Protective Effects of Methanolic Extract of *Digera muricata (L.) mart.* Against Toxicity Induced by Acrylamide in Rat Lung.

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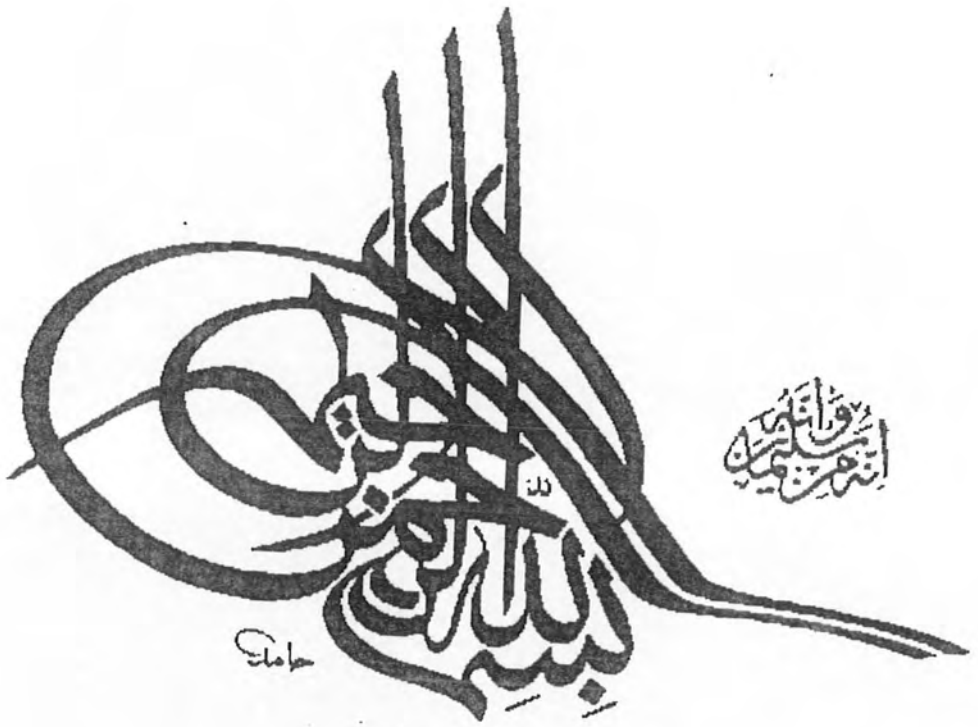
بسم الله الرحمن الرحيم
إنه من سليمان وإنه

DECLARATION

This thesis submitted by ***Sonia Gul*** is accepted in its present form by the Department of Biochemistry, Quaid-i-Azam University, Islamabad as fulfilling the thesis requirements for the degree of Master of Philosophy in Biochemistry/Molecular Biology.

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DEDICATED

To

My Loving

Father

Without his Patience, Support and most of all Love,

The completion of this work would not have been possible

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Sonia Gul

LIST OF CONTENTS

LIST OF ABBREVIATION

Abbreviations	Full Name
ROS	Reactive Oxygen Specie
DMSO	Dimethylsulphoxide
HNO3	Nitric Acid
µg	Microgram
µl	Microlitre
mg	Milligram
ppm	Part per million
Ph	-log[Hydrogen]
OD	Optical density
Tris	Trishydroxymethylaminomethane
MDMP	Methanolic extract of Digera muricata plant
GPx	Glutathione peroxidase
CAT	Catalase
TBARS	Thiobarbituric acid-reactive substances
NIH	National Institute of Health
GSH	Glutathione reductase
SDS	Sodium dodecyl sulphate
DTNB	1,2-dithio-bis nitro benzoic acid
CDNB	1-chloro-2,4-dinitrobenzene
NADPH	Nicotinamide adenine dinucleotide phosphate
DNA	Deoxyribonucleic acid
FAD	Flavine adenine dinucleotide
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid
TBE	Tris borate EDTA
ml	mililiter
NBT	Nitoblue tetrazolium
nmol	nanomolar
EDTA	Ethylene diamino tris acetic acid
PBS	Phosphate Buffered Saline
mmol	Millimolar
HCl	Hydrochloric acid
NaCl	Sodium chloride
H_2SO_4	Time-Tag Engine
dH2O	Distilled water
POD	Peroxidase
APX	Asorbate peroxidase activity
GSH	Glutathione reductase
GST	Glutathione-S-transferase
BUN	Tris tritronX100 EDTA

List of Figure

List of Table

ABSTRACT

Exposure to acrylamide induces acute lung injuries as well as oxidative stress in rats by formation of free radicals. Extracts of medicinal has been shown to exhibit a pharmacological actions against the pulmonary toxicity of acrylamide. The aim of this study was to evaluate the protective effect of exogenous (oral) *Digera muricata* extract treatment on acrylamide-induced oxidative stress and pulmonary toxicity in rats at histological and biochemical level. Forty eight healthy female albino rats, weighting 190-200 g, were provided by the Animal House of National Institute of Health (NIH) Islamabad and were maintained at the Primate Facility maintained at Quaid-i-Azam University, Islamabad. These rats were divided into eight groups with six rats in each group. Among them group I, comprising of six rats were chosen randomly as control, while group II and were administered with dimethylsulphoxide (DMSO) 5.0 ml/kg b.w. orally once a day for four weeks. Group III was given 200 mg/kg b.w. of methanolic extract dissolved in DMSO once a day for four weeks. Rest of the rats were divided into five groups and were treated with aqueous solution of acrylamide 6 mg/kg b.w. intraperitoneally once a day for two weeks. Group IV was sacrificed after the acrylamide treatment to collect the lung tissue. Group V remained untreated as such for the rest of the experiment. Group VI, VII and VIII were given 100, 150 and 200 mg/kg b.w. methanolic extract dissolved in DMSO once a day for two weeks.

Mean body weight was decreased while lung weight of rats treated with acrylamide was statistically ($P<0.01$) induced as compared to the control group. Treatment of methanolic extract reversed the body and lung weight in a concentration dependent manner as compared to the acrylamide group (30 days).

For histopathological evaluation, lung sections were processed and stained for light microscopy. Representative sections taken from lung tissues of acrylamide treated rat showed the changes in microanatomy by rupturing the alveoli and damaging the cells causing the aggregation of blood capillaries. While in extract treated groups these deleterious histopathological alternation were reduced or absent resulting from acrylamide induced lung injuries. Intense repairing effects of the 150 and 200 mg/kg of the methanolic extract of *Digera muricata* against the acrylamide were observed in the lungs of rats. Antioxidant status in lung tissues was estimated by determining the activities of peroxides (POD), catalase (CAT), reduced glutathione (GSH) and the level of lipid peroxidation (TBARS), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and glutathione-S-transferase activity (GST). Treatment of rats

with acrylamide decreased the activity of CAT, POD, SOD, protein and GSH contents while increased the TBARS and H_2O_2 contents in lung tissues of rats as compared to control group.

Total protein content of tissue was also measured. To determine the DNA damage in lung tissues; DNA fragmentation and DNA ladder assay was performed. Rats with acrylamide decreased the protein contents in lung tissues of rats as compared to control group. An increase in protein content was observed with the methanolic extract of *Digera muricata* in lung tissues of rats in a dose dependent manner.

DNA fragmentation and DNA ladder assay revealed DNA damage in lung tissues of rats treated with acrylamide as compared to control group. Co-administration of the extract of *Digera muricata* decreased the DNA fragmentation% as compared to the acrylamide group in a dose dependent manner which confirms the restoration. In case of acrylamide (15 days and 30 days treated rats) DNA samples showed a peculiar type of continuous pattern of DNA fragmentation which was absent from the lungs of control rats. Our results demonstrated that *Digera muricata* may contain antioxidant metabolites which effectively ameliorated acrylamide induced pulmonary toxicity.

INTRODUCTION

INTRODUCTION

1.1. Morphology and histology of lung

To meet the needs of oxygen and to remove carbon dioxide, higher animals have developed specialized respiratory organ; the lungs. The lungs are essential respiratory organ of air breathing vertebrates. The two lungs are located in the chest cavity on either side of the heart and are fully covered in double membrane called pleura. The main function of lungs is to transport oxygen from the atmosphere into the blood stream, and to release carbon dioxide from the blood stream into the atmosphere. Lungs not only perform the function of respiration but, also play a roll in homeostasis. They also provide soft and protective layer around the heart.

Human lungs are more efficient, as is required by higher metabolic rate of these homeotherms. The mammalian lung is more compartmentalized than reptile lungs. Its gross appearance is more like dense foam rubber and spongy (Mclaughlin *et al.*, 1961).

Of more functional importance is the nature of the respiratory tree. The morphology of the "respiratory tree" of mammals is essentially a successive series of dichotomously branching tubes. There is a fluid- dynamic basis to the complex pattern of branching.

The trachea is supported by rings of hyaline or fibrous cartilage. Elastic connective tissue joins ring to ring and completes the tube where cartilage is absent. The resultant structure is ideal for holding the airway open yet allowing the tube to twist as the neck is turned, change length with swallowing. The trachea is lined with ciliated epithelium. Smooth muscles and mucous glands are present in the walls.

Inspired air is drawn in through the trachea and the trachea divides into right and left primary bronchi, each of which enters its lung. All internal bronchi are supported by cartilage which however, may be in plates rather than rings. With in the lungs, the bronchi divide into a number of secondary bronchi that further divide and ramify into many smaller tertiary bronchi, called bronchioles which are membranous, ciliated and non cartilaginous where gaseous exchange begins. The bronchioles are just one millimeter in diameter. Glands in the bronchiolar mucosa and cartilage in the walls are totally absent. Pseudostratified columnar ciliated type cells called goblet cells are present in the lining of epithelium of larger bronchiole. The epithelium changes its form as the

bronchiole divides. Goblet cells then disappear and epithelium of terminal bronchiole becomes cuboidal or low columnar and also bears cilia. But there are also sprinkled non-ciliated cells called Clara cells are present which have dome shaped apices project out into the lumen of the bronchiole. Secretory granules are present on the apical region of Clara cells which secret a material called surfactant.

Surfactants are given their name due to their effect on surface tension. The surface tension should make the lungs collapse, but this tendency is minimized by the presence of phospholipids surfactants. Surfactants are phospholipids and are also present on the inner surface of the alveoli (Georke and Clements, 1986). Surfactants are found in the lungs of all vertebrates, mammals, birds, reptiles and amphibians.

The final extensions of the terminal bronchioles are small sac like alveoli which forms alveolar duct system, which are the clusters of about 20 hemisphericle alveoli all opening into common terminal chamber. The alveoli are primary site of gas exchange in the lungs. In each lung of a human for example, there are about 150,000,000 alveoli, each of about 150 to 300 micron in diameter. The total surface area of alveoli is about 80 m square.

The pulmonary alveoli are very thin walled cup shaped structures. The alveoli are richly supplied with blood capillaries where gaseous exchange occurs between the blood and air. The blood is separated from the air only by the endothelium of capillary. The pulmonary alveoli lined by two types of cells:

- Squamous alveolar cells (type I pneumocyte) which forms 95% alveolar surface.
- Cuboidal great alveolar cells (type II pneumocytes) which forms 5% of alveolar surface.

The adjacent alveoli are separated from each other by inter alveolar septum which consist of blood capillaries which lies between the two squamous epithelial layers. The capillaries have wide lumina and anastomose freely to form an extensive network. Inter alveolar septa is perforated by one or more pores which connect the adjacent alveoli so it is not completely partitioned. The alveolar network contains alveolar macrophages, mast cells, wandering leucocytes and reticular and elastic fibres.

In inter alveolar septum, the alveolar macrophages (dust cells) are present along with the cuboidal cells. These alveolar macrophages engulf the foreign particles like dust particles and inspired bacteria and also destroy them. Hence they protect the lungs from pulmonary infection (Brown *et al.*, 1993)

1.2. Acrylamide

Acrylamide is an important synthetic industrial chemical and has been commercially available since 1950s. Acrylamide is the substituted olifinic monomer, a white and odourless chemical and is crystalline solid at room temperature and is highly soluble in water (Budavari *et al.*, 1989).

It has the following physical data:

- Formula: = CH_2-$CHCONH_2$
- Synonym = Propenamide
- Appearance = Colourless and odourless crystalline solid
- Molecular mass = 71.09
- Solubility in water = 2155 g/L water
- Melting point = 84.5 °C,
- Boiling point = 192.6°C at atmospheric pressure

1.2.1. Reactivity

Acrylamide is a reactive chemical, which is used as monomer in the synthesis of polyacrylamides used e.g., in purification of water, and in the formulation of grouting agents. Acrylamide is primarily reactive through its ethylenic double bond. Polymerisation of acrylamide occurs through radical reactions with the double bond. Acrylamide could also react as an electrophile by 1,4-addition to nucleophiles, e.g., SH- or NH_2-groups in biomolecules. Acrylamide is metabolized in the body to glycidamide, a reactive compound formed through epoxidation of the double bond.

1.2.2. Absorption and distribution

Animal studies show that acrylamide is rapidly and well absorbed by intravenous, intraperitoneal, subcutaneous, intramuscular, oral, tran mucosal and dermal routes (Kuperman, 1958). In rats, absorption of acrylamide following oral administration is

virtually complete. However, only about 25% of a dose applied to the skin is absorbed over the subsequent 24 hours (Dearfield *et al.*, 1988).

Following absorption, acrylamide is rapidly distributed throughout the total body water. Tissue distribution is not significantly affected by dose or route of administration. Highest concentrations are found in red blood cells. Despite the prominence of neurological effects, acrylamide is not concentrated in nervous system tissues (Miller *et al.*, 1982). Acrylamide readily crosses the placenta (Edwards, 1976).

1.2.3. Toxicity

Acrylamide is biotransformed to its epoxide, glycidamide. Both acrylamide and glycidamide , are genotoxic in a variety of in vitro and in vivo test systems. Acrylamide increased DNA synthesis in the target tissues for tumor development in lungs, thyroid, testicular mesothelium and adrenal medulla in experimental rats.

The toxicological effects of acrylamide have been studied in animal models. Exposure to acrylamide leads to DNA damage and at high doses neurological and reproductive effects have been observed. Carcinogenic action in rodents has been described but carcinogenicity to humans has not been demonstrated in epidemiological studies, although it cannot be excluded. The International Agency for Research on Cancer (IARC) has classified acrylamide as "probably carcinogenic to humans" (Group 2A). Neurological effects have been observed in humans exposed to acrylamide. Properties, use and toxic effects of acrylamide are reviewed by IARC, (1994)

1.2.4. Biological half-life

In blood, acrylamide has a half-life of approximately 2 hours. In tissues, total acrylamide (parent compound and metabolites) exhibits biphasic elimination with an initial half-life of approximately 5 hours and a terminal half life of 8 days (Edwards, 1975; Miller *et al.*, 1982).

1.2.5. Metabolism

Acrylamide undergoes biotransformation by conjugation with glutathione (Edwards, 1975; Miller *et al.*, 1982) or reduction by microsomal cytochrome P-450 (Kaplan *et al.*, 1973) with glutathione conjugation probably being the major route of detoxification. The metabolites are non-toxic (Edwards, 1975).

Greater than 90% of absorbed acrylamide is excreted in the urine as metabolites. Less than 2% is excreted as unchanged acrylamide. Smaller amounts are excreted in the bile and faeces (Miller *et al.*, 1982). Approximately 60% of an administered dose appears in the urine within 24 hours (Miller *et al.*, 1982).

1.2.6. Acrylamide in food

According to Swedish National Food Administration and a research group at the University of Stockholm, acrylamide had shown to be formed in foods during heating. The chemical mechanism for acrylamide formation in heated foods, a multitude of reaction mechanisms are involved, depending on food composition and processing conditions. Several plausible mechanistic routes may be suggested, involving reactions of carbohydrates, proteins/amino acids, lipids and probably also other food components as precursors. Acrolein is one strong precursor candidate, the origin of which could be lipids, carbohydrates or proteins/amino acids.

According to joints FAO/WHO standards programme codex committee on food additives and contaminants studies conducted to date suggest that acrylamide formation is particularly likely in carbohydrate-rich foods cooked (baked or fried) at temperature of above approximately 120°C One potential pathway that has been identified involves a chemical reaction between the amino acid asparagine and certain reducing sugars, both of which are found naturally in foods. Acrylamide has not been detected in boiled foodstuffs. Acrylamide is known as a component in tobacco smoke (Rotterdam, 2004).

1.2.7. Formation

The main rout of formation of acrylamide is the Maillard reaction (MR) during processing of food. The MR takes place between proteins (amino acids) and carbohydrates (reducing sugars) and the final products are the brown nitrogenous polymers and co-polymers at high temperature or if the food is over cooked (SpirosGrivas *et al.*, 2002)

1.2.8. Defense system against xenobiotics toxicity

A lot of current research labors express the role of active oxygen species (AOS) in the pathogenesis of various organs like liver, kidney, thyroid, pancreas, adrenal gland etc. these active oxygen species are also responsible for lungs toxicity including lungs cancer, pulmonary fibrosis, adult respiratory distress syndrome, chronic bronchitis, emphysema

and pleural diseases (Prior, 1992). The enhanced production of free radicals and oxidative stress can be induced by a variety of factors such as ionizing radiation or exposure to drug and xenobiotics (Szymonik-Lesiuk *et al.*, 2003).

The lungs have developed defences to AOS mediated damages by producing wide range of antioxidant enzyme. The antioxidant family includes catalases, peroxidases, dismutases (Keller *et al.*, 1991). To prevent the damage caused by oxygen-free radicals, tissues have developed an antioxidant defense system that includes nonezymatic antioxidants (glutathione, uric acid, bilirubin, vitamins C and E) and enzymatic activities such as that of superoxide dismutase, catalase, and glutathione peroxidase.

The antioxidant enzymes protect the macromolecules of the tissue like lipids, proteins and DNA from the damage by inactivating the oxidants. The superoxide dismutase which are the product of highly conserved gene convert the free oxygen radical to H_2O_2. This molecule which is itself toxic for the cells is broken down to release hydroxyl radical (*OH), a reactive species which is more toxic either *O2 and H_2O_2. The enzymes responsible for converting H_2O_2 to other harmless substances are catalase and glutathione peroxidase (GSH-Px). Thus this enzyme family may act in a sequential fashion to dismutate the one toxic oxygen species to another which then can be rapidly broken down to non toxic byproducts (Schinina *et al.*, 1987). Catalase is also an antioxidant enzyme and primarily located in the cytosolic peroxisomes. Its function is to detoxify H_2O_2 to oxygen and water (Freeman *et al.*, 1986). Glutathione peroxidase is a cytosolic enzyme and also eliminates H_2O_2, but in comparison to catalase, GSH-Px has a wider range of substrate including lipid peroxides. The kinetics of this enzyme is very complex, but it though to have a greater affinity for H_2O_2 as compared to catalase. Glutathione peroxidase primarily functions to detoxify low level of H_2O_2 in the cells (Heffner, 1989). The dismutase like copper-zinc superoxide dismutase is a containing copper for its catalytic action and zinc as a protein stabilizer. This enzyme may be localized in the cytoplasm and nuclear matrix or in the peroxisomes. This enzyme also acts as a scavenger for the oxygen free radical (Marklund, 1992; Crapo *et al.*, 1992; Leung *et al.*, 2006). The antioxidant enzymes may act in a coordinate manner to defend living tissues from oxidant damage. As the production of antioxidant enzymes are under the genetic control, but some exogenous chemicals or their metabolites my react with the

DNA and bring the changes in the biosynthetic pathway of the production of antioxidant enzymes. So the alterations in the antioxidant enzymes also alter the defense mechanism of the cells and tissues.

Antioxidant therapy by some natural or synthetic antioxidants prevents the lung fibrosis and other lung injuries caused by bleomycin and may protect other disease related with interstitial pulmonary fibrosis (Arslan *et al.*, 2002).

1.3. Repairing of tissue damages by medicinal plants

Due to failure and severe toxicity of cancer chemotherapy, several alternative medicine approaches including herbal phytochemicals are increasingly being claimed to be safe and effective. Because of lack of relevant scientific evidence, this concern represents a common public opinion about potential benefits of herbals and alternative medicines in cancer control. However, current advances in drug development have revealed cancer preventive and curative efficacies of many phytochemicals (Surh *et al.*, 2003).

The use of plant based natural products as chemopreventive agents is drawing a lot of attention and considered to be practically beneficial in certain cell/tissue based systems and animal model systems. It is necessary to provide scientific proof to justify the use of a plant or its active principles for medicinal purposes (Ammon and Wahl, 1991). Modern drugs, plants and plant extracts must be characterized after their pharmacological screening for their pharmacokinetic and pharmacodynamic properties, including toxicity (Kelloff *et al.*, 1994). Cancer chemoprevention is defined as the use of chemicals or dietary components to block, inhibit, or reverse the development of cancer in normal or preneoplastic tissue. A large number of potential chemopreventive agents have been identified, and they function by mechanisms directed at all major stages of carcinogenesis (Tanaka, 1994; Morse and Stoner, 1996; Pezzuto, 1997).

All living organism contains antioxidant metabolites and enzymes which prevent oxidative damage to cellular compounds such as lipids, proteins and DNA. Researches have found a correlation between oxidative damage and the occurrence of diseases. A large number of plants and their products and several plants derived dietary components have been evaluated as potential chemoprotective agents in the living cells. Medicinal plants have played a significant role in maintaining human health and improving the

quality of human life for thousands of years. Much enthusiasm have been generated to explore the potential use of naturally occurring anti-inflammatory and antioxidant compounds to attenuate the xenobiotic compounds induced inflammatory reactions.

Curcumin (CCM) is a dietary antioxidant derived from turmeric (*Curcuma longa*) and has been known since ancient times to possess therapeutic properties. It has been reported to be a potent anti-inflammatory, antioxidant, and anti-carcinogenic compound (Huang *et al.*, 1998; Singletory *et al.*, 1998; Kawamori *et al.*, 1999; Antunes *et al.*, 2001; Khar *et al.*, 2001).

Ginkgo biloba (GB) tree is considered to be a potent hepatoprotective agent against the carbon tetrachloride induced liver injury. Histopathological studies showed that CCl_4 caused steatosis and hydropic degeneration of the liver tissue. *Ginkgo biloba* pre-treatment exhibited protection, which confirmed the results of biochemical studies. All the effects of *Ginkgo biloba* were comparable with those of *silymarin*, a proven hepatoprotective. The results of these studies indicate that simultaneous treatment with GB protects the liver against CCl_4 induced hepatotoxicity (Ashoke *et al.*, 2001).

Recent research indicates that the extracted oil of *Nigella sativa* have the medicinal effects and repair the liver injury induced by carbon tetrachloride. It also increased the reduced antioxidant enzyme level in the CCl_4 treated rats (Kanter *et al.*, 2004). Kujawska *et al.* (2007) studied the ethyl ether extract of *Aquileja vulgaris* inhibited in vitro micromal lipid peroxidation and showed moderate ability to scavenge superoxide radicals and to chelate iron ions. The extract itself significantly enhanced superoxide dismutase activity.

Digera muricata is used ethnopharmacologically in renal disorders (Anjaria *et al.*, 2002) aperient, refrigerant (Hocking, 1962). However, it has not been evaluated against the toxicity induced by acrylamide in lungs of rat. On account of increasing importance of plant metabolites as alternative medicine, the present experiment was designed to study the protective effects of *Digera muricata* extracts against lungs toxicity induced by acryl amide in rat model.

Main objectives of the project

Due to the pathogenic and degeneration effects of several harmful natural and industrial chemicals, there is an immense need to introduce new natural and synthetic

chemoprotective agents which reduces the effects of free radicals and preserve the tissues and macromolecules and keep them in their natural biochemical and physiological states. *Digera muricata* (L.) Mart. commonly called "Tandla" is used as an antidote against the snake bite. This plant is selected to explore its histoprotective and antioxidant activity which is still unknown. This project is designed to know the protective effects of *Digera muricata* on the lungs injuries induced by acrylamide.

Main objectives

The main objectives of present study were to determine the protective effects of the methanolic extract of *Digera muricata* against acrylamide toxicity as underlying;

1. To determine the cytological and histopathological changes in the lungs of rats induced by Acrylamide.
2. To study the DNA damages induced by acrylamide.
3. To estimate the degenerative effects of acrylamide on protein content and of lungs.
4. To determine the activity of antioxidant enzymes in tissue homogenate.
5. To determine the protective effect *Digera muricata* on the genomic DNA of lungs in rats.
6. To determine the chemopreventive and antioxidant activity of *Digera muricata* in lung injuries induced by acrylamide.
7. To examine the histoprotective and cytoprotective effects of *Digera muricata* in lung injuries induced by acrylamide.

REVIEW OF LITURATURE

REVIEW OF LITERATURE

In the lung, the lining epithelium and alveolar macrophages (AMs) constitute the first line of defense from external injuries. Alveolar macrophages are located at the interface between air and lung tissue, being extensively exposed to high oxygen levels, foreign pathogen organisms and pollutants. Under normal conditions, AMs are key cells of the immune system with major phagocytic ability which, in response to stimulation, release various inflammatory factors and reactive oxygen species (ROS) (Hakim, 1993). It has become increasingly evident that these species can function as second messengers and at low levels can activate signalling pathways resulting in a broad array of physiological responses from cell proliferation to apoptosis (Forman and Torres, 2001).

An imbalance in the oxidative metabolism of AMs may cause cell damage which can be relevant in the progress of respiratory diseases (Sporn et al., 1990). The study of the effect of sodium arsenite (As3+) on AMs is critical to the knowledge of the potential human hazard of this toxicant in the lung. Bishayi and Sengupta (2003) observed that the viability of bacteria gradually decreased in control macrophages with time, whereas in macrophages of As3+ exposed mice the viability of *Staphylococcus aureus* gradually increased. As3+ interferes with the ability of macrophages to modulate the immune response, inhibiting cell inflammatory functions. Within this context we evaluated the metabolic alterations of AMs exposed to an As3+-enriched environment, focusing on morphologic and functional cell features and their potential association with arsenic induced diseases.

2.1. Chemicals affecting lungs physiology

There are many experimental evidences that exposure to organic solvents, pesticides, industrial effluents, motor exhausts and several other pollutants create a serious problems for the living tissues and organs. But their main target points are the lungs and liver. Exposure to various organic compounds including a number of environmental pollutants and drugs can cause cellular damage through metabolic activation of highly reactive substances like reactive oxygen species. Free radicals induce lipid per oxidation which destroys the membranes of the cells and create severe pathological satiations (Manna *et al.*, 2006)

Carbon tetrachloride (CCl_4) is the common name for tetra chloromethane, which is a synthetic compound and is highly toxic, carcinogenic for the living tissues. Carbon tetrachloride is a well known hepatotoxins, nephrotoxins, neurotoxins and pulmotoxins (Ogeturk *et al.*, 2005). Carbon tetrachloride also causes effects the other tissues of the body like lungs, thyroid, pancreas, adrenal gland, liver, kidney, and brain (ATSDR, 1993).

Zakria *et al.* (2004) observes the pulmonary lesions produced by carbon tetrachloride in the rats. The most prominent changes in the lungs are the vascular changes. Such changes were represented by the congestion of the blood capillaries that were engorged with erythrocytes. Most of the alveolar septa were thickened revealing the appearance of large number of type II alveolar cells, together with several fibroblasts, neutrophils, lymphocytes and macrophages.

Type II alveolar cells displayed certain degenerative changes in the form of masses of endoplasmic reticulum and scarcity of well recognized intracellular constituents. The endoplasmic reticulum has been severely fragmented into small spherical vesicles; these vesicles have been segregated within electron moderately dense material. Among the most signs of impairment encountered in the pulmonary tissue were inflammatory infiltrative cells consisting of neutrophils, mast cells and monocytes. The plasma cells were clearly affected with colossal quantities of dilated rough endoplasmic reticulum (RER). In addition, occasional degenerative areas were detected in the interstitial accompanied by obvious increase of longitudinally and circularly oriented collagen fibers. Boyd *et al.*(1980) found that oral administration of carbon tetrachloride to rats or mice caused striking decreases in rat lung microtonal cytochrome P 450 activity and in the enzyme-mediated covalent binding of 4-ipomeanol in preparations of rat and mouse lung microsomes. Histological examinations of lungs of animals given doses of CCl_4, orally or by inhalation, revealed striking morphologic changes in Clara cells, including severe dilation of endoplasmic reticulum and occasional cellular necrosis. Because of cytochrome P 450 enzymes are capable of mediating the formation of highly reactive and potentially toxic-free radicals from CCl_4, support the view that pulmonary Clara cells are susceptible to CCl_4 induced injury due to their capacity to metabolically activate the chemical.

2.2. Mecahanism of the acrylamide action

Reactive oxygen species (ROS) are normally generated in aerobic organisms (Halliwell et al., 1982). Natural antioxidative defense mechanisms counteract the production and actions of ROS; thus, under physiological conditions the production and detoxification of ROS are more-or-less balanced (H. Sies,. 1993). A variety of internal or external pathological factors may disrupt this balance, however, leading to conditions of excessive ROS generation and oxidative stress. Oxidative stress plays a significant role in the pathogenesis of cancer and involves numerous mechanisms (D. Dreher and A.F. Junod 1996). All molecules are sensitive to free radical attack (H.Sies,. 1986). The products of oxidative damage to DNA, lipid and protein constitute markers of oxidative damage (L.L. De Zwart et al , 1999); these damaged products usually are harmful and directly or indirectly cause DNA damage (J. Termini , 2000). The most frequently measured product of DNA damage, 8-oxo-2-deoxyguanosine (8oxodGuo), and other oxidized bases are highly mutagenic (R.A. Floyd , 1990 and L.J. Marnett , 2000). Lipid peroxidation is a chain reaction involving numerous by-products, which in turn damage DNA via a number of mechanisms (L.J. Marnett , 2000 and P.C. Burcham, 1998).. Proteins are also easily mutilated by free radicals, with consequential changes in enzyme activities and in the properties of membranes, including permeability, fluidity, efficiency of signaling pathways (E.R. Stadtman, 1992). To prevent the formation of damaged products and thereby diminish alterations in DNA is of basic importance in protection against cancer. The simplest scheme of the complex process of carcinogenesis comprises three main steps: initiation, promotion and progression. Oxidative stress may participate in all three steps of carcinogenesis. At the first step, initiation, free radicals activate several mechanisms contributing directly or indirectly to mutations. Initiated cells which result from these processes are also influenced by free radicals and exhibit either enhanced proliferation and/or inhibition of cell death. During the final step, free radicals may contribute to uncontrolled growth of tumor cells, genomic instability, resistance to chemotherapy, invasion and metastasis (Kolaja , 1998)

Reactive oxygen species (ROS) which are free radicals, cause denaturation of cell membranes, so living organisms have developed a specialized defense mechanism to

reduce the effects of ROS. Endogenous mechanisms by which ROS are generated involves specific cytokines such as Tumor necrosis factor (TNF) and interleukin-l , or after respiratory burst by neutrophils and activated macrophages. Exogenous mechanisms which involves the generation of ROS, includes number of chemical and physical agents such as ozone, paraquat, mineral dusts, nitrogen and sulphur oxides, hyperoxia, tumor initiators and promotrs, endotoxins and ultraviolet radiations. According to the recent researchers these reactive oxygen species play an important roll in the pathogenesis of various lung diseases like pulmonary fibrosis, pleural diseases, emphysema, adult respiratory distress syndrome, chronic bronchitis and lung cancer (Quinlan et al., 1994).

A typical antioxidant, whether it is enzymatic or nonenzymatic, should be able to reduce ROS so that they become less reactive than the parent species (i.e., the conversion of superoxide to hydrogen peroxide by superoxide dismutase in mitochondria. In biological systems, this is typically accomplished by the two-electron reduction of ROS (e.g., the conversion of superoxide to hydrogen peroxide by superoxide dismutase or the conversion of hydrogen peroxide to water by peroxidases). In order for curcumin to scavenge ROS, it must act as a reducing agent by donating keto-enol and/or phenolic protons (Fridovich, 1998).

Free radicals can be neutralized by molecules called antioxidants which eliminate the unpaired condition by accepting or donating electrons. In the process of neutralization, free radical becomes non free radical and antioxidant molecule becomes free radical. This antioxidant molecule may be neutralized by another antioxidant molecule or it may be very large to dilute the unpaired electron or it may have another mechanism to neutralize it. If in radical form, then antioxidant is less reactive than the neutralized radical.

Doelman and Bast, (1990) explains the formation of epoxides, peroxide and lipid peroxy radicals by diene radical, when OH abstract a hydrogen atom from polyunsaturated fatty acids in membranes. And a chain reaction may starts when this radical abstract another hydrogen atom from another polyunsaturated fatty acid. Polyunsaturated fatty acids are present in all biological membranes. Some proteins such as cytochrome p450 can homolytically cleave oxygen-oxygen bond of lipid hydroperoxide (R-O-O-R) which results in the formation of alkoxy and hydroxyl radicals.

So, that lipid peroxy radicals can be formed. By lipid peroxydation alkanes are like ethane and pentane, alkenes, aldehydes, ketones and hydroxyl acids are produced. Calcium influx occurs in endothelial cells of bovine pulmonary artery by destruction if lipid bilayer (Kehrer, 1993).

2.5. Medicinal plants as antioxidants

Medicinal plants have played a significant roll in maintaining human health and improving quality of human life for thousands of years. Antioxidant enzymes and metabolites are present in all living organisms, can prevent oxidative damage to cellular compounds like lipids, proteins and DNA. Researchers have been found that there is a link between plants or their herbal products and chemoprevention. A large number of plants contain antioxidant compounds. These antioxidants are useful agents for the prevention of several cancers. There are data demonstrating benefit in preventing colorectal and lung cancer. These agents prevent Carcinogenesis not only by inhibiting the initiation of cancer by reactive radicals but are also able to reduce the formation of carcinogens such as nitrosamine. So there is correlation between oxidative damage and cancer. The antioxidant activity may reduce the incidence of gastrointestinal cancers by reducing nitrosamine in the gut. Moreover antioxidant vitamins such as vitamin A and E may reduce the risk of lung cancer by reducing the amount of smoking-related exposure to free radicals (Greenwald et al., 1988).

Allium herbs/vegetables include plants in the garlic and onion family. These herbs and vegetables contain anticancer organisulfur compounds that include diallyl disulphides. These organosulphur compounds can inhibit H-Ras-transformed tumours by inhibiting the association of the gene product with the cell membrane. China demonstrated a large reduction in gastric cancer with a high intake of *Allium* vegetables due to antioxidant properties of the thiol-containing compounds in them (Zheng et al., 1992).

Green tea is fairly a well studied herb have catechins. Catechins are polyphenolic compounds that are not only potent antioxidants but also inhibit neucleoside transport and these both factors are beneficial in cancer prevention, it may have therapeutic value in the treatment of cancer in conjunction with antimetabolites. In addition, polyphenols in green tea may inhibit protein kinase C activation. Protein kinase C regulates cellular growth by

phosphorylating target proteins involved in cellular progression through the cell cycle. Chaparral has anticancer activity that is mediated by the antioxidant nordihydroguaiaretic acid. It has been used to treat cancer in humans. It contain substances that inhibit the cyclooxygenase enzyme which is responsible for oxidative conversion of procarcinogens to carcinogens (Sheng et al., 1997).

Although lung cancer is a leading cause of cancer deaths worldwide (H.I. Pass et al., 2005) and *Ganoderma lucidum* is widely used in the treatment of cancer in traditional Asian medicine (J.W.M. Yuen, et al 2005)., this is the first report on the effects of *Ganoderma* extracts on lung cancer cells.

Samarth et al., (2006) evaluated the chemopreventive and antimutagenic effects of an aqueous extract of *Mentha piperita* Linn on benzo[*a*]pyrene-induced lung carcinogenicity and mutagenicity in Swiss albino mice. Polycyclic aromatic hydrocarbons comprise an important class of environmental genotoxins, and the most extensively studied member of this class of chemicals is BP. This compound is ubiquitous in the environment, is mutagenic in both prokaryotic and eukaryotic test systems, and is an animal carcinogen (27 –28). BP is converted to a highly-reactive electrophile by enzymes involved in drug metabolism. The genotoxic metabolite produced by the cytochrome P450 (CYP) system has been identified as the *anti* isomer of BP 7,8-diol-9,10-epoxide. Three enzymatic reactions are required for its formation: initial epoxidation to yield the 7,8-epoxide, hydrolysis of this epoxide to yield the (–)-*trans*-7,8-diol, and finally a second epoxidation of the diol to produce BP-7,8-diol-9,10-epoxide (*anti* isomer) (Yang,S.K. et al 1977). The present study demonstrated that oral administration of ME has chemopreventive and antimutagenic effects against BP in Swiss albino mice. The ME produced significant reductions in the lung tumor incidence and tumor multiplicity and significant increases in body weight and the weight of the lungs in Swiss albino mice treated with BP as newborn.

The ROS produce deleterious effects by initiating lipid peroxidation directly or by acting as second messengers for the primary free radicals that initiate lipid peroxidation (Das andU.N. 2002). Thus, the enhanced hepatic LPO in BP-treated animals may be due to the generation of ROS exacerbated by decreased efficiency of host antioxidant defense mechanisms. The liver, which is rich in GSH, supplies this

antioxidant to various extrahepatic tissues via a distinct GSH transport system (Locigno,R. and Castronovo,V. 2001). GSH maintains the integrity of the liver when the organ is challenged by a wide variety of xenobiotics, ROS and toxic compounds (Lu,S.C. 1999). The depletion of GSH resulting from increased utilization to scavenge lipid peroxides may shift the redox status towards oxidative stress. The GSH content in liver and lung tissues observed in present study was significantly elevated suggesting a protective role of ME.

MATERIAL & MATHODS

MATERIALS AND METHODS

Herbs are staging a comeback and herbal 'renaissance' is happening all over the globe. The herbal products today symbolize safety in contrast to the synthetics that are regarded as unsafe to human and environment. Although herbs had been priced for their medicinal, flavouring and aromatic qualities for centuries, the synthetic products of the modern age surpassed their importance, for a while. However, the blind dependence on synthetics is over and people are returning to the naturals with hope of safety and security. The present study was conducted to evaluate the protective effects of *Digera muricata* on the toxicity induced with acrylamide in rat heart

3.1. Plant collection

Digera muricata (L.) Mart. locally also named as "Tandla" or "Lulur" at maturity were collected, shade dried and used as plant material. Plants were identified and submitted vide accession # 125127 in the Herbarium of Pakistan at Quaid-i-Azam University Islamabad, Pakistan.

3.2. Preparation of plant extract

Air dried aerial parts of *D. muricata* (1.50 kg) were ground and extracted with methanol and n-hexane of 5.0 litres successively, with occasional shaking and filtered. The residue re-extracted twice with the respective solvent and dried under vacuum. Methanolic extract of *Digera muricata* plant yielded 19g green viscous material.

3.3. Animals and treatment

Forty eight healthy female albino rats, weighing 190-200 g, were provided by the Animal House of National Institute of Health (NIH) Islamabad and were maintained at the Primate Facility maintained at Quaid-i-Azam University, Islamabad. Food and fresh water was available to the rats. These rats were divided into eight groups with six rats in each group. Among them group I, comprising of six rats were chosen randomly as control, while group II and were administered with dimethylsulphoxide (DMSO) 5.0 ml/kg b.w. orally once a day for five weeks. Group III was given 200 mg/kg b.w. of methanolic extract dissolved in DMSO once a day for five weeks. Rest of the rats were divided into five groups and were treated with aqueous solution of acrylamide 5 mg/kg b.w. intraparetoneally once a day for two weeks. Group IV was sacrificed after the acrylamide treatment to collect the lung tissue. Group V remained untreated as such for the rest of the experiment. Group VI,

VII and VIII were given 100, 150 and 200 mg/kg b.w. methanolic extract dissolved in DMSO once a day for two weeks.

3.4. Dissection procedure of animals

After completion of five weeks all the animals were kept on normal feed without any treatment for one day before the dissection of animals. Animals were given chloroform anesthesia and then start the dissection from ventral side, just below the diaphragm. First of all, the respiratory system (lungs and trachea) has been separated carefully, measures the weight by electrical balance and then washed in ice cold saline, dried with blotting paper and. After weighting the lungs were treated in two groups.

1. One lobe of lung from all tissue were cut off and stored in fixative sera (Absolute alcohol 60%, Formaldehyde 30%, Glacial acetic acid 10%), for histology.
2. The other lobe of lungs was treated with liquid Nitrogen and stored at -70°C.

3.5. Chemicals

Reduced glutathione (GSH), glutathione reductase, gamma-glutamyl p-nitroanilide, glycylglycine, bovine serum albumin (BSA), 1,2-dithio-bis nitro benzoic acid (DTNB), 1-chloro-2,4-dinitrobenzene (CDNB), reduced nicotinamide adenine dinucleotide phosphate (NADPH), flavine adenine dinucleotide (FAD), glucose-6-phosphate, 2,6-dichlorophenolindophenol, thiobarbituric acid (TBA), picric acid, sodium tungstate, sodium hydroxide, trichloroacetic acid (TCA) were purchased from sigma.

3.6. Histological procedure of tissues

Dehydration

Fixation is followed by dehydration in following ascending grades of alcohol

80% Alcohol	Overnight at room temperature
90% Alcohol	2-4 hrs at room temperature
100% Alcohol	2-4 hrs at room temperature

Following the dehydration fixed lungs tissue were transferred to cedar wood oil until they become clear and transparent at room temperature.

Embedding

Following steps were under taken.

Benzol 1	10 minutes at room temperature
Benzol 2	10 minutes at room temperature
Benzol + paraplast (1:1)	20 minutes at 60 °C
Paraplast 1	12 hrs at 60 °C
Paraplast 2	12 hrs at 60 °C
Paraplast 3	12 hrs at 60 °C

Embedded tissues were then transferred into melted wax in a boat. Bubbles were removed and wax was allowed to solidify. A block of wax containing tissue was prepared. Paraffin wax of blocks were trimmed with the help of sterilized knife or scalpel and then mounted on wooden block for sectioning.

Microtomy

Paraffin embedded tissue mounted on wooden blocks or plastic cassettes and cut thin slices of 3µm-5µm using microtome. The thin section embedded in wax strip is firstly shift to cold water where the long ribbon of wax is divided into portion equal to the size of glass slide. The sections along with wax stripe is than shift to the water bath temperature 60 °C. In water bath the wax stripe becomes smooth and then shift on clean glass slide. Slides were incubated at 65 °C in oven to melt the extra wax and fix the tissue on slide. These glass slides were now placed in incubator for overnight.

3.7. Staining Procedure

After removing wax from tissue they were stained by Eosin hematoxylin staining and silver nitrate. Eosin and Hematoxylin stain were prepared by following procedure.

Hematoxylin

Following chemicals were used in hematoxylin preparation.

Hematoxylin	2 g
Ethanol absolute	100 ml
Ammonium alum	3 g
Distilled water	100 ml
Glycerol	100 ml
Sodium iodide	0.24 g
Acetic acid	10 ml

Hematoxylin was dissolved in ethanol. Thoroughly prepare ammonium alum

solution in distilled water and boiled. After the addition of hematoxylin solution to ammonium alum solution, carefully added the glycerol and sodium iodide and then added acetic acid one by one in given amount, mix thoroughly.

Eosin

It is prepared by dissolving 1g of eosin in 100 ml of 70% of ethanol.

Staining Methodology

For staining by hematoxylin and eosin method following steps were taken.

Hydration

These slides were deparafinized in xylene as follows

Xylene 1	2-5 minutes at room temperature
Xylene 2	2-5 minutes at room temperature

Paraplast removed thyroid tissues were hydrated in graded solutions of alcohol in descending order.

100% Alcohol	2-5 minutes at room temperature
90% Alcohol	2-5 minutes at room temperature
70% Alcohol	2-5 minutes at room temperature
50% Alcohol	2-5 minutes at room temperature
30% Alcohol	2-5 minutes at room temperature
Hematoxylin	1-2 dips
Wash in tap water	5-10 minutes until the tissues were blue in colour.

Dehydration

Now cross sections were dehydrated in ascending order in alcoholic grades for 3-5 minutes.

30% Alcohol	2-5 minutes at room temperature.
50% Alcohol	2-5 minutes at room temperature.
70% Alcohol	2-5 minutes at room temperature.
90% Alcohol	2-5 minutes at room temperature.
Eosin	1-2 dip.
90% Alcohol	1 dip.
100% Alcohol	2-5 minutes at room temperature.
Xylene	5-10 minutes.

After staining the slides were mounted with Canada Balsam. Cover slips were placed on the slides and they were placed in incubator overnight. Extra Canada

Balsam was removed by xylene.

Light microscopic study

3-5µm thick sections were studied under compound microscope (DIALUX 20 EB) at 10x, 40x, 100x and magnifications and photographs were taken.

3.8. DNA Extraction

DNA was isolated by using the methods of Bin Wu *et al.* (2005), Barone *et al.* (2006) and Gilbert *et al.* (2007).Tissues used for DNA isolation were first treated with Liquid Nitrogen and stored at -70 °C up to start the DNA extraction. The reagents used in DNA isolation are given below.

Reagents

Chloroform

EDTA, 0.5 M

Ethanol, absolute

Isoamyl alcohol

Phenol

Phosphate Buffered Saline (PBS), 1X

Proteinase K

RNase A

Sodium dodecyl sulfate (SDS) solution, 10%

Preparation of Required Solution.

DNA buffer (Tris-EDTA)

1 M Tris pH 8.0	20 ml
0.5 M EDTA	20 ml
Sterile water	100 ml

Proteinase K (10 mg/ml)

Dissolve 100 mg Proteinase K in 10 ml TE for 30 min at room temperature (RT)

Aliquot and store at –20 °C

RNase A (20 mg/ml)

Dissolve 200 mg RNase A in 10 ml sterile water, boil for 15 min, and cool to room temperature. Aliquot and store at –20 °C

10xTBE (Tris Borate EDTA)

Tris	109 g
Boric acid	55g

EDTA 9.3g

Distilled Water 1000ml

Loading dye

0.25% Bromophenol Blue

4 g sucrose in water

Distilled water to 10 ml

Ethidum Bromide

10 mg/ml water

Agrose gel

1.5% agrose in 1xTBE

Composition of DNA Lysis buffer

10 mM Tris HCl (PH.8)

25 mM EDTA

100 mM NaCl

0.5% SDS

0.1 mg/ml protein kinase

Procedure

1. 70mg of tissue in a patri dish and washed with DNA Buffer.
2. When the tissue becomes air dry it was grinded in aluminum foil by using pestle and Mortar.
3. Put the grinded tissue into number of sterile 15 ml plastic falcon tubes.
4. Put the 1 ml lysis buffer of given composition to each tube and regrind the tissue in the presence of lysis buffer with the help of glass rod.
5. Add 100 µl proteinase K (10 mg/ml) and 240 µl 10% SDS, shake gently, and incubate overnight at 45 °C in a water bath.
6. If there are still some tissue pieces visible, add proteinase K again, shake gently, and incubate for another 5 hr at 45 °C.
7. Add 2.4 ml of phenol, shake by hand for 5-10 min, and centrifuge at 3000 rpm for 5 min at 10 °C.
8. Pipette the supernatant into a new tube; add 1.2 ml of phenol, and 1.2 ml chloroform/isoamyl alcohol (24:1); shake by hand for 5-10 min, and centrifuge at 3000 rpm for 5 min at 10 °C.
9. Pipette the supernatant into a new tube; add 2.4 of ml chloroform/isoamyl

alcohol (24:1), shake by hand for 5-10 min, and centrifuge at 3000 rpm for 5 min at 10 °C.

10. Pipette the supernatant into a new tube; add 25 µl of 3 M sodium acetate (pH 5.2) and 5 ml ethanol, shake gently until the DNA precipitates.

11. Pipit out the solution gently and the DNA thread is not disturbed.

12. Wash the DNA in 70% ethanol to dissolve all the impurities and dry it in the laminar flow.

13. Dissolve the DNA in T E buffer and put the RNAs to each tube having concentration at 4 °C on a rotating shaker.

14. Measure the DNA concentration in a spectrophotometer at 260 and 280nm.

3.9. DNA ladder assay

DNA Ladder was determined using the procedure of Wu *et al.* (2005) and Baron *et al.* (2006).

1. Total DNA was extracted by from thyroid was extracted sequentially using a Phenol-chloroform isoamyl alcohol mixture (25:24:1 v/v/v).
2. Proteins were removed and DNA was purified by using 70% alcohol.
3. Resolving Agarose gel Electrophoresis was performed using a 1.5% gel containing 1.0ug/ml ethidium bromide.
4. Depending upon experiment 5ug of total DNA per well was loaded.
5. DNA standards (0.5 µg per well) were included to identify the size of the DNA fragments.
6. Electrophoresis was performed for 45minuts 100 V, and DNA was observed under ultraviolet fluorescent lighting.

3.10. DNA fragmentation assay with diphenylamine reaction

DNA fragmentation from the lungs tissue extract was determined using the procedure of He *et al.* (2001) and Wu *et al.* (2005).

Materials

Lung tissue 100 mg

TTE solution TE buffer pH 7.4 (AI) with 0.2% tritonX-100 (4 °C)

Trichloroacetic acid (TCA) at 5% and 25%

Diphenylamine (DPA) solution preparation

a) 2g solid Diphenylamine (Recrystalyze in 100% ethanol)

b) 90ml glacial acetic acid

c) 10ml con H_2SO_4

Refrigerated cell centrifuge

Heating Plate

Spectro photometer (Smart spac™ Plus)

Water bath (20 °C-100 °C)

Methodology

1. Grind 0.1g of lungs tissue and mixed it in TTE solution.
2. Deliver1.0ml of cell suspension in tubes labeled B
3. Cells centrifuged at 200xg at 4 °C for 10 min
4. Supernatant transferred carefully in new tubes labeled S
5. Pellet added in tube B 1.0 ml TTE solution and were vortex vigorously
6. To separate fragmented DNA from intact chromatin, tubes were centrifuged at 20,000xg for 10 min at 4 °C
7. Supernatant transferred carefully in new tubes labeled T
8. Small pellet was added in tube B and 1.0 ml TTE solution
9. 1.0 ml of 25% TCA added to all tubes T,B and S and vortexes vigorously
10. Then allowed precipitation to proceed overnight at 4 °C.
11. After incubation precipitated DNA recovered by pelleting for 10 min at 18,000xg at 4 °C
12. Supernatants was discard by aspiration
13. DNA was hydrolyzed by adding 160µl of 5% TCA to each pellet and heating 15 min at 90 °C in a heating block.
14. To each tube 320µl of freshly prepared DPA solution was added, then vortexes. Allowed to develop color for about 4 hr at 37 °C.
15. Optical density was read at 590,600,620 nm with a spectrophotometer (Smart spac™ Plus, catalog # 170-2525).

3.11. Biochemical studies

3.11.1. Protein estimation.

The total soluble protein of homogenate and supernatant of lungs tissue was determined by the method of Lowry *et al.* (1951)

REAGENTS

Phosphate buffer

Potassium dihydrogen phosphate (0.1M) 80 ml

Disodium hydrogen phosphate	(0.1M)	420 ml

Standard protein

Bovine serum albumin	1 mg/ml

Alkaline copper solution

In this solution (50:1) fifty volume of solution (A) and one volume of solution (B) was added.

Solution A

Sodium carbonate	2 g
Sodium potassium tartarate	1 g
Sodium hydroxide	0.4g
Distilled water	100 ml

Solution B

Copper sulphate	0.5 g
Distilled water	100 ml

Folin ciocalteau phenol reagent

Folin cicalteu phenol+dH2O (1:1) were prepared immediately before use.

Procedure

a) 50-80 mg of lungs tissue mixed in 4 volume of phosphate buffer and homogenized in ice bath throughout.
b) The homogenate is then centrifuged at 10,000 rpm for 20 minutes at 4 °C.
c) Supernatant is decanted and saved for protein assay.
d) The saved supernatant is diluted in 5 folds if required in 0.1M phosphate buffer (pH, 7.4).
e) 1ml alkaline copper solution was then added to each sample tube and mix thoroughly.
f) After 10 minutes 0.1ml of 1:1 folin ciocalteau phenol reagent added to each tube.
g) Thoroughly mixed on vortex and incubated for 30 minutes at room temperature.
h) Then optical density was noted at 500, 595 and 650nm at smart spec™ spectrophotometer.
i) For standard curve bovine serum albumin was dissolved 10mg/ml in phosphate buffer of same concentration used for samples and then its further

serial dilutions were used and make slandered curve from it O.D values.

j) The concentration of soluble protein was calculating in mg/ml by comparing the standard O.D values with sample valve.

k) The amount of protein was expressed as mg/ml of tissue homogemate in phosphate buffer.

3.11.3. Extraction of antioxidant enzymes

Lungs tissue of 20-30 mg weighted and was grinded in 1.5 ml of 50mM phosphate buffer (pH 7.8) and centrifuged at 15,000xg for 20 minutes at 4 °C. The supernatant was stored at 4 °C and was used for the determination of activities of enzymes.

3.11.3.1. Superoxide dismutase (SOD) (EC No.1.15.1.1)

The activity of SOD was assayed by using the protocol of Beyer and Fridovich (1987) to measure its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT). The reaction solution (3ml) contained. Reagents used in the process are:

50µM NBT

1.3µM riboflavin

13mM methionine

75nM EDTA

50mM phosphate buffer (pH 7.8)

20-50µl enzyme extract

a) The one set of test tubes containing the reaction solution were irradiated under a light (15W fluorescent lamps) at 78µM $m^{-2}s^{-1}$ for 15 min

b) While the other set of test tube was wrapped in aluminum foil for 15 minutes with out light (in dark).

c) The third tube contains only reaction chemicals and has not any enzyme extract.

d) The absorbance of three types of tubes were noted at 650nm on a (SmartSpec™ Plus Spectrophotometer catalog # 170-2525).

e) One unit of SOD activity was defined as the amount of enzyme which caused 50% inhibition of photochemical reduction of NBT.

f) Data were recorded from five randomly selected rats of each group.

3.11.3.2. Peroxidase activity (POD) (EC No.1.11.1.7)

The activity of POD was assayed by using the protocol of Chance and Maehly.

(1955) using the guaiacol oxidation method.

The POD reaction solution (2.2 ml) contained the following reagents:

50mM phosphate buffer (pH 5.0)

20 mM guaiacol

0.1ml 40 mM H_2O_2

0.1ml enzyme extract

Changes in absorbance of the reaction solution at 470nm at (SmartSpec™ Plus Spectrophotometer catalog # 170-2525) were determined every 20 seconds.

One unit POD activity was defined as an absorbance change of 0.01 units min^{-1}

Data were recorded on six rats randomly selected rats from each group.

3.11.3.3. Catalase activity (CAT) (E.C. No.1.11.1.6)

Activities of CAT were measured using the method of Manna *et al.* (2006) with some modification.

The CAT reaction solution 2.2ml contained

2ml 50mM Phosphate Buffer (pH 7.0)

0.1ml 5.9mM H_2O_2

0.1ml Enzyme Extract

The reaction was initiated by adding the H_2O_2 to reaction solution. Changes in absorbance of the reaction solution at 240 nm at (SmartSpec™ Plus Spectrophotometer catalog # 170-2525) were noted after every 30s. One unit CAT activity was defined as an absorbance change of 0.01 unit min^{-1}. Data were recorded on six rats from each group.

3.11.3.4. γ-glutamyl transpeptidase assay (γ-GT)

This was determined by the method of Orlowski and Meister (1973) using glutamyl *p*-nitroanilide as substrate. The reaction mixture in a total volume of 1.0 ml contained 0.2 ml 10% homogenate which was incubated with 0.8 ml substrate mixture (containing 4 mM glutamyl *p*-nitroanilide, 40 mM glycylglycine and 11 mM $MgCl_2$ in 185 mM Tris–HCl buffer, pH 8.25) at 37 °C. Ten minutes after initiation of the reaction, 1.0 ml of 25% TCA was added and mixed to terminate the reaction. The solution was centrifuged and the supernatant fraction was read at 405 nm. Enzyme activity was calculated as nM *p*-nitroaniline formed/min/mg protein using a molar extinction coefficient of 1.74 x 10^3/M cm.

3.12.3.5. Estimation of lipid peroxidation (TBARS)

The assay for microsomal lipid peroxidation was done following the method of Wright *et al.* (1981) as modified by Iqbal *et al.* (1996). The reaction mixture in a total volume of 1.0 ml contained 0.58 ml phosphate buffer (0.1 M, pH 7.4), 0.2 ml microsomes, 0.2 ml ascorbic acid (100 mM), 0.02 ml ferric chloride (100 mM). The reaction mixture was incubated at 37 ◦C in a shaking water bath for 1 hr. The reaction was stopped by addition of 1.0 ml 10% trichloroacetic acid. Following addition of 1.0 ml 0.67% thiobarbituric acid, all the tubes were placed in boiling water-bath for 20 min and then shifted to crushed ice-bath before centrifuging at 2500xg for 10 min.The amount of malonaldehyde formed in each of the samples was assessed by measuring optical density of the supernatant at 535 nm using spectrophotometer (Milton Roy 21 D) against a reagent blank. The results were expressed as nmol MDA formed/min/mg tissue at 37 °C using molar extinction coefficient of 1.56x105/M cm.

3.11.3.6. Assay for glutathione reductase activity (GSH)

Glutathione reductase activity was determined by method of Carlberg and Mannervik (1975). The reaction mixture consisted of 1.65 ml phosphate buffer (0.1 M, pH 7.6), 0.1 ml EDTA (0.5 mM), 0.05 ml oxidized glutathione (1 mM), 0.1 ml NADPH (0.1 mM) and 0.1 ml 10% PMS in a total volume of 2 ml. Enzyme activity was quantitated at 25 ◦C by measuring disappearance of NADPH at 340 nm and was calculated as nmolNADPH oxidized/min mg protein using molar extinction coefficient of 6.22 x103/M cm.

3.11.3.7. Assay for glutathione-S-transferase activity (GST)

Glutathione-S-transferase activity was assayed by the method of Habig *et al.* (1974). The reaction mixture consisted of 1.475 ml phosphate buffer (0.1 M, pH 6.5), 0.2 ml reduced glutathione (1 mM), 0.025 ml CDNB (1 mM) and 0.3 ml PMS (10% (w/v) in a total volume of 2.0 ml. The changes in the absorbance were recorded at 340 nm and enzymes activity was calculated as nM CDNB conjugate formed/min mg protein using a molar extinction coefficient of 9.6×103/M cm.

3.12. Statistical analysis

To determine the effect of treatment on body weight, lungs weight, soluble protein contents, DNA fragmentation and activity of antioxidant enzymes was carried out by the two way analysis of variance (ANOVA) by computer software MSTATC and Minitab 14 demo. Level of significance among the various treatments was

determined by LSD at alpha 0.05% with 95% confidence interval.

RESULTS

RESULTS

This study was conducted to determine the protective effects of methanolic extract of the *Digera muricata* plant against the injuries of lungs induced by acrylamide at histological, biochemical and molecular level in rats. Different parameters were used to study the deteriorating effects of acrylamide on histology and biochemistry of lungs. Moreover the repairing of lungs by using the crude extract of "Tandla" weed (*Digera muricata* (L.) Mart. in methanol was studied. The results obtained are described below.

4.1. Variation in lungs and body weight among experimental groups

Protective effects of various fractions against acrylamide administration in rat on percent increased in body weight and lung weight are shown in (Table 4.1). Administration of acrylamide significantly increased ($P<0.01$) lung weight and relative lung weight while decreased the percent increase in body weight when compared to control group. However, co-treatment with of methanolic extract of *D. muricata* at different concentration ameliorated the acrylamide intoxication effects and significantly reduced ($P<0.01$) the lung weight and relative lung weight while significantly ($P<0.01$) increased the percent increase in body weight as compared to acrylamide group.

4.2. Histopathology

Histopathalogical features of all experimental groups were observed after the hemotoxylene and eosin stains. There were significant variations in all groups.

4.2.1. Control group

In case of control group lungs show normal morphology. Alveoli have thin walls with interalveolar septum (Fig. 4.1). The alveolar walls have type I and type II pneumocytes. At the junction of alveolar walls fibroblasts were also prominent. The alveolar macrophages were also prominent attached with the inner alveolar membrane and sometime hanging in the alveolar space. Blood capillaries were surrounding around the alveolus showing the normal blood circulation, and blood cells were also present inside the capillaries. The alveolar bronchiole showed their normal shape and was without the cartilage. Inner epithelium of alveolar bronchiole have pear shaped Clara cells were pointed toward the cavity of the bronchiole.

Table 4.1. Variation in lungs weight and body weight among experimental groups of rat.

Group	Treatments	% increase in Body weight	Lung weight (g)	Relative lung weight
I	Control	35.00±0.87++	1.63±0.14++	0.01634±0.00148++
II	200 mg/kg extract	35.79±0.55++	1.61±0.12++	0.01566±0.00128++
III	DMSO	32.52±0.92++	1.62±0.13++	0.01667±0.00131++
IV	Acrylamide (6 mg/kg) 15days	30.85±0.96**++	1.81±0.16**	0.01816±0.00161**
V	Acrylamide 30 days	30.08±0.81**++	1.98±0.09**	0.01980±0.00095**
VI	Acrylamide and extract 100 mg/kg	31.64±0.73++	1.88±0.13++**	0.01830±0.00132++**
VII	Acrylamide and extract 150 mg/kg	32.82±0.69*++	1.78±0.01++*	0.01756±0.00017++*
VIII	Acrylamide and extract 200 mg/kg	35.92±0.77**++	1.67±0.05*	0.01630±0.00057++

Mean ±SE (n=6 number)

*,** indicate significance from the control group at P<0.05 and P<0.01 probability level, respectively.

+,++ indicate significance from the acrylamide group at P<0.05 and P<0.01 probability level, respectively.

Table 4.2 Effect of various fractions on histopathology of lungs

Group	Treatments	A B	DCT	DEF	CBC	ABC	DIEAB	PE	PF
I	Control	-	-	-	-	-	-	-	-
II	200 mg/kg extract	-	-	-	-	-	-	-	-
III	DMSO	+++	+++	+++	+++	+++	+++	+++	++
IV	Acrylamide (6 mg/kg) 15days	-/+	-	-	-/+	-	+/-	-	-
V	Acrylamide 30 days	-	-	-/+	-	-/+	-/+	-	-
VI	Acrylamide and extract 100 mg/kg	-/+	-	-	-	-	-/+	-	-
VII	Acrylamide and extract 150 mg/kg	-	-	-	-	-	-	-	-
VIII	Acrylamide and extract 200 mg/kg	-	-/+	-/+	-/+	-/+	-/+	-/+	-

-, normal; +/-, mild; +++, severe disruption

AB (alveolar breakage), DCT (degeneration of connective tissue), DEF (damages of elastic fiber), CBC (congestion of blood capillaries), ABC (aggregation of blood cells), DIEAB (disorganized inner epithelium of alveolar bronchiole, PE (pulmonary edema), PF (pulmonary fibrosis)

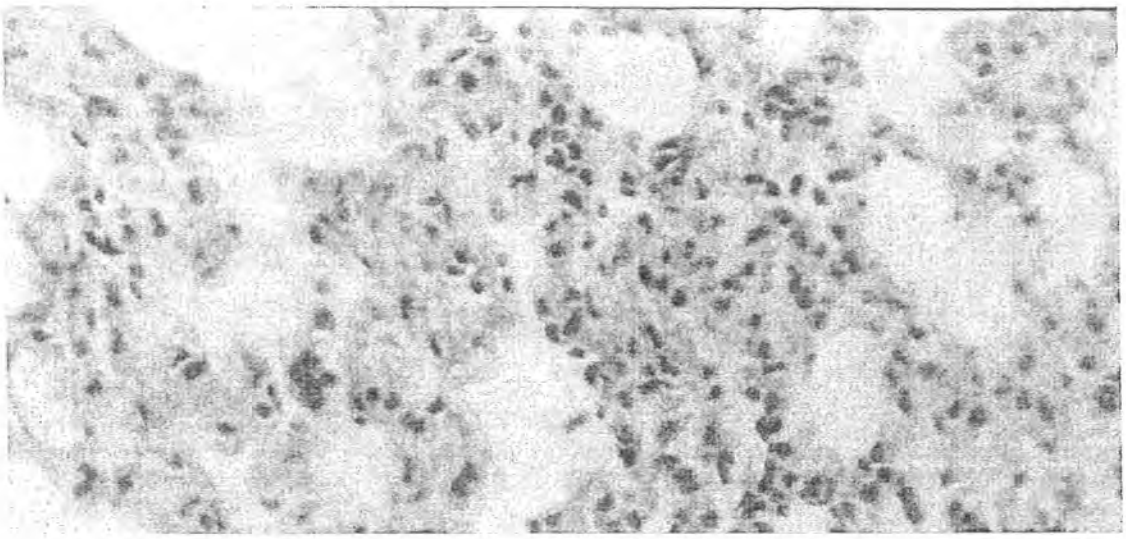

Fig . 4.1. Control group. Rat lungs showing the well developed alveolar sacs, alveolar walls along with alveolar septum, alveolar macrophages, Pneumocyte I and II are also prominent in control group.

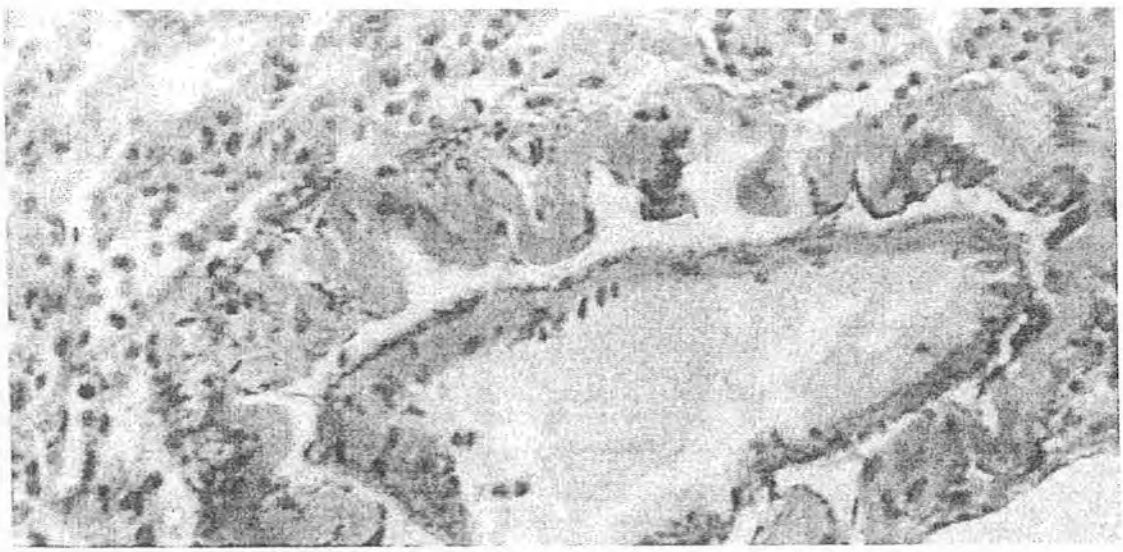

Fig . 4.2. Control group. Structure of alveolar bronchiole having appendages like non ciliated Clara cells, towards the inner side of terminal bronchiole

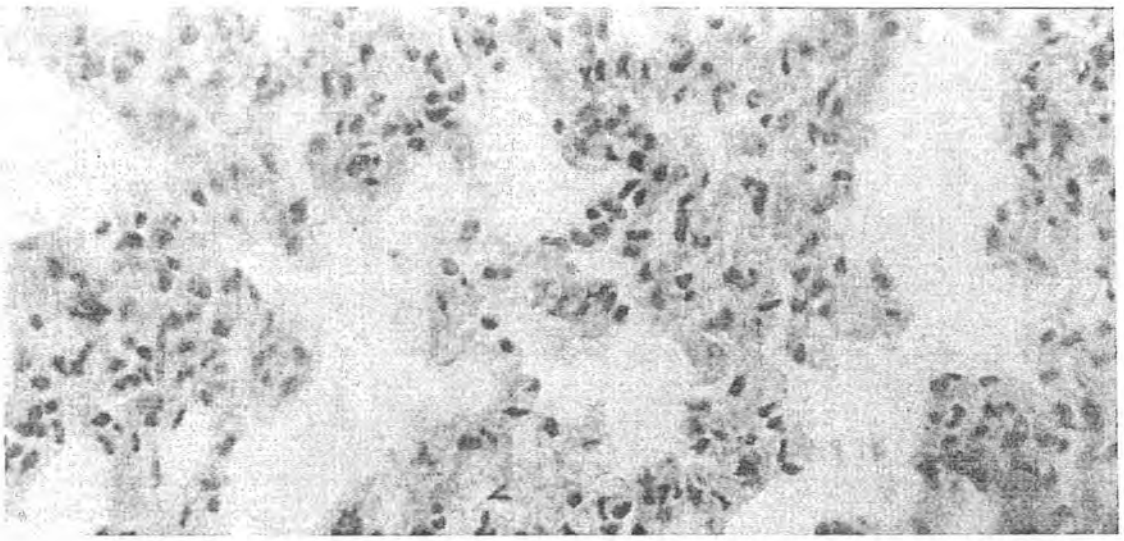

Fig 4.3. Control DMSO. Alveolar sacs are prominent with inter alveolar septum.

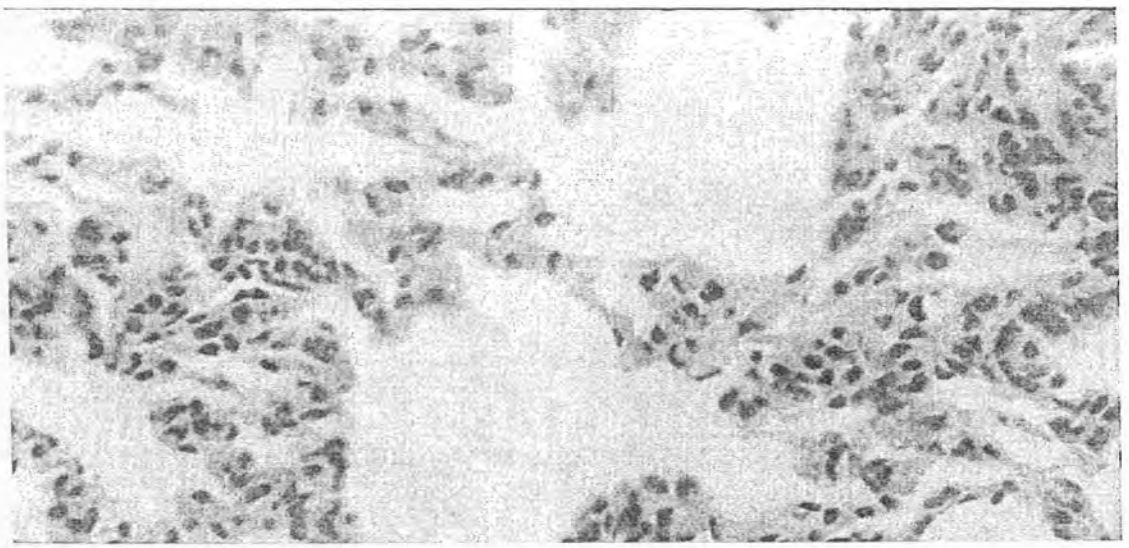

Fig. 4.4. Methanolic extract of *Digera muricata* 200mg/kg treated rats showing normal histology with minor glomerular changes.

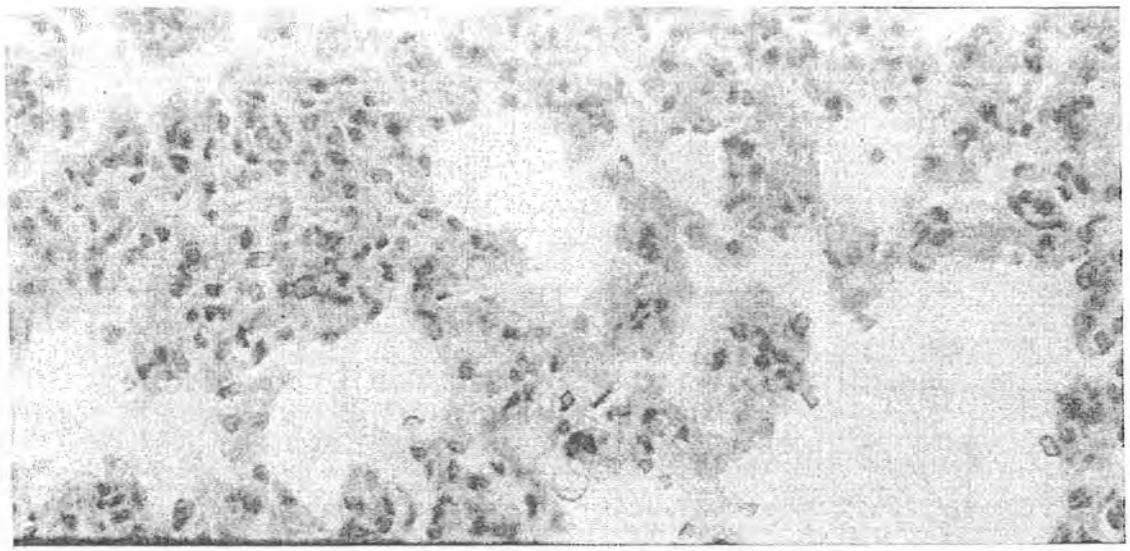

Fig. 4.5. Acrylamide (15 days) . Ruptured alveolar walls along with interstitial edema and aggregation of pulmonary parenchyma can be seen.

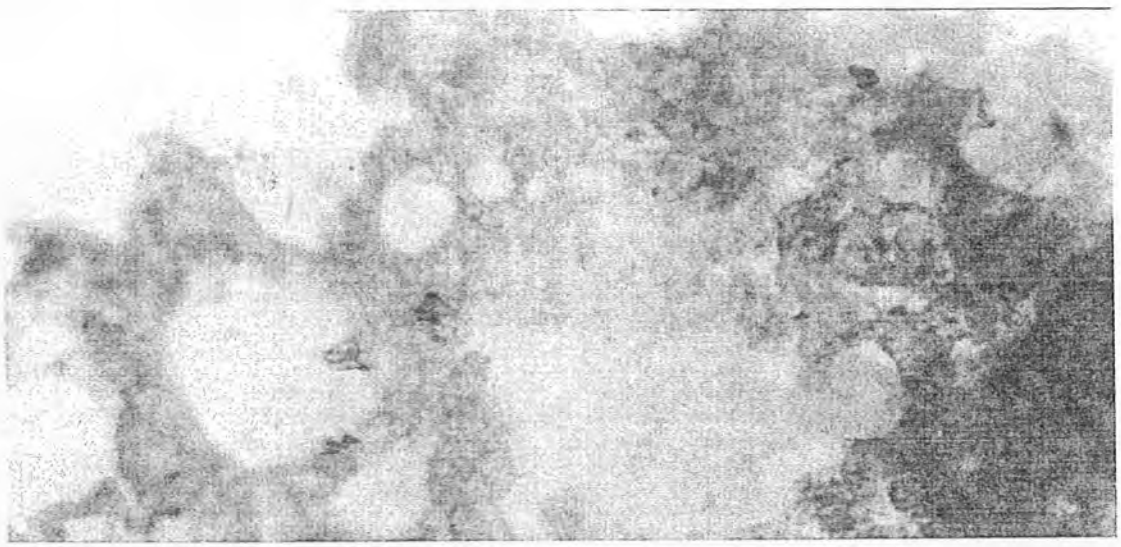

Fig.4.6. Acute lung injuries with disorganized structure. Fibroblasts, Blood cells and demaged blood copillaries.

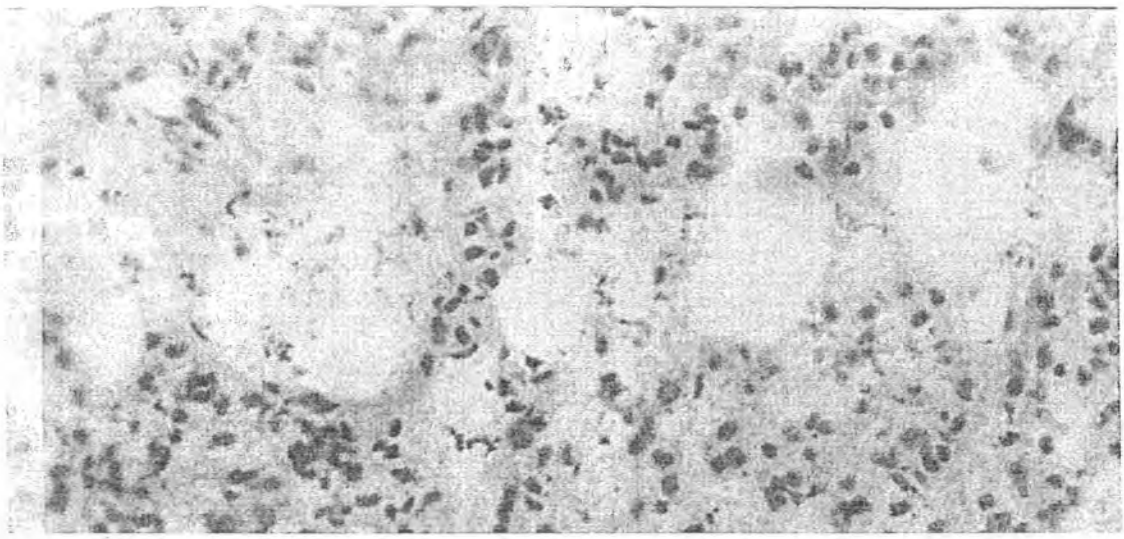

Fig.4.7. Acrylamide (30 days) Aggregation of fibroblasts and along with collagen fibers and ruptured alveolar walls showing the pulmonary edema and interstitial hemorrhage due to high dose of acrylamide.

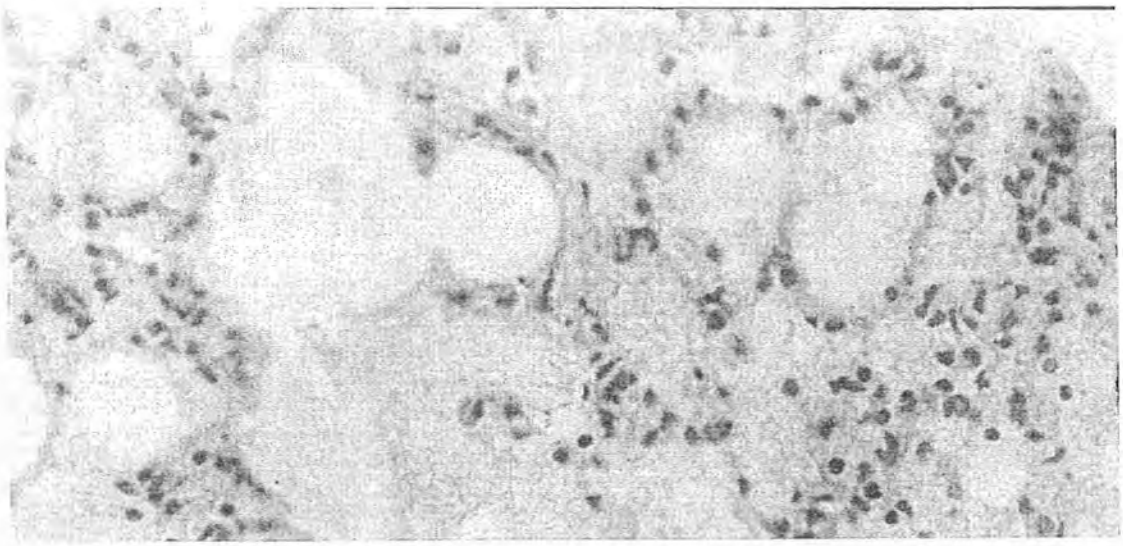

Fig 4.8. Intense damages due to toxicity of acrylamide

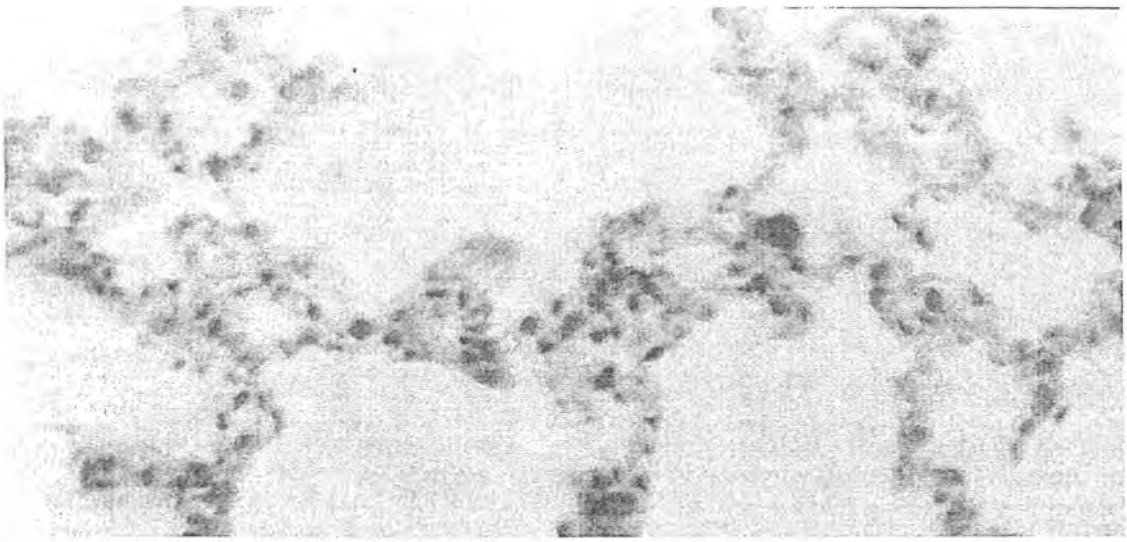

Fig.4.9. . Control DMSO. Alveolar sacs are prominent with inter alveolar septum.

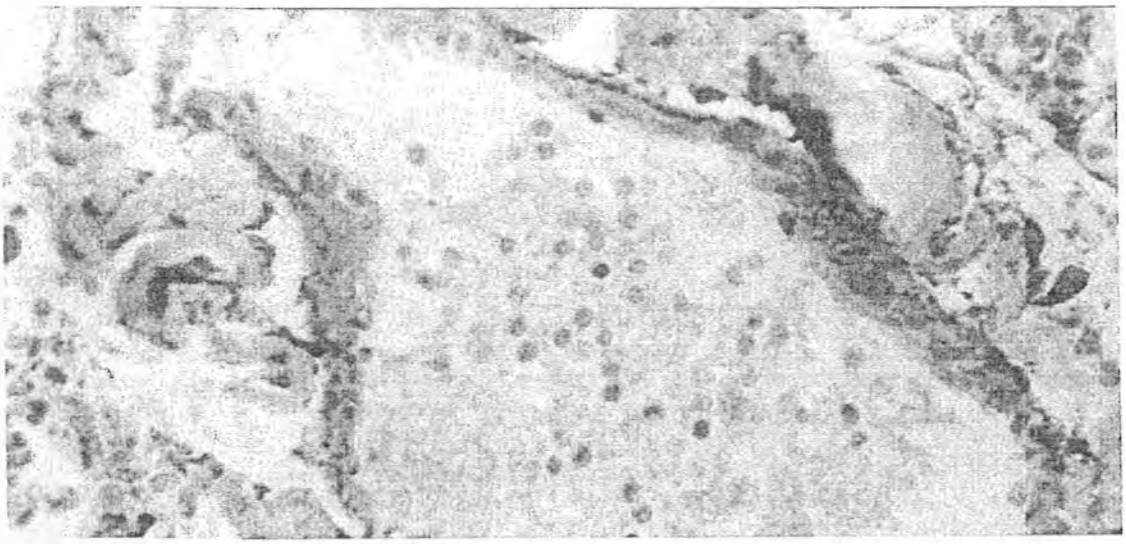

Fig.4.10. Alveolar bronchiole has centrally collapsed inner epithelial layer (Columnar epithelium. CE) and outer muscular layer, and inner non ciliated clara cells re also disorganized.

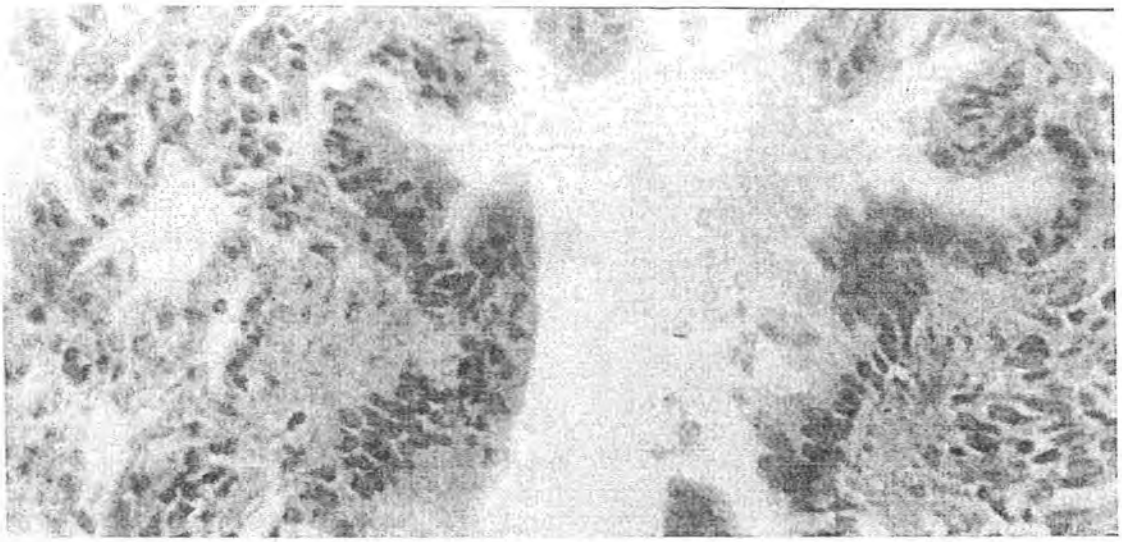

Fig.4.11. Alveoli and alveolar bronchiole with non-ciliated clara cells and toward the inner side of terminal bronchiole.

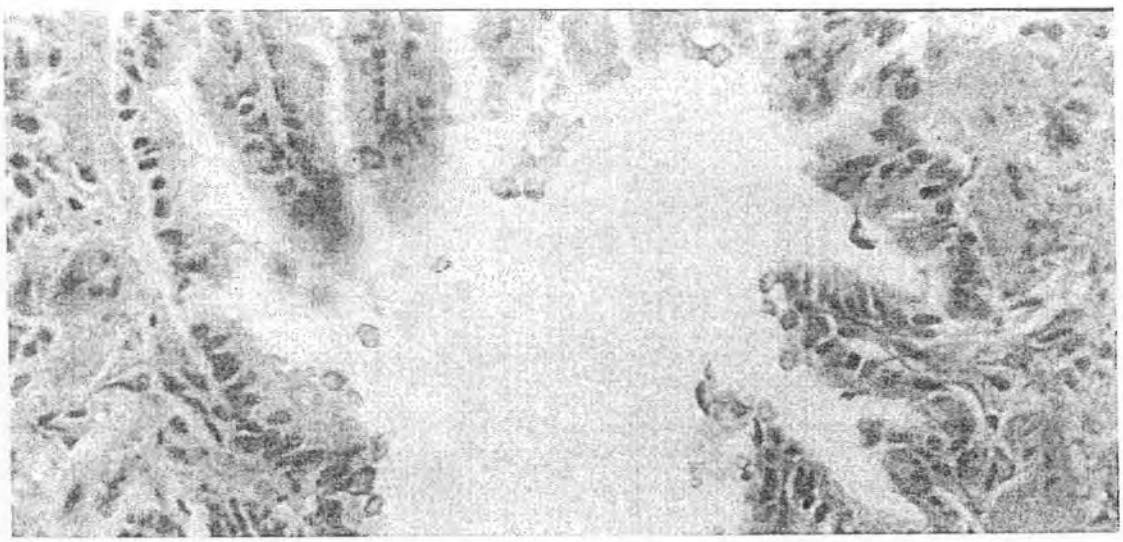

Fig.4.12. Alveoli and alveolar bronchiole with non-ciliated clara cells and toward the inner side of terminal bronchiole.

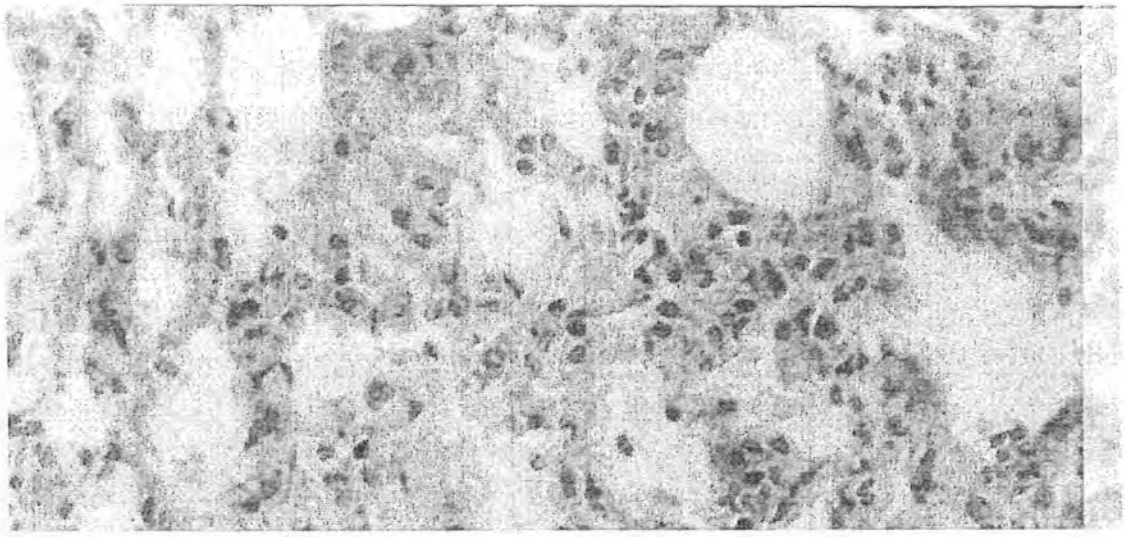

Fig.4.13. Acrylamide (15 days) + 100mg/kg MDMP.This picture shows normal and regular alveolar spaces, but there is aggregation showing the protective and repairing action of *Digera muricata.*

4.2.2. Acrylamide treated group

The histology of acrylamide treated group was totally different from control group. There were severe injuries in the overall section like disruption of alveolar septa, degeneration of the connective tissues and elastic fibres and the congestion of blood capillaries which were also blocked with the large aggregation of blood cells. Most of the alveolar septa become hard and thickened revealing the appearance of large number of type II pneumocytes alveolar cells, together with several fibroblasts, neutrophills, and macrophages. The alveolar bronchiole had the constricted inner epithelium and moved toward the inner side reducing the volume of lumen and shortened the air breathing passage. The inner non ciliated cells "Clara cells" also showed the wilting and rupturing at some places. The epithelium of alveolar bronchiole had the congested inner cytoplasmic contents. The alveolar macrophages were also disrupted and some are present in the mild condition (Fig. 4.2)

4.2.3. Methanol extract treated group

In case of methanol extract treated group, showed somewhat more or less normal histological characteristics in the lungs which were simultaneously treated with achrylamide and methanolic extract of *Digera muricata.* This group had the normal alveolar spaces but some inter alveolar septa were thickened indicating the some remnant effects of acrylamide induced injuries. The blood capillaries were normal in shape but their diameter was comparatively less as compared to control group due to which some erythrocytes were gathered at some places. The terminal respiratory bronchiole also had the normal features, but the inner epithelium was slightly constricted towards the inner lumen like normal. The non ciliated cells of the columnar epithelium of respiratory bronchiole were also normal, but some are in mild condition. The alveolar macrophages are also normal. The other cells like fibroblast and pneumocyte type I in the inter alveolar septa had the normal structure with connective tissues and elastic fibres, but there was some deposition of collagen fibres. The alveolar pneumocyte II were normal in their shape but larger in number showing the protective effects of methanolic extract of *D. muricata*. So, treatment of methanolic extract of *D. muricata* preserved the normal morphology of lungs and showed somewhat normal features (Fig. 4.).

Table 4.3 Effect of *D. muricata* on protein and antioxidant enzymes in lung tissues.

Group	Treatments	Protein µg/mg tissue	SOD U/mg protein	POD U/min	CAT U/min
I	Control	1.20±0.03++	7.60±0.45++	8.01±0.68++	2.11±0.23++
II	200 mg/kg extract	1.23±0.11++	7.50±0.32++	7.60±0.42++	2.01±0.31++
III	DMSO	0.51±0.10**	4.01 ±0.29**	6.21±0.19**	0.71±0.10**
IV	Acrylamide (6 mg/kg) 15days	0.60±0.31**	5.42±0.31**	6.50±0.24**	0.81±0.26**
V	Acrylamide 30 days	1.00±0.11+	7.30±0.43++	6.80±0.28+	1.01±0.11+
VI	Acrylamide and extract 100 mg/kg	1.5±0.08++	7.27±0.38++	7.3±0.19++	1.80±0.07++
VII	Acrylamide and extract 150 mg/kg	2.10±0.24++	7.50±0.38++	7.80±0.21++	2.11±0.24++
VIII	Acrylamide and extract 200 mg/kg	1.90±0.29++	7.71±0.24++	7.80±0.45++	1.91±0.29++

Mean ±SE (n=6 number)
*,** indicate significance from the control group at P<0.05 and P<0.01 probability level, respectively.
+,++ indicate significance from the acrylamide group at P<0.05 and P<0.01 probability level, respectively.

Table 4.4 Effect of various fractions of the acrylamide and extract of *D. muricata* on the GST and γ-GT contents in the lungs of rat.

Group	Treatments	GST nmol /min/mg protein	γ-GT nmol /min/mg protein
I	Control	166.00±1.93	0.040±0.004++
II	200 mg/kg extract	161.00±2.05++	0.038±0.002++
III	DMSO	165.10±1.57++	0.039±0.005++
IV	Acrylamide (6 mg/kg) 15days	132.00±2.59**++	0.078±0.005++
V	Acrylamide 30 days	94.20±1.93**++	0.080±0.003**
VI	Acrylamide and extract 100 mg/kg	129.50±2.35**++	0.073±0.003**
VII	Acrylamide and extract 150 mg/kg	150.00±1.83*++	0.062±0.003++*
VIII	Acrylamide and extract 200 mg/kg	158.00±2.71++	0.048±0.006++

Mean ±SE (n=6 number)
*,** indicate significance from the control group at P<0.05 and P<0.01 probability level, respectively.
+,++ indicate significance from the acrylamide group at P<0.05 and P<0.01 probability level, respectively.

4.3. Estimation of protein

Table 4.3 depicts the level of protein in the tissue of normal and experimental groups of rats. There was found significant difference of protein contents of rat lungs in different treated groups. Treatment of acrylamide showed a significant decrease in the protein concentration as compared to the control groups. The treatment with different concentrations of methanolic extract of *D. muricata* significantly increases the protein concentration as compared to acrylamide treated rats.

4.4. Antioxidant enzymes

Nature has provided metabolic systems to various organisms to reduce the oxidative damage to cells and tissues. During metabolic processes various oxidant metabolites are formed as a by product in the cells. Antioxidant enzymes are present to reduce the metabolites as a scavenging process. Following studies were conducted to establish the activities of various antioxidant enzymes which are given as under.

4.4.1. Effect of *D. muricata* extract on CAT

CAT activity was decreased ($P<0.01$) with acrylamide treatment in the lungs tissues as compared to the control group (Table 4.3). Toxicity of acrylamide was erased with the treatment of *D. muricata* extract and the CAT reversed towards the normal level in a dose dependent way. Methanolic extract of *D. muricata* itself was unable to change the CAT activity of lung tissues as against the control group.

4.4.2. Effect of *D. muricata* extract on POD

Treatment of acrylamide decreased ($P<0.01$) the POD activity in the lungs tissues versus the control group of rats. *D. muricata* extract reversed the POD activity towards the normal level and this effect was more pronounced at the higher level of the extract. Treatment of MDMP alone did not change the POD activity in the lungs tissues (Table 4.3).

4.4.3. Effect of *D. muricata* extract on SOD

SOD activity was decreased in the lungs tissues with the acrylamide treatment as against the control group (Table 4.3). Activity of this enzyme was increased with the treatment of *D. muricata* in a dose dependent manner as compared to the control. However, SOD activity remained unchanged in the lungs tissues with the treatment of *D. muricata* alone as against the control group.

4.4.4. Effect of *D. muricata* on GST

GST activity was decreased ($P<0.01$) with acrylamide treatment in the lungs tissues as compared to the control group (Table 4.4). Toxicity of acrylamide was ameliorated with the treatment of methanolic extract of *D. muricata* and the GST returned towards the normal level in a dose dependent way. However, *D. muricata* extract itself did not change the GST activity of lungs tissues as against the control group.

4.4.5. Effect of *D. muricata* on γ-GT contents

γ-GT activity was decreased in the lungs tissues with the acrylamide treatment as against the control group (Table 4.4). Activity of this enzyme was increased with the treatment of *D. muricata* in a dose dependent manner as compared to the control. However, SOD activity remained unchanged in the heart tissues with the treatment of MDMP alone as against the control group.

4.4.6. Effect of *D. muricata* on GSH contents

Treatment of acrylamide decreased ($P<0.01$) the GSH contents in the lungs tissues versus the control group of rats. Various doses of the extract reversed the GSH contents towards the normal level and this effect was more pronounced at the higher level of the extract (200 mg/kg). Treatment of the extract alone did not change the GSH contents activity in the lungs tissues (Table 4.5).

4.4.7. Effect of *D. muricata* on H_2O_2 contents

Treatment of acrylamide increased ($P<0.01$) the H_2O_2 contents in the lungs tissues versus the control group of rats. Various doses of the extract reversed the H_2O_2 contents towards the normal level and this effect was more pronounced at the higher level of the extract (200 mg/kg). Treatment of the extract alone did not change the H_2O_2 contents activity in the lungs tissues (Table 4.5).

4.4.8. Effect of *D. muricata* on TBARS contents

Treatment of acrylamide increased ($P<0.01$) the TBARS contents in the lungs tissues versus the control group of rats. Various doses of the extract reversed the TBARS contents towards the normal level and this effect was more pronounced at the higher level of the extract (200 mg/kg). Treatment of the extract alone did not change the TBARS contents activity in the lungs tissues (Table 4.5).

Table 4.5 Effect of various fractions of the acrylamide and *D. muricata* extract on the GSH, TBARS H_2O_2 contents and DNA fragmentation% in the lungs of rat.

Group	Treatments	GSH µmol /g tissue	TBARS nmol /min/mg protein	H_2O_2 nmol /min/mg tissue	DNA Fragmentation%
I	Control	0.14±0.009++	10.50±0.38++	20.40±0.68++	44.95±1.38++
II	200 mg/kg extract	0.13±0.003++	9.51±0.40++	21.30±0.95++	44.48±0.93++
III	DMSO	0.14±0.005++	11.50±0.65++	21.16±0.62++	45.78±0.80++
IV	Acrylamide (6 mg/kg) 15days	0.07±0.005**	16.90±0.50++**	32.80±0.96**	57.67±0.83**
V	Acrylamide 30 days	0.08±0.004**	15.11±0.50++**	29.64±0.99**	58.70±1.42**
VI	Acrylamide and extract 100 mg/kg	0.09±0.007++**	14.50±0.49++**	26.81±0.94++**	53.58±0.97++**
VII	Acrylamide and extract 150 mg/kg	0.10±0.006++**	12.40±0.86++*	23.14±0.55++**	48.78±1.02++*
VIII	Acrylamide and extract 200 mg/kg	0.11±0.002++*	11.50±0.70++	22.18±0.73++*	46.81±1.40++

Mean ±SE (n=6 number)

*,** indicate significance from the control group at $P<0.05$ and $P<0.01$ probability level, respectively.

+,++ indicate significance from the acrylamide group at $P<0.05$ and $P<0.01$ probability level, respectively.

4.5. Effect of fractions on DNA fragmentation%

Effect of various fractions of *Digera muricata* on DNA fragmentation% (Table 4.4) is shown. Acrylamide administration to rats significantly increased the DNA fragmentation% in the lung tissues. Co-treatment of various doses of the extract ameliorated the effects of acrylamide intoxicity, DNA% fragmentation were markedly reversed towards the normal range.

4.6. Effect of fractions on DNA damages (Ladder assay)

Toxicity of acrylamide induced DNA damages in the lung tissues of rats. DNA banding pattern are shown in (Fig. 4.). DNA ladder assay showed the similar banding pattern in control and DMSO groups indicating no damages. However, acrylamide group showed extensive DNA damages. There was much variation present in the genomic DNA extract from the lung tissue of all experimental group in rat. In case of control group the genomic DNA gave single sharp band of given size. In case of acrylamide treated rats DNA sample shows a peculiar type of continuous pattern of DNA fragmentation of up to 100bp, which was absent in the control group. DNA extracted from the lungs tissue of the plant extract treated rats markedly repair the DNA damage and the tailing of the DNA fragmentation was not detected from the gel. Extract group showed much parallel with the intact group. In the case of acrylamide + 150 mg/kg group there was slightly tailing of the genomic DNA present show the partial repairing of DNA damage caused by the acrylamide treatment. Treatment of the extract at 200 mg/kg, gave almost the similar DNA banding pattern to that of the control group shows that the DNA fragmentation was significantly improved by the *Digera muricata* indicated the protected effect of plant extract.

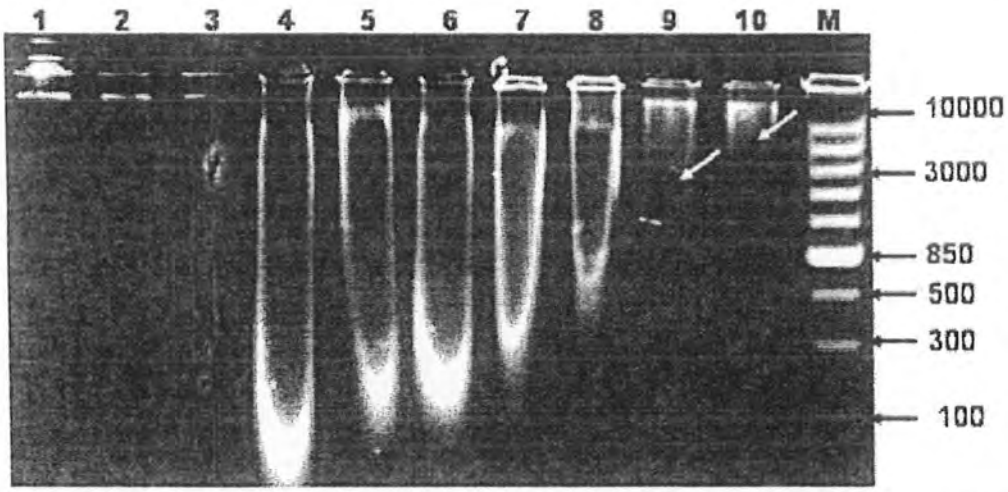

Figure 4. Agarose gel (1.5%) showing DNA damages induced with acrylamide and ameliorating effects of *D. muricata* extract in rat. Lanes from left (1) control, (2) DMSO, (3) extract 200 mg/kg, (4;5) acrylamide, (6;7) extract 100 mg/kg, (8;9) extract 150 mg/kg, (10) 200 mg/kg and (11) DNA marker.

DISCUSSION

DISCUSSION

The present study was conducted to investigate the medical importance of a common herbaceous plant *Digera muricata* which is commonly known as "Tandla". The methanolic extracts of "Tandla" play a significant role to repairs the injuries of lungs induced by acrylamide treatment. A very significant improvement is observed in different parameters of this experiment which are as follow.

In the present study increases in lungs weight was observed in acrylamide group as compared to the control group contrasting to body weight in the same group. The extract treated group erases the effects of acrylamide damage to lungs and maintain the weight of lungs compared to control group. This increase in the lungs weight is due to the increase number of the cancerous cells in acrylamide group. These results are related with the study of Lin and Lin (2006) where they found that by administration of toxic dose like CCl_4 cause significant decrease in body weight of rats as compared to sham control group, while the body weight of acryl amide treated rats decreases as compared to control group. The recovery in lungs weight of extract treated group shows the curative effect of *Digera muricata* extract.

Histological features of the lungs elucidated the significant variations among the treatments. This study indicates the treatment of acrylamide to the rats produce a severe damage in the different cells tissues of the lower respiratory system. In acrylamide treated group, the lung tissues are highly damaged with destructed alveolar septa and congested blood capillaries with several inclusions in their lumen. Due to the constriction of blood capillaries, blood cells were also gathered at various places in the lungs tissues producing the edemic conditions. The most of the alveolar septa become hard with increased number of type II alveolar cells. The fibroblast was also increased in number which is also responsible for the accumulation of collagen fibers at the junction of various alveolar walls which become hard and are ruptured. Alveolar macrophages were present various places in alveolar septa,but they were also severely or moderately damaged. These observations are similar with other researchers. Zakaria *et al.* (2004) reported the similar changes in the lungs tissues of the rates induced by carbon tetrachloride. Yao *et al.* (2005) also explain the same pathological changes in the lungs tissues including the pulmonary fibrosis induced by bleomycin. In this histological study of the lungs, several

lungs cells had undergone the process of necrosis and apoptosis. Non ciliated cells (Clara cells) in the inner epithelium of the bronchiole also show the morphological changes as these were dilated towards the lumen of the bronchiole and making the lumen narrow. These changes were also reported by Boyd *et al.* (1980). They study the morphological and biochemical changes in the Clara cells induced by carbon tetrachloride. This histoprotective effects of the methonolic extract of *Digera muricata* is found to be very effective in the present study to minimize the tissue damage induced by carbon tetrachloride. *Digera muricata* extract in methanol repaired the alveolar walls and also prevent the damage to the membranes by preventing the lipids peroxidation caused by free radicals. It also maintained the normal morphological characteristics of the cells in the lungs like alveolar type I and II, fibroblasts and macrophages. Clara cells in the inner epithelium of the bronchiole were also normal and have the dome shape appearance. The same observations were made by Zakaria *et al.* (2004) where they reported the histoprotective effects of curcumin (dietary product obtained from *Curcuma longa*) against the carbon tetrachloride induced lung damage. They observed that curcumin found to modulate the inflammatory oxidant activity of CCl_4 and restores the normal histological of the lung tissues. Xiaolu *et al.* (2004) investigate the Chinese herbal medicines and reported that ethanolic extract of *Scutellaria barbata* is very effective to start apoptosis in the human lung cancerous cells and inhibit the growth of human cancer cell lines A549. Lionis *et al.* (2005) also studied the protective effects of medicinal plants. They found that treatment of extracts of *Coridothymus capitatus*, *Salvia fruticosa*, and *Salvia pomifera* significantly decrease the lipid peroxidation in cultured lungs cells exposed to iron or ozone.

Now scientists are trying to investigate the protective effects of medicinal plants to minimize the oxidative damage of free radicals. Nan *et al.* (2002) investigate the protective effects of aqueous extract from the roots of *Rhodiola sachalinensis* on the liver injury induced by repetitive administration of carbon tetrachloride in rats. Immunohistological findings indicated that aqueous extracts from the roots of *Rhodiola sachalinensis* inhibited hepatic stellate cell activation which is a major step in collagen accumulation during liver injury. Immunuhistological findings indicated RSE treatment

inhibited hepatic stellate cell activation, which is a major step for collagen accumulation during liver injury.

There are number of studies also present in which the specific chemicals have been used which are either plant derived such as curcumin (Zakria *et al.*, 2004) kolaviron (Oluwaqtosin *et al.*, 2000) or synthetic such as Liv.52 and Kumarysava (Kataria and Singh 1997). In all above mentioned studies the acrylamide causes the centrilobular necrosis vacularization and macrovascular fatty changes. In some of the studies the hydrophobic changes in the centrilobular hepatocytes and single cell necrosis surrounded by neutrophills congestion of central veins and cynocyte with mild to moderate degree of fatty changes was observed which was recovered up to different levels by the treatment of phytochemicals.

In the present study the main effect of acrylamide was the prominent fatty changes congestion in the blood vessel clearing of cytoplasm with foamy appearance and nuclear degeneration in some area occur which were significantly recovered by the methanolic derived crude extract of *Digera muricata*. This study revealed that extract tested; of *Digera muricata* was found to possess significant protective effect against lung injuries induced by acrylamide which may be attributed to the individuals or combined action of phyto constituents present in it. The component of the extract responsible for this mechanism of effect however was not yet investigated. The present findings provide scientific evidence to the ethano medicinal use of this plant.

All the organisms have the special defense mechanism to attenuate the effects of toxic compounds in the tissues. The defense system consists of several antioxidant enzymes which are the scavenger of free radicals because free radicals cause the peroxidation of the macromolecules of the cells. The results obtained in this study indicated that activity of superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) and glutathione-S-transferase (GST) was significantly decreased, in the acrylamide treated group as compared to the control group. These findings suggested that the acrylamide toxicity decrease the production of SOD, POD, (GST) and (GSH) which in turn increases the level of reactive oxygen species. Then these reactive oxygen species cause the tissue damage. These results are related with the work of Szymonik- Lesiuk *et al.* (2003). They studied that the level of antioxidant enzymes in the tissues of heart, liver,

brain and kidney treated with acrylamide was markedly decreased in the level of SOD and POD. Nkosi *et al.* (2006) also reported the same results that the level of antioxidant enzymes decreases in the liver tissues treated with acrylamide. Tirkey *et al.* (2005) also reported the oxidative stress induced by acrylamide in liver and kidney of rats which ultimately decrease the level of antioxidant enzymes like SOD and CAT. This research is mainly focused on the anti-inflammatory and antioxidant effect of a medicinal plant *Digera muricata.* The simultaneous treatments of acrylamide and methanolic extract of *Digera muricata* attenuate the oxidative stresses in the liver tissues and increase the level of antioxidant enzymes. These findings suggested that treatment of methanolic extract *D. muricata* decreases the level of free radicals by increasing the level of their scavengers like SOD, POD, and CAT. These results are related with the findings of Yao *et al.* (2006). They found that activity of SOD is decreased in the bleomycin treated rats as compared to sham control group, while on the other hand losartan increase the activity of SOD and decrease the level of TBARS in the liver tissues. Similar results were also reported by Manna *et al.* (2006) where they found that acrylamide treatment decrease the activities of SOD and CAT in the liver and kidney tissues while the activities of these antioxidant enzymes increases with the simultaneous treatments of CCl_4 and aqueous extract of the bark of *Terminalia arjuna.* They concluded that bark of the *Terminalia arjuna* has the antioxidant activity and repair the tissues damaged by acrylamide. Liu *et al.* (2006) reported that the extract of *Hibiscus sabdariffa* (L.) has protective effects against the acrylamide induced lipid peroxidation and also maintain the level of antioxidant enzymes. Topdag *et al.* (2005) also proved that the level of SOD in the cancer cells remain low as compared to normal cells suggesting that decreased activities of antioxidant enzymes are responsible for cancer development in the liver tissues. Bhandarkar and Khan (2003) reported that extract of *Nymphaea satellata* (a medicinal plant) flowers reduce the acrylamide induce liver injury in rats. The extract of *Nymphaea stellata* flowers also increase the activities of antioxidant enzymes like glutathione peroxidase, superoxide dismutase and catalase which were decreased by the toxic effects of acrylamide.

Results obtained in this study shows that acrylamide treated group cause extensive DNA damage in lung tissues while the post treatment with extract of *Digera*

muricata erase most of the damage induced with acrylamide. These results indicate that the acrylamide and pollutants cause the degradation of the DNA by generating free radicals. Similar finding have also been reported by (Khan *et al.*, 2009) where they found the protective role of *Digera muricata* in erasing the damaging action of CCl_4 at various metabolic cycles, protesomal protein degradation and the repair of DNA damage. According to them administration of *Digera muricata* extracts to CCl_4 intoxicated rats protected the renal tissues and decrease the percentage of fragmented DNA that was also revealed in DNA ladder. Similarly Maniere *et al.* (2005) found that the chemicals such as acrylamide, and other xenobiotic compounds induce the production of reactive oxygen species which cause the oxidative damage to DNA, with formation of DNA adducts and genetic mutations (Collins, 2004). Also found acrylamide that induce the DNA damage in the hepatocytes of the rats and also found that organo-solenocynates prevent the acrylamide induced DNA damage. Ostling and Johanson (1984) also reported that damage of hepatocellular DNA and abnormal expression of hepatic extracellular matrix were observed in the acrylamide treated rats and inhibit hepatocellular degeneration, necrosis and DNA damage but also inhibited the lesions of extracellular matrix induced by acrylamide.

Conclusion

This study substantiated the scientific evidence in favour of *Digera muricata* pharmacological use in pulmonary disorders in folk medicine. The plant *Digera muricata* possesses the antioxidant as well as anticancer compounds. In addition this study suggested to isolate and to evaluate the active compound present in this plant.

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