levels of these enzymes to near normalcy ($P < 0.05$) as compared to those Group II CP-injected rats. In *F. hispida* alone administered rats (Group III) versus controls, no significant changes were observed.

Effect of *Ficus hispida* on the levels of cardiac enzymes

In CP administered rats (Group II), the increase in serum marker enzyme activities was accompanied by concomitant decreased activities ($P < 0.05$) of these enzymes in the heart tissue (Table 2), which depict the damage of heart in Group II animals. Activities of these enzymes in the cardiac tissue were restored to near normal levels ($P < 0.05$) in *F.hispida* treated rats (Group IV). This may be due to the protection offered by *F. hispida* against tissue damage and oxidative stress induced by cyclophosphamide.

Effect of *Ficus hispida* on the levels of GSH and MDA in cardiac tissue

Injection of CP induced a significant ($P < 0.05$) increase in the level of lipid peroxidation (LPO), measured in terms of MDA (Fig. 8), which was paralleled by significant ($P < 0.05$) reduction in the level of GSH (Fig. 7) in the heart tissue of Group II animals as compared to normal controls. Glutathione plays an important role in the regulation of variety of cell functions and in cell protection from oxidative injury. Depletion of GSH results in enhanced lipid peroxidation, and excessive lipid peroxidation can cause increased GSH consumption, as observed in the present study. In this study, the treatment with *F. hispida* (Group IV) significantly ($P < 0.05$) counteracted the CP-induced lipid peroxidation and restored the level of GSH to near normal level in Group IV rats as compared to that of Group II animals.

Effect of *Ficus hispida* on the levels of cardiac tissue antioxidants

Activities of glutathione-dependent antioxidant enzymes (GPx, GST and GR) and anti-peroxidative enzymes (SOD and CAT) were significantly ($P < 0.05$) lower in the heart tissue of Group II CP-injected rats as compared to that of Group I normal control rats (Table 3). The observed reduction in the activities of GPx, GR and GST in CP-induced myocardial damage might be due to decreased availability of its substrate, reduced glutathione (GSH). In the present study, the treatment of Group IV rats with *F. hispida*, significantly ($P < 0.05$) reversed all these CP-induced alterations in the activities of antioxidant enzymes (SOD, CAT, GPx, GST and GR) to a near normal status. The normal rats receiving *F. hispida* alone (Group III) did not show any significant change when compared with control rats, indicating that it does not *per se* have any adverse effects.

Effect of *Ficus hispida* on the levels of serum TNF-α and NO

Fig. 9 and Fig.10 illustrate the serum levels of TNF-α and NO respectively. There was a significant ($P < 0.05$) rise in the levels of TNF-α and NO in the serum of CP intoxicated rats as compared to that of control rats. The administration of *Ficus hispida* leaf extract to Group IV animals restored the levels of these enzymes to near normalcy ($P < 0.05$).

HISTOPATHOLOGICAL STUDIES (Plate 3)

A. Control (T.S.)

- ► Section depicts normal architecture of myocytes.

B. Cyclophosphamide-Induced (T.S.)

- ► Section depicts abnormal cardiac muscle with karyorrhexis, pyknotic nuclei and leucocytic infiltration.

C. *Ficus hispida* alone (T.S.)

- ► Section depicts almost normal architecture.

D. Cyclophosphamide + *Ficus hispida* treated (T.S.)

- ► Section depicts a significant recovery in the cardiac fibres.

DISCUSSION

High-dose cyclophosphamide was introduced as a mainstay of numerous preparative regimens for haemopoietic stem-cell transplantation and its potential to cause myocardial damage was soon recognized. Santos *et al*.[43] reported the first human fatality of cyclophosphamide (CP) cardiotoxicity as a complication of bone marrow transplantation. Several studies implicate that high-dose cyclophosphamide is associated with cardiotoxicity.[92-94] The pharmacokinetics and metabolism of CP have been extensively studied.[95] CP requires bioactivation to form 4-hydroxy-CP and also aldophosphamide, which spontaneously degrades by β-elimination, to form stoichiometric amounts of phosphoramide mustard and the toxic by-product acrolein.[96] Acrolein is a highly reactive α, β-unsaturated aldehyde, and its formation from CP was first demonstrated by Alarcon and Meienhofer.

The aetiopathogenesis of CP induced cardiotoxicity is not yet fully unraveled. However, toxicity of CP was postulated to be mediated by oxidative stress[48] which may have deleterious effects on the heart. Moreover, it is thought to involve direct endothelial damage, with extravasation of plasma proteins, high concentration of cyclophosphamide and erythrocytes into the myocardial interstitium and muscle cells, resulting in damage of myocardial cells.[97,98] Due to the damage, the enzymes (CPK, LDH, AST and ALT) leak from the necrotic heart cells to the serum, which are important measures of cardiac injury. These enzymes are not specific for myocardial injury individually; however, evaluation of these enzymes together may be an indicator of myocardial injury. In CP-administered rats, the activities of these marker enzymes were elevated in serum with a concomitant decrease in the heart tissue. FH treated rats showed near normalcy in these enzyme levels. This might be attributed to the membrane stabilizing effect of the phytoconstituents like oleanolic acid and β-sitosterol, present in the FH[51,99].

Reactive oxygen/nitrogen species (ROS/RNS) include superoxide anion, hydroxyl radical, alkoxyl radical, peroxyl radical, hydrogen peroxide, singlet oxygen, nitric oxide and peroxynitrite. Superoxide anion itself is not a strong oxidant, but it reacts with protons in water solution to form hydrogen peroxide (H_2O_2), which can serve as a substrate for the generation of hydroxyl radicals and singlet oxygen. The prevalent free radical states, or so-called oxidative stress, initiate the oxidation of polyunsaturated fatty acids (PUFA), proteins, DNA, and sterols. The reaction of nitric oxide (NO) with superoxide anion forms peroxynitrite,[100] a potent cytotoxic oxidant eliciting lipid peroxidation and cellular damage.[101] Thus, free radicals generated through cyclophosphamide metabolism, cause membrane damage by initiating LPO which leads to impairment in the integrity and function of myocardial membranes. The obtained data reveal that CP exposure produced a marked oxidative impact as reflected by elevated LPO, measured in terms of MDA level in the heart tissue. FH treated rats showed decreased MDA level, due to significant inhibition of LPO which is in line with earlier studies[49]**.** This might be due to the presence of oleanolic acid, hispidin and β-sitosterol which have been reported to possess anti-lipid peroxidation and/or free radical scavenging properties.

TNF-α, the principal cytokine that mediates acute inflammation after high dose CP administration, was reduced by *Ficus hispida* treatment, which indicates its protective effect against CP-induced inflammation. Earlier, it was documented that FH exert effective anti-inflammatory activity.[66] The presence of phenanthroindolizidine alkaloids and other phytoconstituents might be attributed for the activity.

Cells are equipped with an impressive repertoire of antioxidant defensive system. The present study shows that the free radical-induced increase in LPO is accompanied by concomitant decline in the activities of cellular antioxidants. This may be due to the inactivation of cellular antioxidants by lipid peroxides and ROS. SOD is inhibited by hydrogen peroxide (H_2O_2) while GPx and CAT by an excess of superoxide radical. In fact, the heart has a greater susceptibility to

oxidative stress than other tissues due to its inherent decreased detoxifying. The decrease in endogenous antioxidant enzymes might predispose the cardiac tissue to increased free radical damage, because SOD catalyzes the dismutation of superoxide anion to hydrogen peroxide, while CAT and GPx are involved in cellular detoxification and can convert H_2O_2 into water and oxygen. GPx is the most important hydrogen peroxide-removing enzyme existing in the membrane. If the activity of CAT or GPx is not adequate to degrade H_2O_2, more H_2O_2 could be converted to toxic hydroxyl radicals and may contribute to the CP–induced oxidative stress. Administration of FH replenished the antioxidant levels, which might attributed to the free radical scavenging/antioxidant properties of its phytoconstituents described elsewhere in this report.

Reduced glutathione (GSH), the first line of defense against ROS, is a readily available source of endogenous sulfhydryl (-SH) groups. CP exposure caused a dramatic decline in GSH level, which may be ascribed to the direct conjugation of CP's metabolites with free or protein bound -SH groups[47,102], thereby interfering with the antioxidant functions. The activities of CAT, SOD and GPx were significantly reduced in GSH depleted condition due to pronounced oxidative stress and accumulation of H_2O_2, making the cells more vulnerable to oxidative stress. FH treatment restored the GSH level to near normalcy. One of the reasons for this restorative effect might be the presence of triterpenoid constituents in FH.

CP treated rats displayed decreased activities of GSH metabolizing enzymes, GST and GR which is consistent with the previous report.[103] Many investigators have suggested that GST offers protection against LPO by promoting the conjugation of toxic electrophiles with GS. GR is a flavoprotein that permits the conversion of oxidized glutathione (GSSG) to reduced glutathione (GSH) by the oxidation of NADH to NAD^+. Inactivation of GR in the heart, leads to accumulation of GSSG which in turn inactivates enzymes containing -SH groups and inhibits protein synthesis. Impairment of these enzyme activities may thus be doubly detrimental to the myocardial tissue. *F.hispida* treatment restored the normal activities of these enzymes, thereby confirming its protective action.

On histological examination, the cardiac sections from cyclophosphamide-treated rats portrayed karyo-pyknosis of myocyte nuclei and leucocytic infiltration. *F.hispida* treated rats exhibited significant recovery from cyclophosphamide-induced myocardial damage, presenting a normal cardiac architecture.

Previous studies suggest that oleanolic acid has protective effect against cyclophosphamide-induced toxicities.[54] Recent evidence suggests that oleanolic acid has a significant cardioprotective effect[51]. Intriguingly, literature citations show that phytosterols like β-sitosterol exert antioxidant, cardioprotective properties.[104,105] A recent report suggests the possibility of GSH replenishing effect of β-amyrin.[106] Prodigious amounts of literature data suggest that triterpenoids, flavonoids, tannins possess significant antioxidant/cardioprotective effects.[107-110] Hence, it is suggested that presence of the aforementioned active ingredients in *F.hispida* leaf extract might be responsible for the abrogation of CP elicited cardiotoxicity.

SUMMARY AND CONCLUSION

To summarize, the results of the present study indicate that cyclophosphamide exposure results in the pronounced oxidative stress and tissue damage. Administration of *Ficus hispida* leaf extract protects the cardiac tissue by scavenging the free radicals, which is evidenced by the normalization of the biochemical parameters. These observations support the hypothesis that *Ficus hispida* has potential for its evaluation as a cardioprotective agent against CP-induced oxidative myocardial injury. Further studies for the protective role of *Ficus hispida* in cyclophosphamide-induced toxicities are currently under investigation.

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