Cardioprotective Potential of *Raphanus sativus* Seeds Against Carbon Tetrachloride Induced Injuries in Rat

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DEDICATED

TO

MY DEAREST GUL JEE,
ABU JEE AND BHAYYA

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List of Abbreviations

ALP	Alkaline phosphatase
ALT	Alanine transaminase
ANOVA	Analysis of variance
ANT	Anthracyclin
AST	Aspartate transaminase
ATP	Adenine Triphosphate
$BaCl_2$	Barium chloride
B. bron.	*Bordetella bronchioseptica*
CAT	Catalase
CCl_4	Carbon tetrachloride
CDNB	1-Chloro-2,4-Dinitrobenzene
Cef.	Cefixime
CFU	Colony forming unit
CK	Creatine kinase
Cs A	Cyclosporin A
CVD	Cardiovascular Diseases
CYP-E1	Cytochrome
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DOX	Doxorubicin
DTNB	1,2-Dithio-Bis Nitro Benzoic acid
EDTA	Ethlene Diamine Tetra Acetate
FAD	Flavine Adanine Dinucleotide
E. coli	*Escherichia coli*
E. aero	*Enterobacter aerogens*
gm	Gram
GSH	Glutathione reductase
GSH-Px	Glutathione peroxidase
GST	Glutathione-S-transferase
γ-GT	γ-Glutamyl Transpeptidase

HCl	Hydrochloric acid
HDL	High Density Lipoproteins
HNO	Nitrosyl hydride
HNO_3	Nitric oxide
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulphuric acid
IARC	International Agency for Research on Cancer
L	Litre
LDH	Lactate Dehydrogenase
LDL	Low Density Lipoproteins
MIC	Minimum inhibitory concentration
mg/ml	Milligram per milliliter
MDA	Malondialdehyde
$MgCl_2$	Magnesium chloride
MIC	Minimum inhibitory concentration
M. luteus	Micrococcus luteus
mm	Millimeter
NaCl	Sodium chloride
NADP	Nicotine amide Dinucleotide Phosphate
NADPH	Nicotine amide Dinucleotide Phosphate reduced
NH_3	Ammonia
NH_4	Ammonium ion
nMol.	Nanomolar
OD	Optical density
ppm	Part per million
P. piketti	*Pseudomonas piketti*
Resp.	Respectively
ROS	Reactive Oxygen Species
RNS	Reactive Nitrogen Species

R. sativus	*Raphanus sativus*
Roxi.	Roxithromycin
RSAE	*Raphanus sativus* aqueous extract
RSBE	*Raphanus sativus* butanolic extract
RSEE	*Raphnus sativus* ethyl acetate extract
RSHE	*Raphanus sativus* n-hexane extract
RSME	*Raphanus sativus* methanolic extract
rpm	Revolutions per minute
TBA	Thiobarbituric acid
TBARS	Thiobarbituric Acid-Reactive Species
TCA	Tricarboxylic Acid Cycle
TCM	Traditional Chinese Medicine
TGF	Transforming growth factor
TNF	Tumor necrosis factor
WHO	World Health Organization
>	Greater than
<	Less than
%	Percent
µl	Microlitre
^{o}C	Degree Celsius

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ABSTRACT

Carbon tetrachloride is a perilous compound, well-known to cause injuries almost in all body tissues. After being activated by CYP-450 enzyme system, CCl_4 produces an oxidatively stressful condition in the cell by producing reactive trichloromethane radical. This radical is proficient in causing lipid per oxidation of the polyunsaturated fatty acids in cell membrane and causes enzymatic deactivation hence destroying both, cellular structure and function.

This study aims to verify the counter effect of *Raphanus sativus* seed extract against CCl_4-induced cardiotoxicity along with the *in vitro* antibacterial activity of different fractions of *Raphanus sativus* against five bacterial strains. The experimental design comprises of thirty male Sprague Dawley rats, divided into five groups with six rats in each group. All groups were given separate doses on alternative days. Group 1 was given corn oil orally. Group 2 received CCl_4 (10%) intraperitoneally. Group 3 was given CCl_4 (10%) intraperitoneally + RSME (100mg/kg bw) orally. Group four was given CCl_4 (10%) + RSME (200mg/kg bw) orally. The last Group 5 was given only RSME (200mg/kg bw). After a starvation period of 24 hrs, the rats were sacrificed. The blood was collected from heart by cardiac puncture. Serum from blood and the supernatant from heart tissue homogenate were subjected to biochemical analyses.

The study deciphered the antioxidant potential of *Raphanus sativus* seed extract; clear from the close-to-normal values of serum markers such as alkaline phosphatase (ALP), alanine transaminase (ALT), aspartate transaminase (AST), low density lipoproteins (LDL), high density lipoproteins (HDL), triglycerides, total cholesterol, γ-glutamyl transpeptidase (γ-GT), creatine kinase (CK), lactate dehydrogenase (LDH) and tissue markers; catalase (CAT), superoxide dismutase (SOD), peroxidase (POD), malondialdehyde (TBARS), glutathione peroxidase (GSH-Px), glutathione-S-transferase (GST) and glutathione (GSH) when coadministered with CCl_4. The effect of 200mg/kg extract is more pronounced than the 100mg/kg extract. Histologial examinations of the heart tissues second this ameliorative effect.

The five different fractions of the *Raphanus sativus* extract were tested for their *in vitro* bactericidal activity using agar well disc diffusion method; against five

bacterial strains including one Gram positive *Micrococcus luteus*, and four Gram negative *Escherichia coli*, *Bordetella bronchioseptica*, *Pseudomonas piketti* and *Enterobacter aerogens*. The ethyl acetate (RSEE) and butanolic (RSBE) extracts showed commendable activities. Methanolic (RSME) and aqueous (RSAE) extracts had an intermediate while n-hexane (RSHE) extract had a low antibacterial activity. All fractions were inactive against *E. coli.*

Hence all the results prove that *Raphanus sativus* has a tremendous potential to be considered for compound extraction for anticancerous pharmaceutical products.

INTRODUCTION

HEART: ANATOMY

The mammalian heart is a hollow muscular organ having a conical shape. Its function is to provide a propulsive force to move the blood through circulation. Its size is about a clenched fist. Parietal pericardium encloses the heart and protects it by forming the wall of pericardial cavity which contains in it, a watery lubricating fluid called pericardial fluid (Graff, 1995).

1.1.1. Heart Wall

Heart wall is composed of three layers:

- Thick layer of cardiac muscles; the myocardium,
- Externally covered by the Epicardium and
- Internally lined by Endocardium.

The outer epicardium (also known as visceral pericardium) functions to reduce the friction during hear beat. The middle myocardium is responsible for contraction while the inner endocardium reduces the friction when blood flows through the heart (Snell, 2008).

1.1.2. Heart Chambers

Human heart consists of four chambers; two auricles and two ventricles (separated by atrial and ventricular septa respectively). The right side of the heart i.e. the right auricle and the right ventricle, pump the deoxygenated blood to the lungs (pulmonary circulation). On the other hand, the left side i.e. the left auricle and the left ventricle pump the oxygenated blood to the whole body (systemic circulation) (Graff, 1995).

The atrial portion of the heart is thin walled while the ventricular portion is thick walled; walls of left ventricle being three times thicker than the right ventricle. Ventricle comprises of two parts, lower rough part and upper smooth part (Snell, 2008).

1.1.3. The Heart Cycle

The heart cycle begins when blood inside the heart first enters into the right ventricle from the right atrium, and then enters into the lungs where it undergoes a cleaning process/oxygenation. Now it returns back to the heart by the left atrium, enters the left ventricle and gets distributed by aorta to the whole body (Snell, 2008).

Right atrium→ right ventricle → lungs → left atrium → left ventricle → whole body

An average beating of a human heart is 100,000 times per day, pumping more than 4,300 gallons of blood throughout the entire body. All of the 5.6 liters (6 quarts) of human blood circulates through the body three times every minute indicating the circulation efficacy (http://thevirtualheart.org).

1.2. HISTOLOGY:

The skeleton of the heart comprises of fibrous rings surrounding the atrioventricular and atrial orifices, along with adjoining masses of fibrous tissue. The valve cuspus is being supported by this heart skeleton which separates the muscular walls of the atria from those of ventricles and provides attachment for muscle fibers along with the electrical insulation of the atria from ventricles. Actin and myosin fibers are organized to form sarcomeres while the sarcoplasmic reticulum and T tubules are not as organized as in skeletal muscles (Snell, 2008).

Intercalated discs are joined with the cardiac muscle cells to allow the action potential to move from one cell to the other. The cardiac muscle cells work as a close unit, having a slow onset of contraction time depending on the time required for calcium to move to and from the myofibrils. Glucose and fatty acids are the main source of aerobic production of ATP for energy in cardiac muscles (Secley *et al.*, 1998). The heart muscles are supplied with blood by two coronary arteries arising from the base of aorta; left coronary artery and right coronary artery (Bavelander and Ramaley, 1979).

1.2.3. Conducting system of the heart

Cardiac muscle fibers form the:

- Sinoatrial node (lies between the opening of the superior vena cava and the auricle; in the right atrial wall)
- AV node (at the superior limit of the interventricular septum; in the left wall of the right atrium).
- AV bundle (arises from the AV node and descends in the interventricular septum).

Sinoatrial node (pacemaker) produces contractions spreading through the atrial walls, reach the AV node, and then the left and right bundles (Hildreth *et al*., 2009).

1.3. Cardiotoxicity

As the name indicates, the term cardiotoxicity refers to the condition when there is damage to heart muscles and electrophysiology dysfunction. As a result, the heart weakens and is not as effectual in pumping and circulating blood through the body. Cardiotoxicity is commonly seen with chemotherapy treatment or other medications. Severe cardiotoxicity leads to a medical condition called cardiomyopathy that is often a result of chemotherapeutic medications or by a group of diseases or disorders that lead to damaging heart muscles. This leads to turbulences in heart pumping efficacy and subsequent heart failure (Hamilton, 2005).

1.4. CARBON TETRACHLORIDE:

Carbon tetrachloride is an organic compound having the molecular formula CCl_4. It is a colorless, nonflammable liquid with a "sweet" smell (Budavari, 1996). In molecular form, it contains four atoms of chlorine, present symmetrically at four corners of a regular tetrahedron, attached by a covalent bond to a carbon atom placed at the center. It becomes non-polar due to this symmetry.

1.4.1. Physical Data (CRC, 1994; HSDB, 1995).

- Formula CCl4
- IUPAC name Tetra Chloromethane
- Synonyms Methane tetrachloride, Per chloromethane
- Industrial name Carbon tet
- Molecular mass 71.09
- Melting point -22.92 °C

- Boiling point 76.72 °C
- Solubility in water 785–800 mg/L at 25 °C

1.4.2. History

Carbon tetrachloride does not occur naturally and is manufactured artificially. A French chemist Henri Victor Regnault in 1839 attempted first to make CCl_4 by reacting chloroform and chlorine (Regnault, 1839), but now it is produced from methane (Budavari, 1996).

$CH_4 + 4\ Cl_2 \rightarrow CCl_4 + 4\ HCl$

Before 1950s, the process used was the chlorination of carbon disulfide at 105 to 130 °C (Rossburg *et al.*, 2006).

$CS_2 + 3Cl_2 \rightarrow CCl_4 + S_2Cl_2$

1.4.3. Reactivity

The non-polar nature of carbon tetrachloride makes it a solvent for non-polar compounds such as oils and fats. It is almost nonflammable at low temperatures but forms poisonous gas phosgene under high temperatures in air. Due to the lack of C-H bonds, Carbon tetrachloride does not easily undergo free-radical reactions. It is volatile to some extent and gives off vapors with a smell just like other chlorinated solvents (IARC, 1972, 1979, 1982).

1.4.4. Uses

Carbon tetrachloride was previously used as a dry-cleaning agent and as a fire extinguisher (McGregor and Lang, 1996). It was also used for metal degreasing, as a fabric spotting fluid and as grain fumigant in agriculture (DeShon, 1979). Carbon tetrachloride is used as a solvent to recover tin in tin-plating waste and for manufacturing semiconductors. It is an integral part in petrol additives, refrigerants and also in the production of polymers where it acts as a catalyst. Some pesticides and fluorocarbons require its use as a chemical intermediate. (HSDB, 1995).

1.4.5. Fate & Transport

The carbon tetrachloride reaches the air where it is found as a gas after being released into the environment during its production and use. It can remain in air for several years before its break down to other chemical compounds. Its atmospheric life time is 85 years (Dow and Downing, 2006). Surface water also has small amounts of carbon tetrachloride in it. However its entrapment in groundwater can be for longer periods. Other sources of absorption are the gastrointestinal tract, respiratory tract and skin, the extant depending upon the source of administration (Paul and Rubinstein, 1963). After absorption, it is distributed to all major organs of the body (EPA, 1985), where it temporarily accumulates in body fat.

1.4.6. Metabolism

After carbon tetrachloride gets entry into the body, it is metabolized. Several studies have been conducted to investigate its metabolism in rat, dog, rabbit and human. Much of the absorbed CCl_4 is excreted unchanged from the body but the remaining compound is metabolized via the cytochrome P-450 enzyme system into trichloromethane free radical (Zangar *et al.*, 2000).

This free radical can react with cellular macromolecules to disrupt and denature cellular proteins (McGregor and Lang, 1996), or may bind directly to microsomal lipids to contribute to the collapse of cellular membranous structures and also disrupting cell energy processes and reactions (ATSDR, 1992; Fleming and Hodgson, 1992). The trichloromethane free radical can dismutate anaerobically to form toxic chloroform, can react with itself to form hexachloromethane, and can also form toxic carbon monoxide (McGregor and Lang, 1996). The CCl_3 radical can yield a precursor to carbonyl chloride (phosgene) i.e. trichloromethanol, which then hydrolyses to form carbon dioxide (ATSDR, 1992).

1.4.7. Excretion

Much of the carbon tetrachloride that enters the body by inhalation or by drinking contaminated water leaves the body quickly by breath within a few hours (ATSDR, 1992). Animal studies specify that under differing conditions, 75 percent of carbon tetrachloride excretes during exhalation through lungs, 20-62 percent is

expelled in feces, while only a small quantity comes out in the urine. Most of the carbon tetrachloride is eradicated from the body unmetabolised, but a little amount may change to other chemicals like chloroform, hexachloroethane, and carbon dioxide, after interacting with cellular proteins and other macromolecules resulting in severe intoxication (CDHS, 1987; ATSDR, 1992).

1.4.9 Mode of action

Carbon tetrachloride is a highly carcinogenic compound which when taken in, effects many body organs. It is a classical hepatotoxicant (Munoz *et al*., 1990). But it is also a proven nephrotoxic, neurotoxic and pulmotoxic agent (Ogeturk *et al*., 2005).

Several reviews describing the mechanisms of carbon tetrachloride toxicity have been published (Mehendale, 1989). The leading theory for the mechanism of cellular damage caused by CCl_4 illustrates the contribution of highly reactive free radicals. These free radicals on one hand, initiate lipid peroxidation by binding covalently with the poly unsaturated fatty acids, present in the triglycerols and phospholipids, thus causing damage in cell membrane (Williams and Burk, 1990; Stoyanovsky and Cederbaum, 1996) and finally cell death. While on the other hand, these free radicals react either with each other or other molecules to form hazardous compounds inside the body (McGregor and Lang, 1996). In addition to binding with the cellular proteins and macromolecules, the carbon tetrachloride metabolites bind to DNA and show genotoxic effects (Cortan *et al*., 1994). DNA can also undergo oxidative damaged during the peroxidation resulting in the breakdown of membrane polyunsaturated fatty acids. Disturbances in homeostasis of various cells leading to induced signal transductions related with apoptosis and undesired cell propagation are some other outcomes of DNA damages (Bonventre, 2003; Poirier, 2004) along with the micro or macro mutations in the cell (Khan *et al*., 2009). Other adversaries apart from the DNA damages are gene mutations, chromosomal mutations and deletions, probably through the production of oxidative stress (Jia *et al*., 2002). Sreelatha *et al*. (2009) accounted that the depleted activity of cellular antioxidant enzymes like superoxide dismutase, catalase and glutathione peroxidase whereas the elevated level of peroxidation product TBARS is correlated with the experimental administration of CCl_4 in rats.

1.5. Medicinal Plants

Recently, the interest has been shifted towards finding the curative potentials of medicinal plants against different diseases. The reason behind this is the fact that plant extracts are more natural, less pricey and with no or lesser side effects. Various biologically active compounds like vitamins, flavonoids and polyphenols are believed to be the reason for the biological activity of plants (Abhilasha *et al*., 2011). That is why, WHO has suggested the use of medicines from herbal sources, especially in the developing countries (WHO, 2002b).

According to a study, many modern therapeutic drugs, approximately one quarter to be precise, contain active botanical ingredients. Aspirin, the most prevalent analgesic, had originally been derived from Salix and Spirae species. Not only this but valuable anti-cancerous agents paclitaxel and vinblastine, come exclusively from plant sources (Pezzuto and Taxol, 1996). Thus the global demand for herbal medicines is not only large, but growing (Srivastava, 2000). *Sida rhomboidea* from family Malvaceae is a weed found in India (Puri, 2002). It has considerable antimicrobial, anti-inflammatory (Alam *et al*., 1991), lipid lowering, anti-hyper triglyceridemic and antioxidant (Thounaojam *et al*., 2009) potentials. The Ayurvedic name for it is ‘‘Mahabala’’, used against inflammations, fever, heart diseases, burning sensation, urinary problems, piles (Rao *et al*., 2006).

Panax ginseng, a conventional multi-use herb in Asia, has become the World’s most popular herbal supplement in these years. Ginseng has a wide range of beneficial effects against cancers, diabetes and inflammation, as well as cardiovascular and neuro-protection (Joo *et al*., 2005; Jung *et al*., 2005).

In order to evade the oxidative stress inside the body, the balance of oxidant-antioxidant system must exist (Yamamoto and Yamashita, 1997). Body’s natural balance might get disturbed by the use of several medications. But various common herbs are well-known to have free radical scavenging activities. *Curcuma longa* (Srinivas *et al*., 1992), *Silybum marinum* (Kren and Walterova, 2005), *Azadirachta indica* (Bhanwara *et al*., 2000), *Allium sativum* (Rahman, 2001; Al-Numair, 2009), *Morus alba* (Wattanapitayakul *et al*., 2000), *Ilex paraguariensis* (Filip *et al*., 2000; Schinella *et al*., 2000), *Passiflora alata* (Rudnicki *et al*., 2007) are all reported to have

components with antioxidant potential, thus contributing to cell survival in the face of hazardous free radicals roaming inside the cell.

The clinical plants are not only used as such, in their original forms, but their active ingredients are also purified and isolated. *Dracocephalum tanguticum* (Shu-Qi *et al.,* 2010), *Terminalia arjuna* (Sinha *et al*., 2006), *Andrographis paniculata* (Singha *et al*., 2003), *Cajanus indicus* (Ghosh and Sill, 2007; Sinha *et al*., 2007) are some examples from a long list of plants that have been subjected to alkaloids (Rackova *et al*., 2004), flavonoids (Pietta, 2000) and phenolic compounds (Santos-Gomes *et al*., 2003) extraction.

1.6. RAPHANUS SATIVUS

1.6.1. Scientific Classification

- Kingdom Plantae
- Division Magnoliophyta
- Class Magnoliopsida
- Order Brassicales
- Family Brassicaceae
- Genus *Raphanus*
- Species *sativus*
- Common name Radish

The word *Raphanus* comes from a Greek word meaning "quickly appearing", referring to the quick germination of these plants. The common name of the plant, Radish, comes from Latin word "Radix" meaning root (Zohary and Hopf, 2000).

Radish was one of the important crops in ancient Egypt, and was depicted on the walls of the Pyramids about 4000 years ago. Cultivated radish and its uses were confirmed in China almost 2000 years ago (Li, 1989) and in Japan, radishes were known for some 1000 years (Crisp, 1995).

1.6.3. Plant Description

Raphanus sativus is an annual plant growing up to 0.5 m (1ft 8in). The radish is a root vegetable, commonly found as a small-rooted, short-season vegetable. The

plant has alternate leaves without stipules. Hermaphrodite flowers (having male and female organs both in same flower) with white to purple color that are pollinated by bees (PFAF, 2008). Fruit is named as siliqua which is a peculiar kind of capsule, widely referred to as "pods". The fleshy root is edible with variable shapes and colors (Curtis, 2003). Round and red-skinned variety is most common. Further varieties have pink, white or grey–black skin (Curtis, 2003).

1.6.4. Varieties

There are many named varieties of *Raphanus sativus* (Facciola, 1998) classified into four major varieties: summer, fall, winter and spring. A number of distinct groups have evolved through cultivation over the time. The botanists classified them as follows.

- *R. sativus*. The common radish.
- *R. sativus caudatus*. The rat-tailed radishes.
- *R. sativus niger.* The Oriental and Spanish radishes.
- *R. sativus oleiformis.* The fodder radishes (Riotte, 1978).

Another winter variety found specifically in Asia is Daikon. It is also called the Japanese, Chinese, Oriental radish or mooli (in India and South Asia) (AHD, 2004). It is the radish, we mostly are familiar with. Daikons mostly have elongated white roots but other varieties also exist.

1.6.5. Cultivation Details

Raphanus sativus is a fast-growing easily cultivated plant, fancying a rich light soil and liberal moisture (Phillip and Rix, 1993). They show disinclination to very heavy or acid soils (Thompson and Watson, 1990) and prefer loamy sandy soils with pH range of 6.5–7.0 (Dainello and Frank, 2003). Plants are vulnerable to drought and need regular watering during dry spells (Facciola, 1998).

1.6.6. Edible Uses

According to an estimate, 7 million tons of radishes are produced per year comprising about 2% of the total world production of vegetables (Schippers, 2004). The napiform taproot is most generally eaten portion of Radish (*Raphanus sativus*)

though the whole plant is edible. Tops can be used as a leaf vegetable, either raw or cooked (Thompson and Watson, 1990; Facciola, 1998). Young flower clusters may also be used raw or cooked (Facciola, 1998). Seeds are used in salads after they are soaked in water for 12 hours following a sprouting for about 6 days (Phillips and Foy, 1990). Young seed pods are also used raw (Thompson and Watson, 1990). Seeds also give edible oil (Hedrick, 1972).

1.6.7. Nutritional Aspects

Radishes are a rich source of potassium, ascorbic acid and folic acid. They also contain decent quantities of riboflavin, vitamin B6, magnesium, copper, and calcium (Lewis-Jones, 1982).

1.6.8. Clinical Rating

Raphanus sativus has earned much consideration these years because of its dietary and health-defensive importance. Papi *et al*. (2008) and Barillari *et al*. (2008) reported Japanese *R. sativus* sprouts to have a dose-dependent chemopreventive effect against human colon cancer cells. In another experiment, *R. sativus* root extract significantly repressed the propagation of numerous human cancer cells through stimulation of apoptosis (Beevi *et al*., 2010b). Apart from this, the roots and leaves of the plant have been reported to possess a wide range of pharmacological activities, such as gut stimulatory (Gilani and Ghayur, 2004), hepatoprotective (Zaman and Ahmad, 2004), cardioprotective (Zaman, 2004), antioxidant (Barillari *et al*., 2006) and antiurolithiatic activities (Vargas *et al*., 1999). Furthermore, the freshly squeezed root juice of *R. sativus* L. has been reported to possess antiulcer activity (Alqasoumi *et al*., 2008). According to Beevi *et al*. (2010a), hexane extract of root and methanolic extract of stem and leaf showed considerable protective effect against cell demise and oxidative DNA damage stimulated by H_2O_2 in lymphocytes.

The seeds of *R. sativus* have not been still investigated for their antibacterial activities and the counter effect they may show against tissue injuries caused by different toxic substances have not yet been reported. So this experiment is designed to assess the protective effect of *R. sativus* seed extract on CCl_4-induced heart damages.

Study objectives

The present project is designed to evaluate the antibacterial properties of *Raphanus sativus* seed extract against the cardiac damages, serum levels of different enzymes and hormones and also the histological damages caused by carbon tetrachloride. The main objectives behind this study are;

- To determine the protective effect of *Raphanus sativus* on the genomic DNA of heart tissues in rats.
- To determine the histological changes in heart tissue.
- To study the effect of heart marker enzymes in serum.
- To evaluate the activity of antioxidant enzymes in tissue homogenate.
- To estimate the protein contents.
- To determine the lipid peroxidation in heart tissue.

REVIEW OF LITERATURE:

Cardiotoxicity resulting from damaging insults has been known for a long time. The mechanisms involved in the toxicity have not been clearly understood until this time (James, 2001). Heart is affected by a number of environmental contaminants, chemicals and drugs that dramatically modify the structure and function of various tissues and produce numerous undesirable effects in the heart (Ozturk *et al*., 1997). Oxidative imbalance is a primary or secondary basis of many cardiovascular diseases. A well acknowledged affiliation between free radicals and cardiotoxicity is recognized by many experimental studies.

2.1. Free radicals: Types

A free radical is a molecule or molecular fragment with one or more unpaired electrons in its outermost atomic or molecular orbital and can exist independently (Halliwell and Gutteridge, 1999). Free radicals are highly unstable and extract electrons from other molecules in order to attain stability thus causing damages (Valco *et al*., 2006).

The most important class of radical species generated in the living systems is the one derived from oxygen and are called as Reactive Oxygen Species (ROS). They include superoxide (O_2•−), hydroxyl (OH•), peroxyl (RO_2•), hydroperoxyl (HO_2•), alkoxyl (RO•) and peroxyl (ROO•) radicals. The subclass of the free radicals is Reactive Nitrogen Species (RNS) that originate from the reaction of nitrogen with oxygen, including, nitric oxide (NO•), nitrogen dioxide (NO_2•)and peroxynitrite (ONOO-) (Valco *et al*., 2006; Miller *et al*., 1990). RNS also include nitrosyl hydride (HNO) (Fukuto, 2008). Some non- radicals like hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), ozone (O_3), singlet oxygen, peroxynitrate (ONOO−), nitrous acid (HNO_2), dinitrogen trioxide (N_2O_3), lipid peroxide (LOOH) (Pham-Huy *et al*., 2008) are also proficient to cause oxidative damages so considered as potent oxidants (Miller *et al*., 1990; Valco *et al*., 2006).

2.2. Generation of free radicals

Oxygen on one hand being essential for life; can also exacerbate the damages by creating an oxidatively imbalanced environment within the cell (Shinde *et al*.,

2006). The use of ground state oxygen by the aerobic organisms during normal metabolism produces ROS, some of which are highly poisonous and deadly to cells and tissues (Halliwell, 1991).

Cardiovascular diseases are often related to ROS overproduction whereas the formation of major RNSs nitric oxide and peroxynitrite (diamagnetic molecule) might decrease or increase depending on the nature of heart injury (Lassegue and Clempus 2003; Murdoch *et al*., 2006). Reactive oxygen species (ROS) are the generated during the normal cell metabolic processes in the body: NADPH oxidases being the common factors for ROS generation in which superoxide is generated by the one-electron reduction of dioxygen (Lassegue and Clempus 2003; Murdoch *et al*., 2006). The Nox-dependent ROS signaling has been suggested as an important factor responsible for many pathological developments in heart (Bendall *et al*., 2002).

Mitochondria, the major eukaryotic energy-generating machinery (Heo and Rutter, 2011) are known to have major role in eliciting cell death by distracting the energy metabolism and electron transport, releasing or activating apoptotic proteins, and shifting cellular redox potential (Liu *et al*., 2009; Ravi Kumar *et al*., 2010). Mitochondria form nearly 30% of the total volume of ventricular cardiomyocytes structuring a network around the myofilaments which results in such an arrangement that ATP production and ATP consumption sites come adjacent to each other (O'Rourke *et al*., 2005). Electron transport chain (ETC) in the mitochondrial membrane may be considered as the main source of ROS production (Scherz-Shouva and Elazar, 2011) but under hypoxic conditions, the ETC produces nitric oxide (NO), which can generate reactive nitrogen species (RNS) (Poyton *et al*., 2009).

Another major process responsible for ROS generation is xanthine oxidase reactions. Superoxide and hydrogen peroxide radicals were considered to be derived from XO reactions (Denisov and Afanas'ev, 2005). Thompson-Gorman and Zweier (1990) measured free radical generation mediated by xanthine oxidase in isolated rat heart. Later in 1993, Ashraf and Samra stated that XO is contained in interstitial cells, coronary vessel endothelium, and smooth muscle cells. The results showed that XO activity elevated during ischemia and got more pronounced after reperfusion.

Another known pathway for ROS and RNS generation in heart is of Nitric oxide synthases (NOS) that convert L-arginine to L-citrulline and nitric oxide.

Superoxide is formed under the uncoupling conditions which is one of the strongest free radicals generated first after oxygen is taken into the living cell (Umar and Laarse, 2010). Many investigations suggest arachidonic acid metabolism in platelets as a source of ROS produced by cyclooxygenase and lipoxygenase enzyme systems (Seno *et al*., 2001).

Various other sources of free radicals are UV radiations, X-rays, gamma rays, microwave radiation and metal-catalyzed reactions. Interaction with chemicals, automobile exhausts fumes, smoking of cigarettes, cigars and beedies may also contribute to ROS production. Several investigations show alcoholic intake, certain drugs, fungal toxins, some pesticides and herbicides, some metal ions, and xenobiotics to be the exogenous substances to produce ROS and RNS (Nagendrappa, 2005; Valco *et al*., 2006).

2.3. CCl_4- induced oxidative imbalance

CCl_4 is an established hepatotoxicant that causes hepatic fibrosis (Hernandez-Munoz, 1990) and cirrhosis after the use exceeds 8-12 weeks (Iredale, 2007) and hepatocellular carcinoma (Loguercio and Federico, 2003; Vitaglione *et al*., 2004). Studies proved the toxic nature of CCl_4 against kidney, heart, brain (Jayakumar *et al*., 2008) and testicular tissues (Khan and Ahmed, 2009).

The mechanism of CCl_4 action has been well established. It involves the activation of carbon tetrachloride into its active metabolites through the CYT P-450 enzyme system, mainly located in liver (Sheweita *et al*., 2001). $CC1_4$ is activated by CYP2E1, CYP2B1 or CYP2B2, CYP2A and probably CYP3A (Lee *et al*., 2001; Weber *et al*., 2003) to an intermediate trichloromethyl radical CCl_3 which is further changed to trichloromethyl peroxy radical (CCl_3O_2) in the presence of oxygen (Weber *et al*., 2003). CCl_3 impairs very vital cellular activities by binding with all important cellular molecules such as nucleic acid, lipid and protein, while a more reactive radical, CCl_3O_2 either attacks the polyunsaturated fatty acids (PUFA) to proliferate a lipid peroxidation chain reaction that results in the destruction of polyunsaturated fatty acids, or covalent binding to lipids and proteins.

This affects the mitochondrial, endoplasmic reticulum and plasma membrane permeability, that leads to the loss of cellular calcium appropriation and homeostasis and ultimate cell death (Sevanian and Ursini, 2000; Boll *et al*., 2001).

Once initiated, the process of lipid peroxidation progresses as a chain reaction mediated by free radicals; initiation, propagation, and termination are the three steps (Gago-Dominguez *et al*., 2005). The first step of lipid peroxidation is initiation which starts when hydrogen atoms are withdrawn from polyunsaturated fatty acid of membrane phospholipids by the reactive species (Gago-Dominguez *et al*., 2005). The fatty acid radicals formed in the initiation step will further react with more lipid molecules in the vicinity to generate new free radicals (Niki *et al*., 2005). As with the proteins, the reactive moieties can attack either the protein directly or react with sugars and lipids to generate products that in turn react with the protein (Freeman *et al*., 2009).

Apart from that, CCl_4 is proved to activate the nitric oxide, tumor necrosis factor TNF-alpha, transforming growth factors TGF-alpha and TGF-beta, which lead to cell's self destruction (apoptosis) and fibrosis (Weber *et al*., 2003).

2.5. Free radical-induced cardiac ailments

The heart is predominantly liable to injuries induced by free radicals, due to the lack of adequate free radical detoxifying substances (enzymes) than do the other organs such as liver or kidney (Olson and Mushlin, 1990).

Cardiovascular disease (CVD) is the major cause of mortality worldwide (Ajjan and Ariens, 2009). It has been estimated by The World Health Organization (WHO) that, CVDs and its complications killed approximately 17.1 million people in 2004, and by 2030, the number of casualties will dramatically raise to nearly 23.6 million (WHO, 2009). Coronary artery ischemia–reperfusion (I/R) injury occurs on reinstatement of coronary flow following a myocardial ischemic condition and results in myocardial cell injury and necrosis (Dhalla *et al*., 2000). The ionic turbulences are exacerbated, including calcium overload in the cytosol and mitochondria alongwith the intensification of superoxide and other reactive oxygen species, leading to many structural and functional alterations in cellular biomolecules and activation of signaling pathways that finally result in cell demise (Hoffman 2004).

Myocardial infarction, a common presentation of ischemic heart disease, is defined as myocardial cell death due to extended ischemia (MIR, 2000). It is the severe condition of myocardial necrosis when the balance between coronary blood supply and myocardial demand is disturbed (Boudina *et al*., 2002). Oxidative stress created by free radicals or reactive oxygen species (ROS), as apparent by noticeably increased lipid peroxidative products and transitory inhibition of cellular antioxidant defense system, has been shown to elicit myocardial damage during MI (Padmanabhan and Prince, 2006; Zhou *et al*., 2008).

Atherosclerosis is an artery wall disease showing a progressive loss of endothelial cell function and the accumulation of the foam cells; lipid-laden macrophages (Ross, 1999). The mechanism involved is the oxidative modification of local low density lipoproteins mediated by the reactive oxygen species (ROS) and reactive nitrogen species (RNS) resulting in foam cell formation (Rubbo *et al*., 1996). The studies supporting the idea demonstrate the presence of modified LDL in vivo and the ability of ROS or RNS added to LDL in vitro to convert lipoproteins to a potentially pro-atherogenic form (Salonen *et al*., 1992).

Another condition called Arrhythmia is also associated with systemic and cardiac oxidative stress caused by ROS. Elevations in cellular ROS levels can modify the cardiac sodium channels, abnormal Ca_2^+ handling, mitochondrial function alterations and gap junction remodeling resulting in arrhythmogenic conditions. (Jeong *et al*., 2011).

2.6. Cellular defense mechanism

Oxidative stress refers to the over production of ROS or RNS by either endogenous or exogenous sources and is responsible for several pathologies that are related with alteration in redox regulation of cell signaling pathways (Valko *et al*., 2006). The injurious effects of ROS are counteracted by the radical scavenging activity of antioxidant enzymes together with non-enzymatic antioxidants (Halliwell, 1996).

Superoxide dismutase (SOD) acts as the most effectual intracellular enzymatic antioxidants (Valko *et al*., 2006); the first line guard system against ROS. SOD catalyses the removal of superoxide, generating a comparatively less harmful H_2O_2 radical as the final product of dismutation (Deneke and Fanburg, 1989; Halliwell and

Gutteridge, 1999). Then comes the role of catalase, an enzyme present in peroxisomes. Of all the enzymes, catalase shows the highest turnover rate: almost 6 million molecules of hydrogen peroxide are converted to water and oxygen by a single molecule of catalase each minute (Mates *et al*., 1999). Another antioxidant enzyme is glutathione peroxidase. Two types of this enzyme namely glutathione-S-transferase (GST) and GPx work in correlation. Glutathione metabolism is one of the most vital processes of antioxidative defense mechanisms (Mates *et al*., 1999). GPx in the cytoplasm reduces the H_2O_2 to H_2O along with the oxidation of GSH at the same time. The oxidized GSH is converted back to its reduced form by the enzyme GR which is a flavoprotein enzyme using NADPH (Willcox *et al*., 2004).

On the other hand, the non-enzymatic antioxidants are classified as (a) metabolic antioxidants and (b) nutrient antioxidants. Metabolic antioxidants are the endogenous antioxidants, generated inside the body through metabolic pathways, like glutathione, L-ariginine, lipoid acid, melatonin, coenzyme Q10, metal-chelating proteins, uric acid, transferring, bilirubin etc. (Droge, 2002; Willcox *et al*., 2004). On the other hand, nutrient antioxidants are considered exogenous antioxidants, that are not generated in the body but supplied through diety sources or supplementations e.g. omega-3 and omega-6 fatty acids, trace metals (selenium, manganese, zinc), flavonoids etc (Pham-Huy, 2008). Vitamin E and C are considered both endogenous and exogenous non-enzymatic antioxidants as they are produced within normal cells as well as they can be supplied through diet (Tiwari, 2001).

2.7. Cardiotoxic drugs

Heart is prone to drug toxicity e.g. Anthracycline (ANT) antibiotics, such as daunorubicin, epirubicin or doxorubicin are considered to be the most effective anticancer drugs ever developed. Chemotherapy protocols for both hematological malignancies and solid tumors include anthracyclines as their major components. Though, their clinical use is markedly held back due to the risk of severe cardiotoxicity (Ewer and Yeh, 2006).

It was accounted by Bristow *et al*., (1978) that a series of eight patients developed cardiac dysfunction within four weeks of anthracycline intake. The chronic anthracycline cardiotoxicity results in severe and irreversible damage to left ventricular (LV) cardiomyocytes which causes a disarray or loss of myofibrils,

cytosolic vacuolization, mitochondrial swelling and ultimately non-programmed or programmed cell death. All these conditions manifest the onset of dilated cardiomyopathy that eventually leads to heart failure (Ewer and Yeh, 2006). The proteomic and molecular insight into the mechanism by Sterba *et al*., (2011) showed the altered energy channeling, dysfunction of mitochondrial antioxidant protection and mutilation of mitochondrial energy production leading to damaging cardiomyocytes. Apart from that, numerous autophagic, proteolytic and apoptotic pathways were activated.

Doxorubicin, clinically known as Adriamycin is an anthracycline antibiotic is being used widely for treatment of various sarcomas, breast cancer, lung cancers and leukemia (Fisher *et al*., 2005). In spite of the effectiveness, the clinical use of doxorubicin is restricted due to dose-dependent cardiotoxic effects, resulting in an irrevocable degenerative cardiomyopathy and congestive heart failure (Fajardo *et al*., 2006; Horan *et al*., 2006). Studies reveal that this cardiotoxicity is the result of the oxidative stress generated within the cell. DOX produces O_2^- radical by a redox cycling reaction thus giving rise to more ROS which cause more oxidative events in the cell (Chen *et al*., 2006) that result in tissue-specific mitochondrial damages (Berthiaume and Wallace, 2007), disorders of calcium (Temma *et al*., 1997) or iron (Minotti *et al*., 1999).

Cisplatin (cis-diamminedichloroplatinum) is one of the most extraordinary achievements in 'the war on cancer'. It is exceptionally active against a wide range of cancers and solid tumors showing resistance to other courses of therapy (Yousef *et al*., 2009; Kart *et al*., 2010). But the weak point is the side effects including cardiomyopathy, myocarditis, electrocardiographic changes, arrythmias, and congestive heart failure (Al-Majed *et al*., 2006). Some studies suggest the commencement of platelet arachidonic acid pathway by cisplatin to be the possible reason (Togna *et al*., 2000).

Cyclosporine A (CsA), is being extensively used in transplant surgery as an influential immunosuppressant. Yet, its full clinical utility is limited due to some undesirable effects such as cardiac dysfunctions and hypertension (Bianchi *et al*., 2003; Rezzani, 2004). The mechanism of CsA-induced toxicity, includes the free oxygen radical formation (Rezzani *et al*., 2003), increased synthesis of

vasoconstrictor eicosanoids, induction of the cytochrome P-450 enzyme system and lipid peroxidation (Bianchi *et al*., 2003).

Myocardial infarction in rats is reported to be produced by a synthetic catecholamine and beta-adrenergic agonist Isoproterenol, which causes an unbalance between the cyototoxic free radicals generation and cellular defense systems (Srivastava *et al*., 2007), leading to metabolic and contractile mutilation, changes in membrane permeability following lipid peroxidation and eventually irreparable injury to the myocardium (Devika and Stanely, 2008; Panda and Naik, 2008).

A insecticide of polycyclic chlorinated hydrocarbon of the cyclodiene group Endosulfan that is 6,7,8,9,10,10- hexachloro- 1,5,5a,6,9,9 a-hexahydro- 6,9 methano-2,4,3 benzodiexathiepin-3-oxide is being used for food crops such as coffee, tea, corn, vegetables, cereals and fruits as well as on non-food crops such as tobacco and cotton (Naqvi and Vaishnavi, 1993). As indicated by studies, the possible mechanism for the pesticide toxicity is the oxidative stress generation by the free radicals and lipid peroxidation in humans as well as other organisms (Hazarika *et al*., 2003). Lipid peroxidation has been indicated by the presence of the end product Malondialdehyde (MDA) in the case of cardiotoxicity (Kalender, 2004).

2.8. Plants with Cardioprotective Potential

Medicinal plants have always been a vital source of a wide variety of active natural components with variable biological properties and play an important role in the treatment of various human ailments, including cardiovascular disorders (Saravan *et al*., 2011).

CardiPro, a polyherbal formula comprising of the standardized extracts of selected herbs; *Terminalia arjuna, Boerhaavia diffusa*, *Withania somnifera*, *Emblica officinalis,* and *Ocimum sanctum* (Gyanendra Pandey, 2001a, b, 2002a, b, c). CardiPro has been known to protect against chronic doxorubicin-induced cardiotoxicity (Mohan *et al*., 2006) due to the fact that herbal component of CardiPro is potentially more active antioxidant as compared to the vitamin E and C synthesized artificially (Ghosal, 2000).

Ayurvedic text accounts that an amalgamation of substances is used to improve the preferred action and to eradicate the undesired side effects. Marutham is an Ayurvedic polyherbal formula with components from eight plants including *Allium*

sativum, *Withania somnifera*, *Glycyrrhiza glabra*, *Wedelia calendulaceae*, *Nelumbium speciosum*, *Tinospora cordifolia*, *Emblica officinalis* and *Terminalia arjuna*. This herbal formulation has been repoted to have cardioprotective potential against isoproterinol-induced myocardial infarction (Stanely Mainzen Prince *et al*., 2008).

Complementary and alternative medicine (CAM) has opened new doors for the alternative mode of treatment of several diseases. An excellent example of CAM is Traditional Chinese medicine (TCM). The basic notion of TCM is based on the Five Elements theory (Water, Fire, Metal, Wood and Earth) which defines that a Yin (negative) Yang (positive) balance exists in each healthy organism (Cheng, 2000; Lu *et al*., 2004). Kudzu root; dried root of *Pueraria lobata* is found in South East Asia and is used as a food source, fodder and medicine for thousands of years (Luo *et al*., 2007; Csurhes, 2008). It is often used as the single ingredient or in combination with other herbs e.g. *Ginkgo biloba, Salvia miltiorrhiza, Bacopa monnieri, Silybum marianum* and *Salix alba* to relieve several pathologies including cardiac functions (Amazon, 2011; Healthstore, 2011; TGA, 2011).

Isolation of different new drugs from medicinal plants with potential benefits has increased to a huge extant (Kim *et al*., 2008). *Leonotis leonurus* L. (Lamiaceae), a plant found in South Africa has marrubiin; a diterpenoid, (Scott *et al*., 2004) with an indirect cardioprotective action. It dampens the hypercoagulable and the inflammatory state related with obesity, so playing a cardioprotective role (Mnonopi *et al*., 2011). *Terminalia arjuna* (Family Combretaceae) is a large tree present in India and its bark has cardioprotective nature in the case of ischaemic heart diseases and hypertension (Gupta *et al*., 2001). A compound Arjungenin extracted from *T. arjuna* had revealed a low hindrance against the respiratory oxyburst.

Oleuropein; a phenolic compound present in large amounts in olives and olive oil gives them a bitter taste and pungent smell (Bravo, 1999). It inhibits lipid peroxidation induced by doxorubicin, by declining the oxidative stress and dropping the iNOS in cardiomyocytes thus relieving cardiotoxicity (Andreadou *et al*., 2007).

2.9. Plants with antibaterial activity

Attention has been ever more focused on the medicinal plants as an alternative source of treatment against bacterial attacks (Nazemiyeh *et al*., 2008; Pesewu *et al*., 2008).

Cassia fistula, from family Leguminosae, is an ornamental tree native to Asia, Brazil and many other parts of the globe. Methanol, ethyl acetate, Hexane, water and chloroform extracts of *Cassia fistula* flowers showed a significant zone of inhibition against many Gram-positive bacteria and one Gram-negative bacterium (Duraipandiyan and Ignacimuthu, 2007).

Runyoro *et al*. (2009) characterized four *Ocimum* species (*Ocimum basilicum* (L.), *Ocimum kilimandscharicum* (L.), *Ocimum lamiifolium* (L.), *Ocimum suave* (L.)) of Tanzania for their phytochemicals and antimicrobial properties, found eighty one compounds that possessed antibacterial properties.

There is a growing apprehension that bacteria are becoming more defiant of clinical drugs, hence the discovery of new antibiotics is needed now more than ever to fight against resistant bacterial varieties (WHO 2002a). *Rhodomyrtus tomentosa* (Aiton) is an evergreen shrub indigenous to South East Asia (Latiff, 1992). Rhodomyrtone which is 6, 8- dihydroxy -2, 2, 4, 4- tetramethyl -7- (3- methyl -1- oxobutyl) -9- (2-methylpropyl)-4, 9-dihydro-1H-xanthene-1, 3 (2H) dione was extracted by Limsuwan *et al*. (2009) and was tested. It was significantly active against a wide variety of Gram-positive bacteria and also against those, resistant to antibiotic. Oleic and linoleic acids extracted from the dicloromethane extract of *Helichrysum pedunculatum* leaves were active only against the five Gram-positive bacterial species and did not show any bactericidal activity against all the tested Gram-negative species (Dilika *et al*., 2000).

By using agar well disc diffusion method Shan *et al*. (2007) evaluated the antimicrobial activities of extracts from dietary spices and medicinal herbs against food borne bacteria e.g. *Escherichia coli, Bacillus cereus, Listeria monocytogenes, Staphylococcus aureus* and *Salmonella anatum*. Extracts of these spices and herbs were characterized and found that they possess enormous level of phenolic compounds that are responsible for the antimicrobial activities.

Sakunpak and Panichayupakaranant (2012) tested twenty two edible plants of Thailand to evaluate their antibacterial activity against some gastrointestinal pathogenic bacteria using the disc diffusion and broth microdilution methods. All of them showed resistance against various bacterial strains. Among these tested plants, an antibacterial active compound chamuangone (a polyrenylated benzophenone) was isolated from the leaves of *Garcinia cowa.* Chamuangone exhibited inhibitory effect against all tested bacteria.

MATERIALS AND METHODS

3.1. Plant material

Raphanus sativus (L.) is locally named as "Mooli". The seeds were collected from the local market of Multan (Pakistan).

3.1.1. Preparation of plant extract

2 kg of *Raphanus sativus* seeds were washed with distilled water, air-dried and grinded. Then the finely ground seed powder was soaked in 4 liters of methanol with random shaking. The mixture was left for some days and then methanol was evaporated in the rotary vacuum evaporator to get the crude extract of *Raphanus sativus* seeds.

3.2. Animal modeling and groups

Thirty albino rats (5-6 week old), weighing 150-200g were provided by the animal house of National institute of Health (NIH), Islamabad. The animals were kept in ordinary cages at room temperature of 25±3°C with a 12 h dark/light cycle. Acclimatization was done for two weeks prior to dosing; during that time they had free access to standard laboratory chow containing protein ; 21.05% , fat ; 35% , fiber ; 3.07, ash ; 8.3% ; sand (silica) ; 1.65%, carbohydrates ; 52.062, calcium ; 0.9%, phosphorous ; 0.55 moisture ; 10% (w/w) and fresh water at the Primate facility maintained at Quaid-i-Azam University, Islamabad. The initial weights of rats were recorded.

3.2.1. Animal treatment

Group 1 (control group) was given only corn oil (1ml/kg bw). Group2 rats were administered with 10% CCl_4 in corn oil (1ml/kg bw), intraperitoneally for 18 days alternatively. Group 3 was treated with 10% CCl_4 in corn oil (1ml/kg of bw) intraperitoneally and *R. sativus* seed extract 100mg/kg of rat body weight (in DMSO) orally, using feeding tube. Group 4 received 10% CCl_4 in corn oil (1ml/kg bw) intraperitoneally and *R. sativus* seed extract 200mg/kg body weight of rat (in DMSO) orally. Group 5 was given *R. sativus* seed extract 200 mg/kg of body weight dissolved in DMSO orally by feeding tubes all for 18 days at alternative days.

3.2.2. Animal Dissection

After 24 h of the last dose, all the rats were starved overnight and weighed. Animals were anesthetized with chloroform and then dissected from ventral side of the body. First step was blood collection by cardiac puncture in falcon tubes which was centrifuged to collect the serum. The serum was stored in regular freezer. Heart was removed and washed in ice cold saline, dried with blotting paper and weighed. After weighing, the heart was divided into two portions. The part from all tissues were cut off and stored in fixative sera (Absolutes alcohol 60%, Formaldehyde 30%, Glacial acetic acid 10%), for histological studies while liquid Nitrogen was put in the 2^{nd} portion of tissue and stored at -70 °C.

3.3. Chemicals

Glutathione reductase, bovine serum albumin (BSA), Reduced glutathione (GSH), 1,2-dithio-bis nitro benzoic acid (DTNB), γ-glutamyl p-nitro anilide, reduced nicotinamide adenine dinucleotide phosphate (NADPH), 1-chloro-2,4-dinitrobenzene (CDNB), flavine adenine dinucleotide (FAD), glucose-6-phosphate, 2,6-dichlorophenolindophenol, thiobarbituric acid (TBA), sodium tungstate, sodium hydroxide, trichloroacetic acid (TCA), picric acid, CCl_4 of Sigma Chemicals C.USA, were purchased from local market.

3.4. Extraction of antioxidant enzymes

To establish the enzymatic activities, 70 mg of heart tissues were homogenized in 10 volume of 100 mM NaH_2PO_4 buffer containing 1 mM EDTA, pH 7.4 and centrifuged at 12,000xg for 30 min at 4 °C. Supernatant was taken and used to check the different enzyme levels as follows.

3.4.1. Peroxidase assay (POD)

POD activity was found out by Chance and Maehly method (1955) with few modifications. The POD reaction solution contained 50 mM phosphate buffer (pH 5.0), 20 mM guaiacol, 40 mM H_2O_2 and the enzyme extract. Changes in absorbance of the reaction solution at 470 nm were determined after one minute of incubation. One unit of POD activity was calculated as an absorbance change of 0.01 as unit min.

3.4.2. Superoxide dismutase assay (SOD)

SOD enzyme activity was found by the method of Kakkar *et al.* (1984). Reaction mixture includes phenazine methosulphate (186 μM) and sodium pyrophosphate buffer (0.052 mM, pH 7.0). Supernatant, that came after centrifugation (1500 xg, 10 min followed 10000xg, 15 min) of 10% heart tissue homogenate was added to the reaction mixture. Reaction started by the addition of NADH (780 μM) and ceased after 1 min when glacial acetic acid was added. Quantity of chromogen formed by the reaction was measured by recording color intensity at 560 nm on spectrophotometer. Results were articulated in units/mg protein.

3.4.3. Catalase assay (CAT)

CAT enzyme activity was determined by the method of Chance and Maehly (1955) but with some alteration. The reaction solution for CAT contained 50mM phosphate buffer pH 5.0, 5.9mM H_2O_2 and enzyme exact. Changes in absorbance of the reaction solution at 240 nm where determined after one minute incubation.

One unit of CAT activity was defined as an absorbance change of 0.01 as units/min.

3.4.4. Estimation of lipid peroxidation assay (TBARS)

Lipid peroxidation levels were measured by following the procedure set by Wright *et al.* (1981), and modified by Iqbal *et al.* (1996). The reaction mixture was prepared that contained Phosphate buffer (0.1M, PH 7.4), tissue homogenate sample, ascorbic acid (100 mM), and ferric chloride (100 mM). The reaction mixture was left for incubation at 37 ºC for 1 h time in a shaking water bath. Then 10 % trichloroacetic acid was added to stop the reaction. Next step is the addition of 0.6% thiobarbituric acid, following the boiling of tubes in the water-bath for 20 min; the tubes were then shifted to crushed ice bath before centrifugation at 2500xg for 10 min. The total of TBARS formed in each of the samples was judged by measuring optical density of the supernatant (collected after centrifugation) at 535nm using spectrophotometer against a reagent blank. The results were articulated as nM TBARS/min/mg tissue at 37 ºC using molar extinction coefficient of 1.56 ×10 /M cm.

3.4.5. Glutathione reductase assay (GSR)

Glutathione reductase activity was determined by method of Carlberg and Mennervik (1975). The reaction solution composed of phosphate buffer: (0.1M, pH 7.6), EDTA (0.5 mM), NADPH (0.1mM) oxidized glutathione (1 mM), and 10% homogenate in total volume of enzyme activity was quantitated at 25 ºC by measuring disappearance of NADPH at 340 nm and was calculated as nM NADPH oxidized/min/mg protein using molar extinction coefficient of 6.22 × 10 /cm.

3.4.6. Glutathione-S-transferase assay (GST)

Glutathione-S-transferase activity was assayed by the method explained by Habig *et al.,* (1974). The reaction mixture consisted of phosphate buffer (0.1 M, pH 6.5), (CDNB) (1 mM). The changes in the absorbance were witnessed at 340 nm and enzymes activity was calculated as nM CDNB conjugate formed/min/mg protein by using a molar extinction of 9.6 × 10/M cm.

3.4.7. Reduced glutathione assay (GSH)

Reduced glutathione in tissue was estimated by Jollow *et al.,* (1974) procedure. 10% homogenate sample was precipitated with (4%) sulfosalicylic acid. That were kept undisturbed at 4ºC for 1 hour and then centrifuged at 1200xg for 20 min at 4 ºC .The total volume of assay mixture composed of filtered aliquot, phosphate buffer (0.1M, pH 7.4) and DTNB (5,5-ditgiobis-2-nitrobenzoic acid), (100 mM). The yellow color of the mixture was developed, read immediately at 412nm on a smart Spec TM plus Spectrophotometer and expressed as µM GSH/g tissue.

3.4.8. Glutathione peroxidase assay (GSH-Px)

The accepted procedure by Mohandas *et al*. (1984) was employed for determination of tissue GSH-Px using sodium azide (1 mM), glutathione reductase (1 IU/ml), NADPH (0.2 mM) and 0.01 ml H_2O_2 (0.25 mM) as reaction substrate. Activity of GSH-px was expressed as nM NADPH oxidized/min/mg protein using molar extinction coefficient of 6.22 × 10^3/M cm.

3.5. Histopathological study of heart

To evaluate the changes at tissue level, heart tissues were fixed in fixative (absolute alcohol 60%, Formaldehyde 30%, Glacial acetic acid 10%) and embedded

in paraffin. The next step was to section them at 4µm and subsequent staining with hematoxylin/eosin. The prepared sections were studied under light microscope at 20 and 40 magnifications. Slides of tissues from all treated groups were studied and photographed.

3.6. Sample preparation for biochemical analysis

Blood samples from rats were collected in tubes and left undisturbed. After 30 minutes of standing, the blood samples were centrifuged at 5000 rpm for 10 min at 4°C by cold centrifugation. Serum was collected and acidified with 6% perchloric acid to precipitate the protein and used to determine the enzyme acivities.

3.6.1. Assessment of Alkaline Phosphatase (ALP)

ALP enzyme was determined by using kit from AMP Diagnostics Company.

Principle

Para – Nitro phenyl phosphate + H_2O $\xrightarrow{ALP}$ Phosphate + Para-nitrophenol

Assay Procedure

At a ratio of 4:1, reagent R1 (Diethanolamine (pH 9.8) 1.25mM) and reagent R2 (p-Nitrophenylphosphate 50mM) were mixed to form a mono reagent. The temperature requirement was 37 °C and wavelength was set at 405 nm (400nm – 420nm) with 1 cm optical path against air blank. Sample was mixed with mono reagent (R1 and R2) and absorbance was noticed after 1, 2 and 3 min interval. The enzyme activity was determined by using formula;

ALP activity (U/L) $\Delta = (\Delta A/min \times F)$

$\Delta A/min$ = average of absorbance per min

F = factor (for ALP it is 2557 at 405 nm).

3.6.2. Assessment of serum Bilirubin:

Serum Bilirubin was measured by using kit from AMP Diagnostics Company.

Principle

Sulfanilic acid reacts with sodium nitrate to from diazotized sulfanilic acid. In the presence of DMSO, total bilirubin in the sample reacts with diazotized sulfanilic acid to give azobilirubin. Only direct bilirubin undergoes this reaction to give azobilirubin in the presence of DMSO. Kit from AMP diagnostics Company was used.

Assay Procedure

Working reagent (R1) (Sulfanilic acid, DMSO, HCl), was mixed with working reagent (R2) with sample or calibrator, sample without R2 was designed as sample blank and OD (555 nm) was noted after giving a 5 min incubation at 37 °C. Serum total Bilirubin and direct Bilirubin were calculated through formula.

(OD of sample – OD of sample blank) x F

Where F is standard factor concentration.

3.6.3. Assessment of serum Gamma Glutamyl transpeptidase:

The quantitative determination of γ – Glutamyl transpeptidase was done by using kit from AMP Diagnostics Company.s

Principle:

Gamma Glutamyl transpeptidase transfers a γ – Glutamyl group from γ–Glutamyl–3-carboxy-4-nitroanilide to glycylglycine alongwith the formation of L–γ–Glutamyl glycylglycine and 5-amino-2-nitro-benzoate. The amount of 5-amino-2-nitro-benzote formed, monitored kinetically at 405 nm, is attributed to the enzyme activity present in the sample.

Assay Procedure

Reagent 1 having TRIS, pH 8.2, glycylglycine, was mixed with Reagent 2 containing L-γ – Glutamyl-3-corboxy-4-nitroanilide) in 4:1. That was the working reagent. Working reagent was mixed with sample, distilled water (for blank). After 1 min incubation at 37 °C, OD was taken at 405nm. The enzyme action/activity was calculated as unite per liter (U/L) by using the following formula;

Enzyme activity = (ΔA/min) x 111

Where (ΔA/min) is the average absorbance of samples per min.

3.6.4. Assessment of albumin

Principle

Colorimetric determination of serum albumin was done by using bromocresol green (BCG) at pH 4.20 through kit purchased from AMP diagnostics company.

Assay procedure

Reagent containing succinate buffer, bromocresol green and Brij 35 was mixed with sample or standard (bovine albumin), distilled water used for blank and optical density (OD) (628nm) was taken after incubation (for 5 min.) at 37 °C. Serum albumin was calculated by using formula;

(OD of sample / OD of standard) x n

Where n is standard concentration.

3.6.5. Assessment of serum cholesterol

The quantitative determination of serum cholesterol was done by using kit from AMP Diagnostics Company.

Principle

The cholesterol in the sample forms a colored complex, according to the following reaction:

$$\text{Cholesterol esters} + H_2O \xrightarrow{\text{cholesterol esterase}} \text{Cholesterol} + \text{fatty acids}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{cholesterol oxidase}} \text{4-Cholestenona} + H_2O_2$$

$$2H_2O_2 + \text{Phenol} + \text{4-Aminoantipyrine} \xrightarrow{\text{peroxide}} \text{Quinonimine} + 4H_2O_2$$

The intensity of the color formed is proportional to the amount of cholesterol present in the sample.

Assay procedure

The assay was performed at 37 °C temperature. The wavelength set was 505nm in 1cm optical path. Sample or standard was mixed with reagent and incubated for 5 min. The absorbance of the sample was taken in opposition to the blank within 60min. Serum Cholesterol was measured by using formula;

(OD of sample / OD of standard) x n

Where n is standard concentration.

3.6.6. Assessment of aspartate transaminase (AST)

The assay was performed according to the standard procedure explained alongwith kit used for in vitro determination of AST, according to the method by Vita lab selectra Routine chemistry analyzer (Merck) and other photometric systems.

Principle

L-Aspartate + 2-Oxoglutarate $\xrightarrow{\text{AST}}$ L-Glutamate + Oxaloacetate

Oxaloacetate + NADH + H+ $\xrightarrow{\text{MDH}}$ L-Maltate + NAD^+

The rate, at which NADH is consumed, was measured photometrically. It was directly proportional to the AST activity in the sample. Two reagents were used. Reagent R1 had TrisHCl, L-aspartate, malate dehydrogenase and lactate dehydrogenase in it while reagent R2 contained 2-Oxoglutarate, NADH and Pyridoxal 1-5-Phosphate.

Assay Procedure

The reaction temperature was 37°C and absorbance was recorded at 430 nm with 1 cm optical path against air blank. The reagents R1 and R2 were mixed in 4:1 to be used as working reagent. Then this working reagent was mixed with sample and absorbance was noted against distilled water used as blank, 340nm in 1cm optical pathway. Temperature required is 37 ^{o}C. The enzymatic activity was calculated by using formula:

AST (U/L) = ΔAbs/min×1768.

(Where ΔAbs /min=Average absorbance change per minute).

3.6.7. Assessment of lactate dehydrogenase (LDH)

The quantitative determination of LDH was done by using kit from AMP Diagnostics Company.

Principle

LDH is catalyses the inter conversion of lactate and pyruvate. Under diseased condition in the tissues containing LDH, the cells discharge LDH into the blood stream so that it is identified in higher than normal levels. The non-radioactive colorimetric LDH assay is based on the fact that the tetrazolium salt MTT in a NADH-coupled enzymatic reaction is converted to a reduced form of MTT which exhibits a maximum absorption at 565nm. The intensity of the purple color formed reflects the enzyme activity.

Assay procedure

Working reagent comprises of R1 (Tris buffer, pH 7.2) and R2 (NADH) mixed in 4:1 then this working reagent and sample were mixed to get absorbance, noted at 340nm and 37°C against distilled water blank. The activity of LDH was determined by formula:

Activity (U/L = OD/min×11496)

3.6.8. Determination of serum creatinine

The quantitative determination of creatinine was done by using kit from AMP Diagnostics Company.

Principle

Creatinine + Sodium Picrate $\xrightarrow{\text{Alkali}}$ Creatinine- picrate complex

Creatinine reacts with picric acid in alkaline/basic conditions to form a color complex that shows absorbance at 510nm. The rate of formation of color complex is proportional to the activity of creatinine in the sample.

Assay procedure.

Working reagent had R1 (Alkaline buffer) and R2 (picric acid 40Mm) mixed together in 5:1 ratio which was mixed with sample and absorbance was noted at 510nm and 37°C after an incubation of 60 seconds. Distilled water was used as sample blank. Serum creatinine was measured by using formula;

mg/dl = ΔAbs (unknown)/ Δ Abs (standard) ×concentration of standard (mg/dl)

ΔAbs= Absorbance change between observations (A_2-A_1).

3.6.9. Determination of serum creatine kinase (CK)

Creatine kinase (CK) level was determined by using kit from AMP Diagnostics Company.

Reagents

R1; Glucose; 20mmol/L, NAC; 20mmol/L, magnesium acetate; 10mmol/L, Imidiazol buffer 100 mmol/L, NADP; 2.5mmol/L, HK; 2.4U/L, EDTA; 2mmol/L

R2: CP; 30mol/L, AMP; 5mmol/L, ADP; 2mmol/L, Di

(adenosine-5) pentaphosphate; 10µmol/L, G6P-DH; 1.5U/L

Principle

Creatine kinase enzyme catalyzes the reaction between creatine phosphate and adenosine 5-diphosphate giving out creatine and adenosine-5-triphosphate. The later then converts glucose to glucose-6-phosphate by phosphorylation in the presence of hexoquinase, which is further oxidized to Gluconate-6-P in the presence of reduced nicotinamide-adenine dinucleotide phosphate (NADP); the reaction being catalyzed by glucose-6-phosphate dehydrogenase (G-6-P-DH). The reaction is monitored at 340nm by noticing the rate of increase in absorbance which results from the reduction of NADP to NADPH proportional to the activity of CK present in the sample. The presence of N-acetilcystein allows the favorable activation of enzyme.

$$\text{CP} + \text{ADP} \xrightarrow{\text{CK(AMP,NAC)}} \text{Creatinin} + \text{ATP}$$

$$\text{ATP} + \text{Glucose} \xrightarrow{\text{NAC}} \text{ADP} + \text{G6P}$$

$$G6P + NADP^{+} + H_2O \xrightarrow{G6P\text{-}OH} Glucose\text{-}6P + NADPH^{+} + H^{+}$$

Assay procedure

R1 and R2 were mixed in 4:1 to have the working reagent. Then working reagent and samples were mixed and OD was noted at 340nm and 37°C. Amount of CK was determined by using formula:

CK [U/L] = ΔOD/min×8095

3.6.10. Assessment of alanine transaminase (ALT)

To determine the ALT level, the photometric method, based on the method of the international federation of clinical chemistry was used.

Principle

$$\alpha\text{-ketoglutarate} + \text{L-alanine} \xrightarrow{ALT} \text{Glutamate} + \text{Pyruvate}$$

$$\text{Pyruvtae} + NADH + H \xrightarrow{LDH} \text{Lactate} + NAD$$

ALT transfers an amino group from L-alanine to α-ketoglutarate which results in pyruvate and L-glutamate formation. Lactate dehydrogenase reduces pyruvate and oxidizes NADH to NAD at the same time. The resulting decrease in absorbance is directly proportional to ALT activity.

Assay procedure

Working reagent having composition; L-alanine, α-ketoglutaric acid, LDH, NADH, Buffer (pH 7.5), sodium azide, stabilizers was made by mixing R1 and R2 in 4:1. Then working reagent was mixed with sample and OD was noted at 37ºC and 340nm in 1cm optical pathway after 1minute incubation against distilled water. The activity of enzyme was calculated as unit per liter (U/L) by using the following formula.

ALT (U/L) = ΔAbs/min × 1768. (Where ΔAbs/min = Average absorbance change per minute).

3.6.11. Determination of serum urea

Blood urea nitrogen was determined using kit purchased from AMP Diagnostics Company.

Principle

Urea is converted to ammonia and carbon dioxide by urease through hydrolysis. Ammonia is acted upon by glutamate dehydrogenase and converted to glutamate in the presence of oxyglutarate and NADH. Oxidation of NADH to NAD results in a decrease in absorbance observed at 340nm which is proportional to the concentration of urea present in the sample.

$$\text{Urea} + H_2O \xrightarrow{\text{urease}} 2NH_3 + CO_2$$

$$2NH4^{+} + 2 \text{ alpha ketoglutarate} + 2 \text{ NADH} \xrightarrow{\text{GIDH}} 2\text{L-glutamate} + 2NAD^{+} + 2H2O$$

Assay procedure

Reagent (Tris buffer, alpha Ketoglutarate, urease, GIDH and ADP) was mixed with sample/standard and OD change (340nm) was read between 30 and 90 seconds. Serum urea was calculated by formula;

(OD of sample / OD of standard) x n.

(Where n is standard concentration.)

3.6.12. Determination of High density lipoprotein (HDL)

The *in vitro* determination of HDL was done by kit from AMP diagnostic company.

Principle

Antihuman β-lipoprotein antibody in R1 binds to lipoproteins. The antigen antibody complexes formed with the addition of R2. CHE and cholesterol oxidase in R2 only react with HDL. Hydrogen peroxide produced by the enzyme reactions with HDL yields a blue color. HDL was measured at 593 nm.

Assay procedure

The whole procedure was done at the temperature of 37 ºC with 1 cm optical path at the wavelength of 600 nm against the reagent blank. 2.5 µl of sample and 2.5

µl of calibrator were mixed and after 5 min OD was measured then R2 of 60 µl was added and mixed. The absorbance was checked after 5 min incubation.

The HDL was calculated by the formula;

(OD2-OD1) sample / (OD2-OD1) Calibrator × n

Where n=Calibrator concentration (AMP HDL-Calibrator (BR9812) used for calibration)

3.6.13. Determination of low density lipoprotein (LDL)-Cholesterol

LDL was calculated by the formula:

LDL= TG/5+ HDL– cholesterol

3.6.14. Triglycerides determination

Assay procedure

1 ml of the reagent (Mg^{+2}, Potassium ferrocynate, Glycerol kinase, ATP) mixed with 10 µl distilled water, standard and sample used as blank, standard and sample, incubated at 37 ^{0}C for 10 min and measured OD at 500 nm.

Triglycerides were calculated by the formula;

OD of sample/OD of standar×n

3.7. Antibacterial assay

3.7.1. Requirements for assay

Test samples, nutrient agar (20g/L), McFarland Barium sulphate turbidity standard of 0.5, cultures of bacterial strains, centrifuge machine, sterile normal saline solution (0.9% NaCl w/v), sterile cork borer, micropipette, Petri dishes (14cm), organic solvent (DMSO), thermometer, incubator, standard antibiotics (Roxithromycin and Cefixime-USP) and spirit lamp.

3.7.2. Preparation of samples

Five types of samples were prepared for antibacterial assay:

1. Methanolic extract of *R. sativus* seeds
2. Ethyl acetate extract of *R. sativus* seeds
3. n-Hexane extract of *R. sativus* seeds
4. Butanolic extract of *R. sativus* seeds
5. Aqueous extract of *R. sativus* seeds

150 mg of the plant exract was dissolved in 10 ml of DMSO to have a 15 mg/ml concentration. This stock solution was then used to make further dilutions with DMSO. Solution of Roxithromycin and Cefixime-USP, 2mg/ml in DMSO, were used as positive control while pure DMSO was used as negative control.

3.7.3. Media for bacteria

Nutrient Broth medium (Merck) was used for bacterial growth for the inoculum preparation. Its composition was;

- Peptone from meat= 5gm/L
- Meat extract= 3gm/L

Nutrient agar medium (Merck) was used to carry out antibacterial assay. Its composition was;

- Peptone from meat= 5gm/L
- Meat extract= 3gm/L
- Agar-agar= 12gm/L

3.7.4. Preparation of media for bacteria

Nutrient broth medium was prepared by dissolving 8.0gm/L in distilled water; pH was adjusted at 7.0 and was autoclaved.

Nutrient agar medium was prepared by dissolving 20gm/L in distilled water; pH was adjusted at 7.0 and was autoclaved.

3.7.5. McFarland 0.5 BaSO4 turbidity standard

The standard was prepared by adding 0.5 ml of 0.048 M $BaCl_2$ to 99.5 ml 0.36 N H_2SO_4. Barium sulphate turbidity standard (4 to 6 ml) was taken in screw-capped test tube and was used for the turbidity comparasion (Koneman, 1988).

3.7.6. Microorganisms used

Five bacterial strains were used. One of these five was Gram positive including *Micrococcus luteus* (ATCC 10240). Remaining four were Gram negative, which were *Pseudomonas picketti* (ATCC 49129), *Enterobacter aerogens* (ATCC 13048),

Escherichia coli (ATCC 49129) and *Bordetella bronchiseptica* (ATCC 4617). The organisms were maintained at a temperature of 4 ^{o}C in nutrient agar medium.

3.7.7. Preparation of inocula

Centrifuged pallets of bacteria, from twenty four hour old culture in nutrient broth (SIGMA) of selected bacterial strain, was mixed with physiological saline. Turbidity was corrected by the addion of sterile physiological saline to get McFarland 0.5 $BaSO_4$ turbidity standard (10^8 colony forming unit (CFU)/ml). Then this inoculum was used afterwards for seeding the nutrient agar with the culture.

3.7.8. Preparation of seeded agar plates

Nutrient agar medium was prepared by suspending nutrient agar (MERCK) 20 gm in 1L distilled water; pH was adjusted at 7.0 and was autoclaved at 120 ^{o}C. It was left to cool upto 45 ^{o}C. Then it was seeded with 10ml of prepared inocula to have 10^6 CFU/ml. 75 ml of seeded nutrient agar were put in the petri plates (14cm) and allowed to solidify. 11 wells were made in each plate with sterile cork borer (8mm).

3.7.9. Assay procedure

100ul of test solutions was poured in respective well by using micropipette. Eight concentrations of extract (15mg/ml- 1mg/ml), two solutions for positive control (Roxithromycin and Cefixime-USP) and one for negative control (DMSO) was put into each petri plate. These plates were incubated at 37 ^{o}C for 24 h. After incubation time, the diameter of the clear zones was measured, showing no bacterial growth, around each well. Duplicate plates were prepared for each extract. Mean clear zone of these plates was calculated with standard deviation. Antibacterial activity of all dilutions of extract (RSME, RSEE, RSHE, RSCE and RSAE) was determined against five bacterial strains.

3.8. Statistical analysis

To determine the effects of different treatments, one way analysis of variance was carried out by computer software ANOVA. Level of significance among the various treatments was taken at 0.05% probability level.

RESULTS

Carbon tetrachloride is a toxic compound which when taken in by any means, causes severe damages to almost all tissues of the body. These adverse effects are the result of oxidative imbalance caused by the compound. The intraperitoneal injection of CCl_4 in rats resulted in cardiac enzymatic, biochemical and histological changes which were compensated by the oral administration of *Raphanus sativus* methanolic extract. Results noted after the experiment are as under:

4.1. Alterations in body weights versus heart weights

There was a notable change in body weights of rats among different groups as well as the considerable value as compared with the control group. On the other hand, there had been a %age increase in the heart weights in the group receiving intraperitoneal dose of CCl_4. The co-treatment with different concentrations of *R. sativus* methanolic extract (RSME) resulted in a robust and lucid fixation of the CCl_4 toxicative effects; so that the body weights showed a %age increase and heart weights showed a %age decrease. (Table 4.1)

Table 4.1. Alterations in body weights versus heart weights

Groups	Treatements	%age increase in body weight (gm)	Heart weight (gm)
I.	Control	53.67±4.3**	0.71±0.32*
II.	CCl_4	21.28±2.94	0.89±0.14
III.	100mg/kg RSME + CCl_4	34.83±3.41*	0.81±0.21*
IV.	200mg/kg RSME + CCl_4	49.79±4.02**	0.76±0.27*
V.	200mg/kg RSME	50.39±4.20**	0.73±0.30**

Mean±SD (n=6)

*, ** indicate significance from CCl_4 at $P<0.05$ probability level

4.2. Protective effects of *Raphanus sativus* methanolic extract; tissue homogenate assays

4.2.1. Protective effects of *R. sativus* on cardiac SOD, POD, CAT and TBARS

When compared with the control group, the activities of SOD, POD and CAT were significantly decreased ($P<0.05$) while the TBARS contents were increased, in case of rats treated with CCl_4 (Table 4.2). Those rats, having *R. sativus* methanolic extract co-treatment (100mg/kg bw) showed an increase ($P<0.05$) in these enzymes level along with a decrease in TBARS. The activities were observed closer to the control group in case of higher doses of *R. sativus* extract co-treated with CCl_4 (200mg/kg bw).

4.2.2. Protective effects of *R. sativus* on cardiac GST, GSH-Px, GSH and GSR

Table 4.3 shows that there is a significant reduction ($P<0.05$) observed in cardiac GST, GSH-Px, GSH and GSR levels in the CCl_4 treated group, which was ameliorated ($P<0.05$) by the *R. sativus* methanolic extract (100mg/kg bw) to an extant but the level of these enzymes was brought closer to the control group by *R. sativus* extract (200mg/kg bw) when co-administered with CCl_4.

Table 4.2. Protective effects of *R. sativus* on cardiac SOD, POD, CAT and TBARS

Group	Treatment	SOD	POD	CAT	TBARS
I.	Control	3.1383±0.0646*	9.252±0.577**	4.263±0.474*	3.628±0.556*
II.	CCl_4	1.2583±0.1005	4.855±0.666	2.187±0.575	5.651±0.530
III.	100mg/kg RSME+CCl_4	2.4867±0.1728*	7.805±0.863**	3.437±0.809*	4.508±0.626*
IV.	200mg/kg RSME+CCl_4	3.0817±0.1278**	9.333±0.721**	4.883±0.592**	3.540±0.411**
V.	200mg/kg RSME	3.0467±0.2435**	9.513±1.012**	4.828±0.565**	3.513±0.419**

Mean±SD (n=6). *, ** indicate significance from CCl_4 group at P<0.05 probability level

Table 4.3. Protective effects of *R. sativus* on cardiac GST, GSH-Px, GSH and GSR

Group	Treatment	GST	GSH-Px	GSH	GSR
I.	Control	155.11±4.14**	119.28±4.24**	18.097±0.537**	159.72±3.27**
II.	CCl_4	82.19±3.47	67.80±3.10	10.528±0.465	102.03±2.71
III.	100mg/kg RSME+CCl_4	126.70±5.38*	92.290±2.044*	15.860±0.539*	137.46±3.69*
IV.	200mg/kg RSME+CCl_4	147.33±2.60**	121.72±4.80**	17.385±0.634**	155.25±4.34**
V.	200mg/kg RSME	156.24±5.12**	118.71±2.15**	18.522±0.719**	154.48±5.59**

Mean±SD (n=6). *, ** indicate significance from CCl_4 group at P<0.05 probability level

4.3. Serum analysis

4.3.1. Protective effects of *R. sativus* extract on serum cardiotoxicity markers; AST, ALP and ALT and total cholesterol

An increase ($P<0.05$) in serum cardiac markers AST, ALP, ALT and total cholesterol was observed in the CCl_4-treated group in comparison with the control group. The toxic effect by the CCl_4 was corrected by *R. sativus* extract (100mg/kg bw) co-treatment. An enhanced effect was seen with the *R. sativus* concentrated extract (200mg/kg bw) where it reduced ($P<0.05$) the enzymes level to nearly normal values as shown in Table 4.4.

4.3.2. Protective effects of *R. sativus* extract on serum cardiotoxicity markers; LDH, LDL, HDL and Triglycerides

Table 4.5 indicates that the serum levels of heart marker enzymes LDH, LDL and triglycerides were increased ($P<0.05$) while HDL was decreased ($P<0.05$) by the administration of CCl_4 as compared to the rats from normal group receiving only corn oil. The dose dependent decrease ($P<0.05$) in the level of these enzymes was seen in the rats provided with *R. sativus* methanolic extract (100mg/kg bw). The effect is more noticeable in the case of the group being provided with 200 mg/kg bw of the *R. sativus* extract.

4.3.3. Protective effects of *R. sativus* extract on serum total bilirubin, urea, creatinine and γ-GT

The CCl_4 intoxication resulted in a significant ($P<0.05$) increase in serum levels of total bilirubin, creatinine and γ-GT while a significant decrease ($P<0.05$) in urea as compared to the control group. The levels of the former three were decreased insignificantly, increase in the later was also insignificant ($P<0.05$) with the intake of *R. sativus* extract co-treatment (100mg/kg bw). Therefore, *R. sativus* (200mg/kg bw) extract was effective to bring the values of those factors to a normal control level as indicated in Table 4.6.

4.3.4. Protective effects of *R. sativus* extract on serum cardiac markers: CK and albumin

Comparing with the normal control group, CCl_4 induced a noticeable increase ($P<0.05$) in serum CK levels, while a decrease was observed in the levels of albumin. But these effects were altered by the administration of *R. sativus* (100mg/kg bw) extract and a more improved condition was observed in the case of 200mg/kg bw extract of the plant seeds. In that case, an decrease in the CK and boost up in albumin were quite clear ($P<0.05$) (Table 4.7)

Table 4.4. Protective effects of *R. sativus* extract on serum cardiotoxicity markers; ALT, ALP, AST and total cholesterol

Group	Treatment	ALT (U/L)	ALP (U/L)	AST (U/L)	Total choles.(mg/l)
I.	Control	11.159±0.822*	186.99±8.05**	73.63±2.83*	69.75±4.87*
II.	1% CCl_4	20.968±0.882	370.54±9.50	213.96±3.51	141.32±4.87
III.	100 mg/kg RSME+ CCl_4	17.144±0.345*	325.38±5.30	180.68±6.60	118.14±13.7
IV.	200 mg/kg RSME + CCl_4	14.420±0.383**	254.30±6.08	132.80±5.38*	92.57±3.79*
V,	200 mg/kg RSME	11.635±1.000**	203.12±3.32*	101.45±5.85*	70.69±1.188**

Mean±SD (n=5). *, ** indicate significance from CCl_4 group at P<0.05 probability level

Table 4.5. Protective effects of *R. sativus* extract on serum cardiotoxicity markers; LDH, LDL, HDL and Triglycerides

Group	Treatment	LDH(nM/min/ mg protein	Triglycerides (mg/dl)	HDL (mg/dl)	LDL (mg/dl)
I.	Control	52.91±5.75**	95.85±6.73**	3.862±0.763**	297.24±6.67**
II.	1% CCl_4	100.17±3.82	184.11±20.59	2.64±0.1845	987.95±6.72
III.	100 mg/kg RSME+ CCl_4	84.54±2.73	151.83±10.46	3.16±0.1645*	810.42±8.67
IV.	200 mg/kg RSME + CCl_4	71.420±1.369*	120.52±2.72*	3.489±0.265**	660.12±14.45
V.	200 mg/kg RSME	58.669±2.400**	90.56±1.377**	3.957±0.258**	432.1±24.9*

Mean±SD (n=5). *, ** indicate significance from CCl_4 group at P<0.05 probability level

Table 4.6. Protective effects of *R. sativus* extract on serum total bilirubin, urea, creatinine and γ-GT

Group	Treatment	γ-GT (nM/min/mg protein)	Total bilirubin (mg/dl)	Urea (mg/dl)	Creatinine (mg/dl)
I.	Control	3.9485±0.1862**	0.1626±0.03**	35.896±1.2 **	28.85±0.43*
II.	1% CCl_4	8.1327±0.2403	0.3704±0.02	65.71±3.70	63.82±0.54
III.	100 mg/kg RSME+ CCl_4	6.4913±0.0908	0.24264±0.0	48.121±1.4	37.74±0.47*
IV.	200 mg/kg RSME + CCl_4	5.0531±0.1170*	0.1957±0.00*	40.23±3.71*	31.97±0.47*
V.	200 mg/kg RSME	3.8740±0.0820**	0.1408±0.02**	40.21±8.45*	29.55±0.92**

Mean±SD (n=5). *, ** indicate significance from CCl_4 group at P<0.05 probability level

Table 4.7. Protective effects of *R. sativus* extract on seum albumin and CK

Group	Treatment	Albumin (mg/dl)	CK
I.	Control	3.6523±0.174**	15.946±0.529**
II.	1% CCl_4	1.8952±0.098	31.075±0.756
III.	100 mg/kg RSME+ CCl_4	2.6047±0.05	25.054±0.395
IV.	200 mg/kg RSME+ CCl_4	2.9633±0.133*	19.97±0.310*
V.	200 mg/kg RSME	3.2204±0.042**	16.875±0.456**

Mean±SD (n=5). *, ** indicate significance from CCl_4 group at P<0.05 probability level

4.4. Cardiac histopathological examination

H&E staining method was used for the histological studies of heart muscles. Different changes were observed at the tissue levels which are stated as under;

4.4.1. Histology of control group

The heart tissue from control group, receiving only corn oil depicted the normal architecture of heart muscles. Light microscopy revealed a normal myofibrillar structure with properly striated cells having a branched appearance and a normal continuation with the adjacent myofibrils. Cardiac muscle exhibited resemblance with the skeletal muscle fibers; the former being shorter than the later. Each cardiac muscle had a single, centrally located nucleus and a vast blood supply. Connective tissue was shown to be provided with small blood capillaries.

4.4.2. CCl_4-treated group

Clear picture of cardiotoxicity was manifested in the group injected with carbon tetrachloride. Disruption in the heart cells was quite apparent, with distortions in blood capillaries, intrusions in heart trabeculae and lesions in heart muscles. Mucoid edema and necrosis was also clear in this group.

4.4.3. *R. sativus* co-treatment

The co-treatment with different concentration of *R. sativus* methanolic extract had a markedly positive effect on the cardiac muscle make-up. There was a noticeable recovery with lesser capillary dilation at fewer sites and normal vacuolar structure. In short, the plant- co-treated groups showed repairing sequences of cardiotoxicity with less abnormal findings as compared with the CCl_4 intoxicated group.

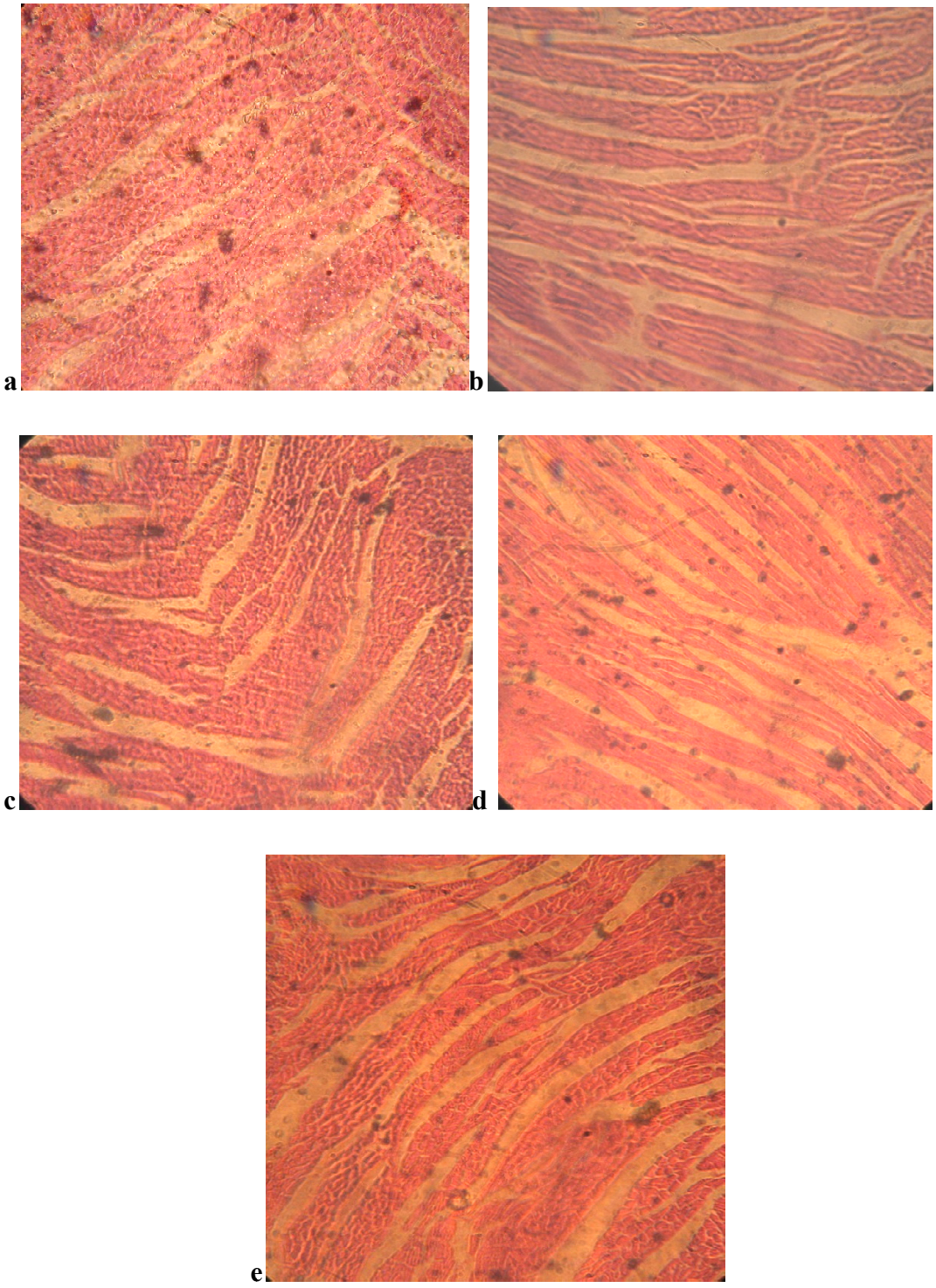

Fig4.1. Preventive effects of RSME on heart histology

Fig4.1a. Control group showing normal structure of heart. **b.** CCl_4 group showing severe cell disruption. **c.** 100mg/kg bw RSME+CCl_4 showing healing in the heart muscles. **d.** 200mg/kg bw RSME+CCl_4 showing almost complete recovery of heart muscles. **e.** 200mg/kg RSME showing similar cardiac architecture as control group.

4.5. Assessment of antibacterial activity of *R. sativus*

Five types of *R. sativus* seed extracts were subjected to antibacterial activity including RSME, RSEE, RSHE, RSCE, and RSAE against five bacterial strains. One was Gram positive; *Micrococcus luteus* (ATCC 10240). Four were Gram negative; *Escherichia coli* (ATCC 1224), *Pseudomonas picketti* (ATCC 49129), *Enterobacter aerogens* (ATCC 13048) and *Bordetella bronchiseptica* (ATCC 4617). The readings were recorded after an incubation period of 24 hours at 37 ^{o}C.

4.5.1. *R. sativus* methanolic extract (RSME)

The RSME showed a decent antibacterial activity against *B. bronchiseptica*; the mean zone of inhibition ranging between 10-23.5mm after 24 hours at the concentration of 3mg/ml to 15mg/ml (Table 4.9). Activity against *P. piketti* was also good; mean zone of inhibition between 9-28mm; the concentration between 5mg/ml to 15mg/ml. While the activity shown against *E. aerogens* and *M. luteus* was not much satisfactory, the mean zones of inhibition ranged between 10-20mm (concentration between 10mg/ml to 15mg/ml) and 13.5mm (15mg/ml) respectively. It was observed that RSME showed no activity against *E. coli*.

4.5.2. *R. sativus* ethyl acetate extract (RSEE)

Table 4.10 shows that the ethyl acetate extract of *Raphanus sativus* seed extract (RSEE) was found very effective against *P. piketti*. The mean zone of inhibition, after 24 hours of incubation, was between 11-32.5mm, the concentration range was 3mg/ml to 15mg/ml. It was also active against *M. luteus* at concentration range between 3mg/ml to 15mg/ml; mean zone of inhibition between 9-23mm. RSEE also showed some activity against *E. aerogens*. At the concentration from 5mg/ml to15mg/ml/ it showed mean zone of inhibition between 15-28mm after 24 hours. The activity against *B. bronchiseptica* was mild; mean zones of inhibition were from 12-21.5mm respectively at the concentrations ranging from 7.5mg/ml to 15mg/ml after 24 hours. No activity was seen against *E. coli*.

Table 4.8. Antibacterial activity of *R. sativus* methanolic extract (RSME) against five bacterial strains

Conc. Mg/ml	Zones of inhibition				
	E. coli	E. aero.	B. bron.	P. piketti	M. luteus
15	-	20	23.5	28	13.5
12.5	-	13.5	20	25	-
10	-	10	19	23.5	-
7.5	-	-	13.5	12.5	-
5	-	-	11	9	-
3	-	-	10	-	-
2	-	-	-	-	-
1	-	-	-	-	-
Cef.	31	35	27	33	23
Roxi.	27	25	25	29	30
DMSO	-	-	-	-	-

E. coli *Escherichia coli* *E. aero.* *Enterobacter aerogens* *M. luetus* *Micrococcus luteus*

B. bron. *Bordetella bronchiseptica* *P. piketti* *Pseudomonas piketti* Cef. Cefixime 2mg/ml

Roxi. Roxithomycin 2mg/ml DMSO Dimethyl sulfoxide mm. Millimeter

*The data represents the mean values of two replicates.

Table 4.9. Antibacterial activity of *R. sativus* ethyl acetate extract (RSEE) against five bacterial strains

Conc. Mg/ml	Zones of inhibition				
	E. coli	E. aero.	B. bron.	P. piketti	M. luteus
15	23	28	21.5	32.5	23
12.5	18	25	19.5	30	21
10	12.3	22.5	17	29	20.5
7.5	9	19	12	27.5	16.5
5	-	15	-	24.5	15
3	-	-	-	11	9
2	-	-	-	-	-
1	-	-	-	-	-
Cef.	26	29	32	34.5	30
Roxi.	27.5	28	31.5	31.5	27
DMSO	-	-	-	-	-

E. coli *Escherichia coli* *E. aero.* *Enterobacter aerogens* *M. luetus* *Micrococcus luteus*

B. bron. *Bordetella bronchiseptica* *P. piketti* *Pseudomonas piketti* Cef. Cefixime 2mg/ml

Roxi. Roxithomycin 2mg/ml DMSO Dimethyl sulfoxide mm. Millimeter

*The data represents the mean values of two replicates.

4.5.3. *R. sativus* butanolic extract (RSBE)

The butanolic extract of *Raphanus sativus* (RSBE) was found very much active against *P. piketti* where the mean zone of inhibition ranged between 10-34mm at the concentration range between 1mg/ml to 15mg/ml after 24 h. A good activity was observed in case of *M. luteus* and *E. aerogens*. Mean zones of inhibition of 15.5-28.5mm and 10-30mm were seen between the concentrations of 3mg/ml to 15mg/ml resp., after 24 hours. Some activity was observed in case of *B. bronchiseptica* which showed mean zone of inhibition between 6.5-20.5mm and the concentration range was 7.5-15mm. No activity was seen against *E. coli* as shown in Table 4.11.

4.5.4. *R. sativus* n-Hexane extract (RSHE)

Table 4.12 presents the mild effectiveness of n-Hexane extract of *Raphanus sativus* seeds (RSHE) *P. piketti* at the concentration between 7.5mg/ml to 15mg/ml showing the mean zone of inhibition from 12.5-27.5mm. A very low effectiveness was seen against *E. aerogens* and *B. bronchiseptica*. The mean zones of inhibition were 17.5-23.5mm and 20-25mm resp., both at the concentration range between 10mg/ml to 15mg/ml. Only a single zone of inhibition of 9.5mm was seen against *M. luteus* at 15mg/ml concentration. RSHE was ineffective against *E. coli*.

4.5.5. *R. sativus* aqueous extract (RSAE)

A good activity was shown by RSAE against *E. aerogens*. The mean zone of inhibition was from 10-33mm at the concentrations between 3mg/ml to 15mg/ml. A low activity was seen against *P. piketti* between 5mg/ml to 15mg/ml concentration; mean zone of inhibition was from 9-29mm after 24 h. The activity shown against *B. bronchiseptica* was only mild. The mean zone of inhibition was from 13.5-24.5mm between 10mg/ml to 15mg/ml. No activity was observed against *M. luteus* and *E. coli* (Table 4.13).

4.5.6. Minimum inhibitory concentration (MIC)

The minimum inhibitory concentration was noted for each extract which is the lowest concentration of an extract at which there was no zone of inhibition against a bacterial strain (Table 4.14).

Table 4.10. Antibacterial activity of *R. sativus* butanolic extract (RSBE) against five bacterial strains

Conc. Mg/ml	Zones of inhibition				
	E. coli	E. aero.	B. bron.	P. piketti	M. luteus
15	-	30	20.5	34	28.5
12.5	-	27	17.5	30	25.5
10	-	25	11	30	24
7.5	-	24.5	6.5	28	22.5
5	-	20	-	25	20
3	-	10	-	22.5	15.5
2	-	-	-	19	-
1	-	-	-	10	-
Cef.	33.5	29	32	37	31.5
Roxi.	28	28	31.5	36	34
DMSO	-	-	-	-	-

E. coli	*Escherichia coli*	*E. aero.*	*Enterobacter aerogens*	*M. luetus*	*Micrococcus luteus*
B. bron.	*Bordetella bronchiseptica*	*P. piketti*	*Pseudomonas piketti*	Cef.	Cefixime 2mg/ml
Roxi.	Roxithomycin 2mg/ml	DMSO	Dimethyl sulfoxide	mm.	Millimeter

*The data represents the mean values of two replicates.

Table 4.11. Antibacterial activity of *R. sativus* n-hexane extract (RSHE) against five bacterial strains

Conc. Mg/ml	Zones of inhibition				
	E. coli	E. aero.	B. bron.	P. piketti	M. luteus
15	-	23.5	25	27.5	9.5
12.5	-	23	22.5	26	-
10	-	17.5	20	23	-
7.5	-	-	-	12.5	-
5	-	-	-	-	-
3	-	-	-	-	-
2	-	-	-	-	-
1	-	-	-	-	-
Cef.	35	35	22	32.5	33.5
Roxi.	30	30	28	29.5	32
DMSO	-	-	-	-	-

E. coli	*Escherichia coli*	*E. aero.*	*Enterobacter aerogens*	*M. luetus*	*Micrococcus luteus*
B. bron.	*Bordetella bronchiseptica*	*P. piketti*	*Pseudomonas piketti*	Cef.	Cefixime 2mg/ml
Roxi.	Roxithomycin 2mg/ml	DMSO	Dimethyl sulfoxide	mm.	Millimeter

*The data represents the mean values of two replicates.

Table 4.12. Antibacterial activity of *R. sativus* aqueous extract (RSAE) against five bacterial strains

Conc. Mg/ml	Zones of inhibition				
	E. coli	E. aero.	B. bron.	P. piketti	M. luteus
15	-	30	24.5	29	-
12.5	-	29	20.5	25	-
10	-	25	13.5	24.5	-
7.5	-	23	-	22.5	-
5	-	20	-	9	-
3	-	10	-	-	-
2	-	-	-	-	-
1	-	-	-	-	-
Cef.	35	35	21.5	31	37
Roxi.	27.5	27.5	25	235	29
DMSO	-	-	-	-	-

E. coli *Escherichia coli* *E. aero.* *Enterobacter aerogens* *M. luetus* *Micrococcus luteus*

B. bron. *Bordetella bronchiseptica* *P. piketti* *Pseudomonas piketti* Cef. Cefixime 2mg/ml

Roxi. Roxithomycin 2mg/ml DMSO Dimethyl sulfoxide mm. Millimeter

*The data represents the mean values of two replicates.

Table 4.13. Minimum inhibitory concentration (MIC)

Extract	**Minimum inhibitory concentration mg/ml**				
	E. coli	E. areo	B. bron.	P. piketti	M. luteus
RSME	-	10	3	5	15
RSEE	-	5	7.5	3	3
RSBE	-	3	7.5	0.7	3
RSHE	-	10	10	7.5	15
RSAE	-	5	10	5	-

E. coli *Escherichia coli* *E. aero.* *Enterobacter aerogens* *M. luetus* *Micrococcus luteus*

B. bron. *Bordetella bronchiseptica* *P. piketti* *Pseudomonas piketti* mm. Millimeter

DISCUSSION

Carbon tetrachloride is a notorious and hazardous compound known to cause severe dysfunctions including hepatic (Okada *et al*., 2003), renal (Ogeturk *et al*., 2005; Khan *et al*., 2009), testicular (Khan and Ahmad, 2009), cardiac and neural (Jayakumar *et al*., 2003) toxicities both in rats and human (Ogeturk *et al*., 2005). This poisonous nature is attributed to the formation of free radicals; capable to interact directly or indirectly with the cellular components resulting in detrimental outcomes. Heart is more prone to the ROS and RNS-induced oxidative damages due to the less amounts of scavenging enzymes (Van Acker *et al*., 2000) which leads to several myocardial injuries including myocardial infarction (Zhou *et al*., 2008) and atherosclerosis (Rubbo *et al*., 1996).

Various remedies had been previously concerned including pharmaceutical products and natural sources; the latter being more effective. Plants have ample amounts of flavonoids (Zhang and Wang, 2002), anthocynins and other phenolic compounds to combat this oxidative stress (Sanchez-Moreno *et al*., 1998).

Raphanus sativus has been known to possess polyphenolic compounds (Beevi *et al*., 2010), glucosinolate (GL), isothiocyanate (ITC) (Nakamura *et al*., 2001; Barillari *et al*., 2006; Papi *et al*., 2008), inositol phosphates, alkaloids, L-ascorbic acid, saponins, reduced glutathione and vitamin B and that probably work cooperatively to contribute to its positive health effects (Nakamura *et al*., 2001; Zielinski and Koztowska 2003; Takaya *et al*., 2003; Zielinski *et al*., 2005).

The underlying hypothesis of the present study was to evaluate the medicinal properties of *Raphanus sativus* as a remedy for the oxidative imbalance brought on by carbon tetrachloride in heart tissues and also to check out the antibacterial activity illustrated by the seed extract of the plant against different bacterial strains. In past, several other chemicals such as Adriamycin (Nagla *et al*., 2008), doxorubicin (Rahimi Balaei *et al*., 2010), cyclosporine A (Bianchi *et al*., 2003; Rezzani, 2004) and isoproterenol (Rathore *et al*., 1998; Srivastava *et al*., 2007) have been mostly used to study the cardiotoxic effects. These studies would be quoted to draw a parallel between the cardiotoxicity upshots of carbon tetrachloride with the other chemical

effects as there is only near-to-none literature available to show the effects of carbon tetrachloride on heart tissues.

In this experiment, the first thing noted was the body weight alteration in the CCl_4 treated group verses the control group. There is a marked decrease in the overall body weights of the rats specified for CCl_4 injections. On the other hand, a %age increase in the heart weights (cardio megaly) is seen in this group in contrast with the group designated as control group. This increase might be due to the augmentation of tumors cells in the heart tissues. A similar set of results was reported by Tikoo *et al*. (2011) where doxorubicin intoxication caused a marked decreased in body weights while an increase in heart weights in female SD rats. The results of Noyan *et al.* (2006) are also similar to our findings, in which they found a significant decrease in the body weight of mice groups treated with CCl_4 intraperitoneally. In the present study, the normalization of heart and body weights is achieved by the RSME co-administration, suggesting its curative effect against CCl_4 poisoning.

As discussed earlier, free radicals are responsible for the pathogenesis of several diseases. The electrons leaked out into oxygen from various cellular pathways including mitochondrial electron transport chain, NADPH oxidase and xanthine oxidase to form ROS and RNS create a stressful atmosphere (Valko et al., 2006). So the body is endowed by defense systems to combat the stress condition produced by these radicals. Enzymes such as SOD, CAT, POD and GSH-Px comprise this defense structure. SODs are metal-containing enzymes that catalyze the reaction to eliminate superoxide radicals forming a comparatively less harmful hydrogen peroxide. Catalase is a heme-containing enzyme present in peroxisomes, serves to eradicate hydrogen peroxide converting it into water and oxygen (Wu and Aruthr, 2003). So these enzymes protect the cell against lipid peroxidation which is a foremost pathogenic element in cardiac ailments; as evident by an obvious amplification in lipid peroxidative products shown to trigger myocardial damage during MI (Padmanabhan and Prince, 2006; Zhou *et al*., 2008). The slowdown of these enzymes might be due to the cross-linkage or the collapse due to the increase lipid peroxidation.

CCl_4 notably elevated the TBARS level as compared with the control group, indicating the production of oxidative stress evoked by CCl_4. These results are in

agreement with the experiment conducted by El-Awady *et al*. (2011), which specified the increase in MDA levels due to the cardiotoxicity caused by cisplatin. The oxidative strain produced by CCl_4 here is counteracted by the co-treatment with RSME so that, an increase in the levels of POD, SOD and CAT and while a decline in TBARS is seen in the groups receiving *R. sativus* extract. Probably by scavenging the products of lipid peroxidation produced excessively by CCl_4 thereby protecting the cardiac tissues and the foremost target of lipid perxidation, the polyunsaturated fatty acids in the cell membranes: an evidence for antioxidant potential of the plant.

Glutathione peroxidase family enzymes are responsible to reduce the hydrogen peroxide radical into water thus converting it to a neutral form by utilizing GSH (Glutathione) as a cosubstrate (Sies et al., 1997; Prabhakar et al., 2006). This enzyme family comprises of several components; glutathione peroxidase and glutathione reductase being the most important members. GPx races with catalase for H_2O_2 as a substrate so is the chief guarding source against low levels of oxidative stress against many xenobiotics and other hazardous compounds (Wu and Aruthr, 2003). Glutathione performs the function of protecting the myocardium from getting injured in the face of free radicals and thus a reduction in cellular glutathione content could weaken the chances of recovery after short period of ischemia (Saravanan and Parkash, 2004).

Significantly lower levels of GSH-Px were found in the rats treated with CCl_4 in comparison with the control group. But a clear boost up is seen in the enzymatic level by the co-administration of RSME thus helping to avoid cell disruption by the free radicals. Our study is in accordance with the one conducted by Selcoki *et al*. (2007) showing the elevation in GSH-Px levels due to the toxicity induced by cyclosporin A which was then attenuated by erdostein co-administration.

Serum marker enzymes are frequently used for the detection and continued management of several disorders. Creatine kinase (CK), an enzyme mostly found in the myocardium is the best known marker for the detection of acute myocardial infarction (AMI). Patients with progressive muscular dystrophy, myocardial infarction or alcoholic myopathy show an elevated level of CK (Adams *et al*., 1994). It has been elucidated by Hamm and Braunwald (2000) and supported by Kasap *et al*. (2007) that CK normally stays in the cellular compartments but escapes out into the plasma due to

the collapse of contractile elements and sarcoplasmic reticulum during injuries in myocardium.

CCl_4 showed its toxic effects thus magnifying the serum CK to a considerable level as compared with the control group. *R. sativus* methanolic extract is effectual in attenuating the cardiac injuries induced by CCl_4 as indicated by the decrease in serum CK level. Similar results were put forth by Ojha *et al*. (2010) where pyruvate attenuated the CK level, intensified by isoproterenol doses.

A noteworthy increase in γ-GT levels has been observed in serum by the intraperitonial injection of CCl_4, while it was normal in the case of control group. This unwanted increase in the enzyme has been abolished by the methanolic extract of *R. sativus*. This study is in agreement with the experiment carried out by Eser and Mustafa (2005). They used doxorubicin as a cardiotoxicity causing agent.

Apart from this, several other serum marker enzymes such as ALT, ALP, AST and LDH are the indicators of cardiac dysfunctions. AST functions to catalyze the transfer of an amino group to a keto group; pyridoxal phosphate acts as a cofactor. The heart wall; myocardium contains copious concentrations of these marker enzymes and once metabolically injured, it releases them into the extracellular fluid (Upaganlawar, 2009). The amount of these enzymes released from the damaged heart tissues into blood marks the onset of cell necrosis indicating the damage in myocardial cell membrane due to disturbances in oxygen or glucose supply. So the damages in cell membranes of cardiomyocytes would elevate the serum levels of AST and ALT (Karthikeyan *et al*., 2003).

The present experiment showed an upgrading in serum ALT, ALP and AST enzyme levels due to CCl_4 intoxication in rats which suggests that there might have occurred disruptions in the myocardium by CCl_4 resulting in the appearance of these enzymes in serum at higher levels. These serum markers had earlier been reported to increase in serum of Wister rats due to the cardiotoxicity caused by isoproterenol. The experiment was conducted to evaluate the antioxidant properties of curcumin; an active component of *Curcuma longa* (turmeric) (Naik *et al*., 2011). The co-treatment with *R. sativus* methanolic extract reduced the level of these enzymes in serum. This improvement is clearly visible in the histoarchitechture of the rats co-treated with *R. sativus* methnolic extract presumably due to the antioxidant capacity of the plant

which helped maintaining the membrane integrity of cardiomyocytes and in turn prevented the leakage of these enzymes into serum.

Increased levels of LDL and cholesterol in blood serum leads to accumulation of undesired deposits in the arteries; thus supporting the onset of coronary heart diseases (Sushamakumari *et al*., 1990) including myocardial infarction. Cholesterol which exists in the human blood in two forms, free sterol form and esterified form, when reaches a higher level than normal, causes a condition called hypercholesterimia. This condition is explained by a boost in serum LDL level due to decline in HDL level. HDL is vital in transporting the cholesterol from tissues to liver for breakdown (Mathew *et al*., 1981). So the higher cholesterol level is an indicator of lower HDL levels.

The end product of protein breakdown is urea, the abnormal accumulation of which sets a condition called Azotaemia. It is caused by nephritic dysfunction due to a decline in cardiac output, increased protein breakdown, dehydration and gastrointestinal ulcerations (Young, 2000).

An increase in serum urea and creatinine is observed in the CCl_4 treated group, clearly picturing the cardiotoxicity resulting in myocardial damages. On the other hand, the *R. sativus* methanolic extract co-treatment resulted in significant lowering of these in serum, which manifests the protective nature of the plant against xenobiotics lethal after-effects. Nagla *et al*. (2008) stated the similar results while studying the protective effects of silymarin against adriamycin-induced cardiotoxicity.

The poisonous effects of CCl_4 on the heart muscles are patently visible in the histological studies. The damages caused to the cardiomyocytes can be elucidated as deformed blood capillaries, invasions in heart trabeculae, vacuolar changes and abrasions in heart muscles. Mucoid edema and necrotic condition is also seen in this group. This histopathological condition is consistent with the findings of Selcoki *et al*. (2007) in which they reported isoproterenol to cause severe cardiac tissue damages. Restructuring of the tissues is seen in the case of co-treatment with *R. sativus* methanolic extract.

The fact that bacteria are growing more and more resistant to the antibiotics has set an alarming situation for the scientists and has compelled them to avert

towards isolation of compounds from more natural sources (WHO, 2002a). Growing interest in medicinal plants is a sign of recognition of the legitimacy of many customary claims regarding the value of natural products in health care (Nair *et al*., 2005).

In the present study different fractions of *R. sativus* were checked to decipher their bacteriocidal activity against five bacterial strains, only one of which was gram positive (*Micrococcus luteus*); the rest being Gram negative, including *Escherichia coli*, *Bordetella bronchioseptica*, *Pseudomonas piketti*, *Pseudomonas piketti* and *Micrococcus luteus*.

The methanloic extract (RSME) was effectual against *B. bronchioseptica* and *P. piketti*. Ethyl acetate extract (RSEE) showed activity against all tested strains; the noteworthy activity was shown against *P. piketti*. The butanolic extract (RSBE) was highly active against *P. piketti*, while a good activity was observed against *E. aerogens* and *M. luteus* and only low activity against *B. bronchioseptica.* The n-Hexane extract (RSHE) was only mildly effective against *E. aerogens, P. piketti* and *B. bronchioseptica* while an unclear activity was seen against *M. luteus*. The activity shown by aqueous extract (RSAE) was good against *E. aerogens* and *P. piketti*.

Solvents with different polar natures were used so that a wide range of compounds from the seed extract could be solublized in them to show maximum diverse activities. It is seen that ethyl acetate being the most polar might have had all the active compounds in the form of RSEE; the reason why it showed activity against all the strains. Moreover, butanol with an intermediate polarity showed a commendable activity as RSBE against some strains which can be explained by the presence of antibacterial compounds extracted by butanol from crude extract. The other fractions with intermediate activity refer to their poor solvent nature for active compounds. Our results match with the findings of Beevi *et al*. (2009) showing ethyl acetate extract of *R. sativus* root, stem and leaf to be most effective of all other extracts against wide range of bacterial strains.

Another aspect is the classification of strains as Gram positive and Gram negative. In this study, only a single Gram positive while four Gram negative strains were used. It is a common observation that Gram negative bacteria are much resistant as compared with the Gram positive strains (Tomas-Barberan *et al*., 1988). This

resistance is attributed to the presence of an additional phospholipopolysaccharide covering over the cell wall attributed only to them (Yao and Moellering, 1995). But interestingly, all the fractions here showed activity against the Gram positive and all the Gram negative bacteria except for *E. coli* which was resistant against all fractions. This dual bacteriocidal activity might be due to the presence of active isothyocinates (Nakamura *et al*., 2001) in *R. sativus* having the ability to disrupt the bacterial membranes thus inactivating them. This antibacterial action has been justified by Narod *et al*. (2004) who found the leaf and stem extracts of *Toddalia asiatica* to be active against both Gram positive and Gram negative bacteria.

The MIC values were noted for each fraction that ranged between 15mg/ml and 7.5mg/ml. The higher MIC values point out towards the scanty concentrations of active antibacterial compounds while the low MIC values indicate the good solvent nature of the organic compounds used. The suppression of active compounds by inter linkage or the presence of non-active compounds like saponins and terpenoids may also add to the higher MIC values of these fractions (Waage and Hedin, 1985).

Conclusion

It can be substantiated from this study, keeping in view the results that CCl_4 is a potent cardiotoxic chemical as confirmed both by histological and biochemical studies. The elevated levels of TBARS and cholesterol while reduced levels of HDL, SOD, CAT and other antioxidant enzymes in heart tissue indicate the cardiac injuries provoked by CCl_4. These adverse effects have been ameliorated to a satisfactory level by the methanolic extract of *Raphanus sativus* showing free radical quenching activity when coadministered with CCl_4; in addition to the antibacterial activity due to the active compounds to thwart the bacterial attack.

Owing to the growing need of alternative medicines, *Raphanus sativus* can be a first-rate addition to the antioxidant and antibacterial agents.

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