

**BIO-CHEMICAL STUDIES ON THE
HEPATOPROTECTIVE EFFECT OF ECLIPTA ALBA
AND PIPER LONGUM**

Thesis Submitted To
**THE TAMILNADU DR. M.G.R. MEDICAL UNIVERSITY
GUINDY, CHENNAI**

As a partial fulfillment of the requirement for the award of the degree of
DOCTOR OF PHILOSOPHY

Submitted by
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Under the supervision of
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ACKNOWLEDGEMENT

My Ph.D. thesis is in the process of being bound. While I have not yet faced the examiners, just completing such an ambitious project is a joy unto itself.

“The joy, satisfaction and euphoria that comes along with the successful completion of any work would be incomplete unless we mention the names of the people who made it possible, whose constant guidance and encouragement served as a beam of light and crowned our efforts.”

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DECLARATION

I hereby declare that the thesis, entitled “**BIO-CHEMICAL STUDIES ON THE HEPATOPROTECTIVE EFFECT OF ECLIPTA ALBA AND PIPER LONGUM**” is submitted to The Tamilnadu Dr. M.G.R. Medical University in partial fulfillment of the requirements for the award of degree of doctor of philosophy is the record of original research work done by me under the supervision and guidance of **Prof. Dr. P. Samudram**, Professor and Head, Department of Biochemistry, Tagore Dental College & Hospital (Formerly Institute of Biochemistry Madras Medical college) and the thesis has not formed the basis for the award of any Degree, Diploma, Associateship, Fellowship or other similar title.

Mrs. R. VASUKI

Date:

CERTIFICATE

This is to certify that the thesis, entitled **“BIO-CHEMICAL STUDIES ON THE HEPATOPROTECTIVE EFFECT OF ECLIPTA ALBA AND PIPER LONGUM”** is submitted to The Tamilnadu Dr. M.G.R. Medical University in partial fulfillment of the requirements for the award of degree of doctor of philosophy is the record of original research work done by **Mrs. R. VASUKI**, under my supervision and guidance and the thesis has not formed the basis for the award of any Degree, Diploma, Associateship, Fellowship or other similar title.

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Co-guide,
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Chennai

Date:

ABBREVIATIONS

ACE	-	Acetyl choline esterase
ACP	-	Acid phosphatase
ATP	-	Adenosine triphosphate
ALP	-	Alkaline phosphatase
ALT	-	Alanine amino transferase
ANSA	-	Aminonaphthol salicylic acid
AST	-	Alanine amino transferase
α	-	Alpha
β	-	Beta
BHT	-	Butylated hydroxyl toluene
CAT	-	Catalase
CCl ₄	-	Carbon tetra chloride
CD	-	Conjugated diene
CDNB	-	1-chloro, 2,4 dinitrobenzene
CHO	-	Cholesterol
dl	-	Decilitre
DNA	-	Deoxy ribonucleic acid
°C	-	Degree celsius
DPPH	-	2,4 dinitro Diphenyl hydrazine
DTNB	-	5,5' -Di thiobis (2nitro benzene)
EDTA	-	Ethylene diamine tetraacetic acid
g	-	Gram
GP _x	-	Glutathione peroxidase
GR	-	Glutathione reductase
GSH	-	Reduced glutathione
GSSG	-	Oxidized glutathine
GST	-	Glutathione -s- transferase
HPLC	-	High performance liquid chromatography

hr	-	Hour
ICD	-	Iso citrate dehydrogenase
KG	-	Kilogram
L	-	Litre
LD ₅₀	-	Lethal dose to kill 50% of animal
LDH	-	low density lipoprotein
LDL	-	Low- density lipoprotein
LOOH	-	Lipid hydro peroxide
LPO	-	Lipid peroxidation
M	-	Molarity
MDA	-	Malondialdehyde
μ	-	Microgram
μg	-	Microgram
μl	-	Microlitre
mg	-	Milligram
min	-	Minute
ml	-	Milliliter
Mm	-	Millimole
N	-	Normality
NADP	-	Nicotineamide adenine dinucleotide phosphate
nm	-	nano moles
No	-	Nitric oxide
NOS	-	Nitric oxide species
OECD	-	Organization of Economic Co-operation and Development
p.o	-	per oral
K+	-	Potassium
ROS	-	Reactive oxygen species
SDS	-	Sodium Dodecyl sulphate
Se	-	Selenium
SEM	-	Standard Error Mean

SOD	-	Superoxide dismutase
TBA	-	Thiobarbituric acid
TBARS	-	Thiobarbituric acid reactive substances
TCA	-	Trichloro acetic acid
TG	-	Triglyceride
V/V	-	Volume / Volume
VLDL	-	Very low density lipoprotein
W/V	-	Weight / Volume
W/W	-	Weight / Weight

INTRODUCTION

The liver is the second largest and one of the most essential organs which is located within the lower part of the rib-cage on the right hand side of the body. It has a huge variety of functions in the body and is the only organ that can repair and re-grow itself after damage. It is a seat of metabolism which is responsible for performing many functions in the body. Bile juice produced by liver gets secreted in the intestines which is required for the fat digestion body. It also works as a guard by keeping check on the cholesterol levels in the blood stream. It also produces certain proteins that are very much required for performing wear and tear function in the body. It also works as an energy store of the body thus providing energy when required in emergency conditions

ANATOMY AND PHYSIOLOGY OF LIVER

The liver is a fairly large organ that consistently lies in the right upper part of the abdomen. From front it has a roughly triangular shape. It stretches from the right upper abdomen across the mid line to halfway across the left upper abdomen (left lobe) and from the right upper abdomen to halfway down the flank on the right (right lobe). Above the liver sits the right diaphragm and the right lung, and below the liver is the intestines. The gall bladder nestles directly below the liver and is attached to it. The gall bladder stores bile from the liver to be squirted into the first part of the small bowel (duodenum) when fat enters the intestine, to help emulsify the fat for absorption.

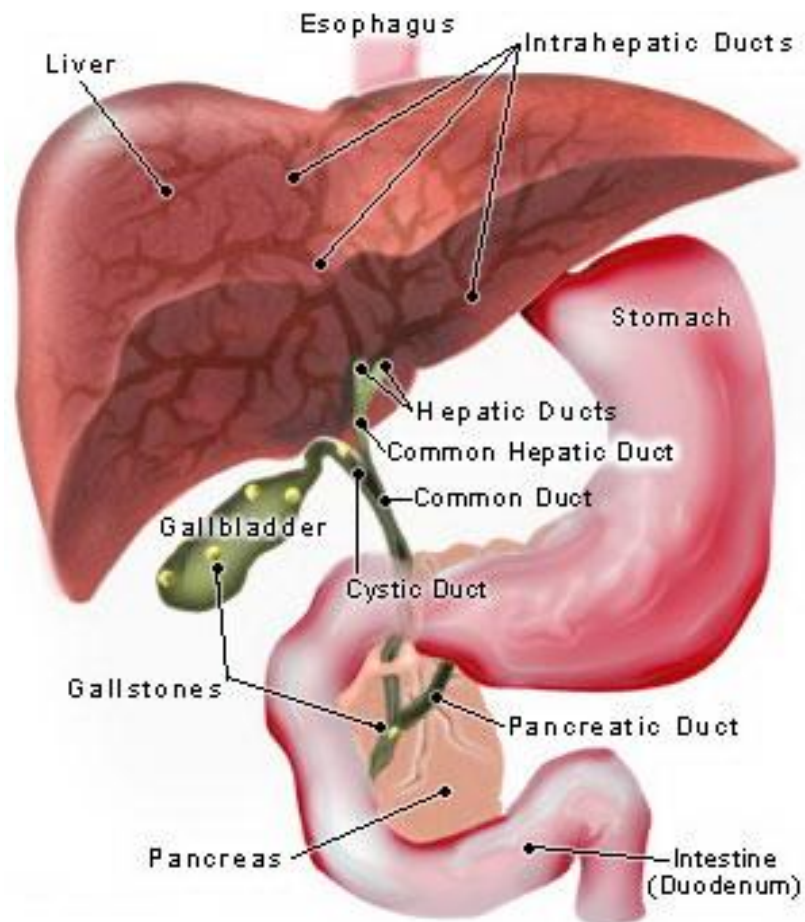


Figure 1. Liver

Blood Flow

A large portion of the output of the heart (about one-third) flows to the digestive system. This blood returns, rich with nutrients and digestive impurities, to the liver through the veins of the portal circulatory system. The liver vascular system is expandable and compressible, and can store large quantities of blood. It normally contains about one-tenth of the blood volume in the body, but can, under certain diseased conditions, expand to hold up to a fourth of the body's blood. Under conditions of stress, on the other hand, its

veins can contract so it holds only a thirtieth of the body's' blood. Thus the liver blood volume can potentially vary by a factor of more than seven. About three-fourths of the blood coming into the liver comes from venous circulation; only about a fourth comes directly from the heart through the arteries. Arterial blood is moved under force, being pumped by the heart, normally under pressure ten to fifteen times that of the blood in the portal veins. Thus most of the blood in the liver is not moved through it by arterial pressure and has a natural tendency to stagnate.

The liver is also extremely permeable to fluids, such that they flow freely into its lymphatic system; the lymph derived from the liver accounts for from one-half to two-thirds of the total lymph in the body. Finally, the liver secretes bile into the small intestine, after storage in the gall bladder. Bile helps to digest fat and is a key player in the symphonic action of the enzymes and hormones that coordinate digestion. Thus the liver is an expandable and contractible reservoir of blood, lymph, and bile. Because it has only weak arterial circulation, these fluids have a tendency to become stagnant and the liver to become congested.

FUNCTIONS OF LIVER

The liver has multiple functions. The liver processes all the blood leaving the stomach and intestines. It breaks down the nutrients and drugs in the blood into forms that are easier for the rest of the body to use or excrete. Amongst other important functions, the liver is responsible for eliminating and detoxifying the poisons that enter our blood stream.

The liver is also very important in the digestion of food and produces bile which is essential in the breakdown of fats, thereby preventing obesity - one of the biggest contributors to bad health. It also regulates blood sugar and stores any excess sugar in a useful 'quick-release' form for when it is needed. The liver clears the blood of old red blood cells, bacteria and other infectious organisms as well as ingested toxins including alcohol. It is responsible for producing a large number of different proteins including hormones and blood clotting factors and is the organ which stores Vitamin A,D, E and K.

The functioning of liver does not really decrease with age so, in the absence of disease, the liver should work optimally right into very old age - helping to keep all body systems functioning normally and supporting overall vitality and protection from illness. The maintenance of optimal liver functioning is therefore of vital importance in the quest for holistic health. In traditional Chinese medicine, for example, a healthy liver is seen as the most critical element in the body's ability to fight disease and function optimally.

LIVER DISEASES

Environmental pollution, fast foods, drugs, alcohol and sedentary lifestyles all contribute to sluggish and diseased livers. The result of a diseased liver are depressed immune systems, constant fatigue, obesity, sluggish digestive systems, allergies, respiratory ailments, and unhealthy skin among many other health problems. Alcohol and many pharmaceutical drugs can affect the metabolism of the liver, and if this continues for long periods of

time, health will be endangered. A common sign of a damaged liver is jaundice, a yellowness of the eyes and skin.

There are many diseases that may affect the liver and they include:

- Liver cancer
- Fatty liver
- Wilson's disease
- Hepatitis
- Hemochromatosis
- Cirrhosis

Like other parts of our body, cancer can affect the liver. Cancer of the liver (primary hepatocellular carcinoma or cholangiocarcinoma and metastatic cancers, usually from other parts of the gastrointestinal tract.

Fatty liver is termed if lipids account for more than 5 percent of its weight. The mechanisms for the development of fatty liver are varied. A reduction in the hepatic oxidation of fatty acids as a result of mitochondrial dysfunction can lead to micro vesicular steatosis. Another mechanism is related to an imbalance between fat uptake and secretion, with high insulin-to-glucagon ratio status leading to macro vesicular steatosis.

Wilson's disease, a hereditary disease which causes the body to retain copper.

Hepatitis, inflammation of the liver, caused mainly by various viruses like hepatitis A, hepatitis B and hepatitis C but also by some poisons, autoimmunity or hereditary conditions.

Hepatitis A or infectious jaundice is caused by a picornavirus transmitted by the fecal-oral route. It causes an acute form of hepatitis and does not have a chronic stage. Worldwide, hepatitis B is another major cause of cirrhosis and hepatocellular carcinoma. Many patients with hepatitis B virus infection fail standard therapy. Hepatitis C virus infection is the leading cause of chronic liver disease and the reason for 30 to 35 percent of liver transplantations (Knolle *et al.*, 1998; Zein *et al.*, 1996).

Haemochromatosis, a hereditary disease causing the accumulation of iron in the body, eventually leading to liver damage.

Cirrhosis is the formation of fibrous tissue in the liver, replacing dead liver cells. The death of the liver cells can for example be caused by viral hepatitis, alcoholism or contact with other liver-toxic chemicals.

In some liver diseases, such as primary biliary cirrhosis, treatment can slow but not stop the progression of liver injury (Poupon *et al.*, 1991). Although each form of liver disease has a distinct natural history, most forms progress slowly from hepatitis to cirrhosis, often over 20 to 40 years (Propst *et al.*, 1995).

Symptoms of a diseased liver

The external signs include a coated tongue, bad breath, skin rashes, itchy skin, excessive sweating, offensive body odour, dark circles under the eyes, red swollen and itchy eyes, acne rosacea, brownish spots and blemishes on the skin, flushed facial appearance or excessive facial blood vessels. Other symptoms include jaundice, dark urine, pale stool, bone loss, easy bleeding, itching, small, spider-like blood vessels visible in the skin, enlarged spleen, and fluid in the abdominal cavity, chills, pain from the biliary tract or pancreas, and an enlarged gallbladder. The symptoms related to liver dysfunction include both physical signs and a variety of symptoms related to digestive problems, blood sugar problems, immune disorders, abnormal absorption of fats, and metabolism problems.

EVALUATION OF DRUG TOXICITY

Most ingested substances are metabolized and chemically altered as they pass through the liver. The liver is vulnerable to injury from some medications, vitamins and herbal remedies (Speeg and Bay, 1995). Prescription and over-the-counter arthritis and pain medications are widely used. Nonsteroidal anti-inflammatory drugs (NSAIDs), which are taken to alleviate headache and a variety of pain symptoms, can cause idiosyncratic liver toxicity. Fatalities associated with NSAID use have been reported (Carson and Willett, 1993). In one study, the use of ibuprofen was associated with a more than 20-fold increase in liver function values in three patients with hepatitis C virus infection (Riley and Smith, 1998). Acetaminophen has

predictable hepatotoxicity and affects the liver in a dose dependent manner. However, acetaminophen hepatotoxicity has been reported with dosages of less than 4 g per day, usually in association with starvation or alcohol ingestion (Zimmerman and Maddrey, 1995).

HERBAL MEDICINES

Herbal drugs have become increasingly popular and their use is widespread.

Ayurveda is accepted to be the oldest treatise on medical system, which came into existence in about 900 BC. The word Ayurveda derived from 'Ayur' meaning life and 'veda' meaning science.

Herbal medicines are prepared from a variety of plant materials- leaves, stems, roots, bark and so on. They usually contain many biologically active ingredients and are used primarily for treating mild or chronic ailments. Naturopathic medicine, traditional Chinese medicine and Ayurvedic medicine all differ in how diseases are diagnosed and which herbal remedies are prescribed. Out of these, the Chinese herbal medicine or the Traditional Chinese Medicine (TCM) has a potential usage similar to the Indian system of medicine.

'Ethnopharmacology' have been recently defined as "the interdisciplinary scientific exploration of biologically active agents traditionally employed or observed by man". The objectives of ethnopharmacology are to rescue and document an important cultural heritage

before it is lost, and to investigate and evaluate the agents employed. Thus, it plays an immense role in evaluation of natural products and more particularly the herbal drugs from traditional and folklore resources. The random screening of plants for food and medicine by our prehistoric ancestors is probably the basis of the botanical pharmacopoeia that exists in virtually all cultures. What of the future for plant based agents? There are many possibilities for research, but priority should be given to tropical infectious and chronic diseases for which current mediations have severe drawbacks, and to the scientific appraisal of plant-based remedies that might be safer, cheaper, and less toxic for self- medication than existing prescription medicines. Man and perhaps some of his closer relatives, has always made use of plants to treat illness, and many of these remedies have real beneficial effects. Licensing regulations and pharmacovigilance regarding herbal products are still incomplete and clear cut proof of their efficacy in liver diseases is sparse.

There is clearly a need for greater education of patients and doctors about herbal therapy, for legislation to control the quality of herbal preparations, and in particular for further randomized controlled trials to establish the value and safety of such preparations in Hepatic disorders.

EXPERIMENTAL HEPATOTOXICITY IN RATS

Carbon tetrachloride- induced hepatotoxicity

Animal studies have revealed that carbon tetra chloride is metabolized in the liver by cytochrome P-450 (Sipes *et al.*, 1977). One of the resulting

products of the metabolic activity is believed to be a trichloromethyl radical that leads to the formation of chloroform, hexachloroethane, carbon monoxide, trichloromethanol, phosgene and carbon dioxide. The radical is thought to induce lipid peroxidation resulting in membrane destruction and the loss of organelle and cell function (Rao and Recknagel, 1968).

Free radicals and hepatotoxicity

The cytochrome p-450 system is encased in phospholipids membrane rich in polyenoic fatty acid. Hence these polyenoic fatty acids are the most likely immediate target for the initial lipid peroxidative attack to occur. The organic fatty acid radical rearranges, yielding organic peroxy and hydroxyl peroxy radicals. The radical destroy the cytochrome p-450 hemoprotein, thus compromising the mixed - function oxygenase activity. The rapid decomposition of the endoplasmic reticulum and its function is a direct result of this lipid peroxidative process (Zangar *et al.*, 2000).

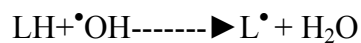
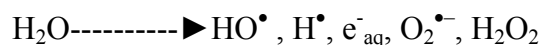
Trichloromethyl free radicals can react with sulfhydryl groups, such as glutathione (GSH) and protein thiols, and the covalent binding of the trichloromethyl free radicals to the cell proteins is considered to be the initial step in a chain of events that eventually lead to membrane lipid peroxidation and finally to cell necrosis (Recknagel *et al.*, 1991). Several mechanisms have been proposed for CCl₄ induced fatty liver and necrosis. Important mechanisms include damage to endoplasmic reticulum, mitochondria lysosomes, disturbances in hepatocellular calcium homeostasis, and lipid peroxidation. All are mediated by free radicals. Lipid peroxidation may be

looked upon as occurring in two steps. Some toxic event initiates lipid peroxidation and organic free radical generated by the initiation process serve to propagate the reaction.

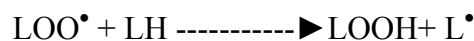
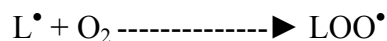
MECHANISM OF LIPID PEROXIDATION

The steps involved in lipid peroxidation are described below and shown schematically (Anjali *et al.*, 2001).

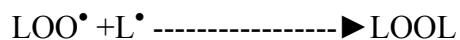
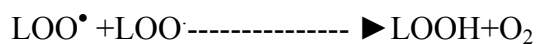
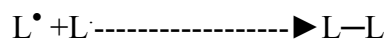
Initiation



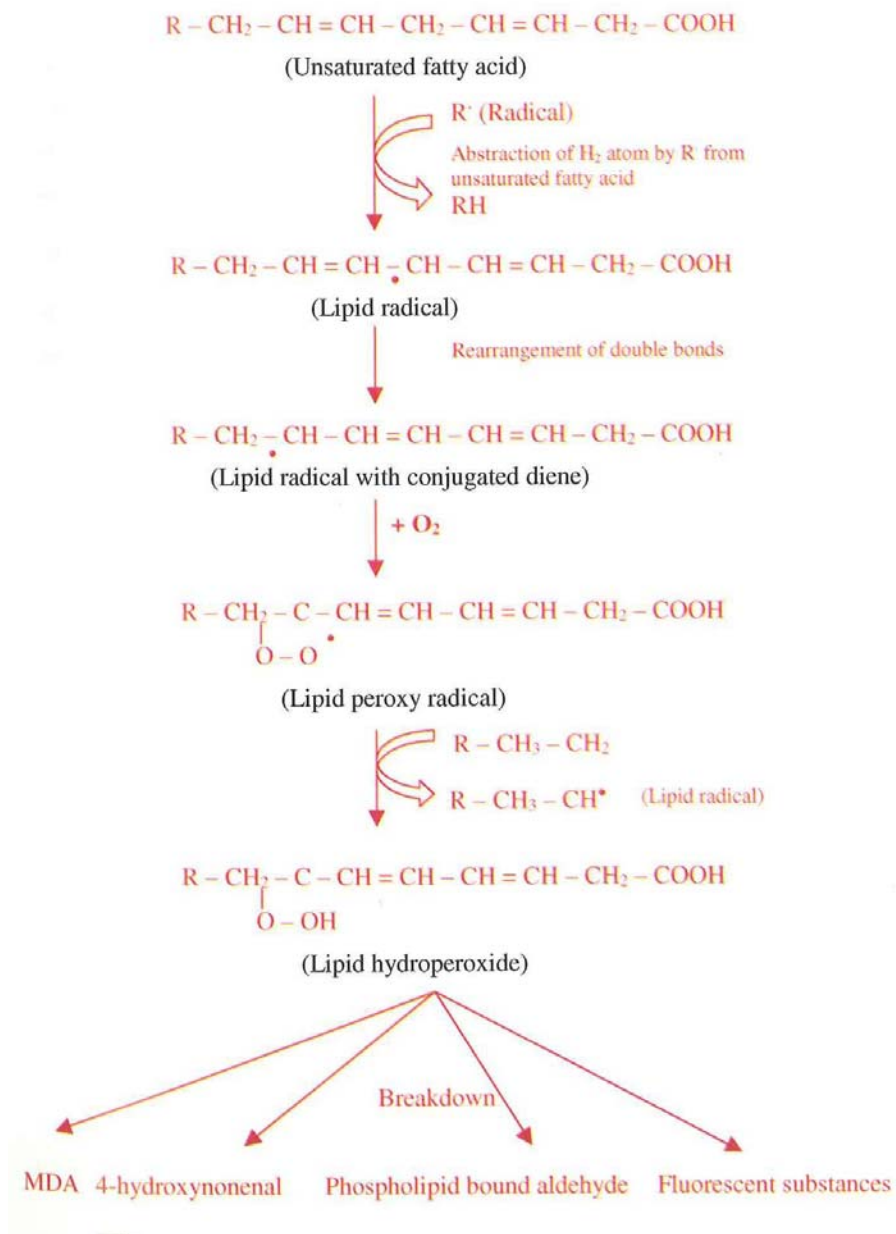
Propagation



Termination



Malondialdehyde is the major reactive aldehyde resulting from the peroxidation of biological membrane polyunsaturated fatty acid (PUFA). Thus MDA is used as an indicator of tissue damage and reacts with thiobarbituric acid and produce red colored products.



Mechanism of lipid peroxidation

Hepatotoxicity following acute exposure to CCl_4 is manifested as necrosis and inflammation mainly in the centrilobular areas of the rodent liver (Germano *et al.*, 2001).

The covalent binding of the radical to cell components initiates the inhibition of lipoprotein secretion and thus steatosis, whereas reaction with oxygen, to form $\cdot\text{CCl}_3$ initiates lipid peroxidation. The latter process results in loss of calcium homeostasis and, ultimately, apoptosis and cell death. The massive production of reactive species may lead to depletion of protective physiological moieties (glutathione and α -tocopherol, etc.), ensuing widespread propagation of the alkylation as well as peroxidation, causing damage to the macromolecules in vital biomembranes (Aldridge, 1981). The reactive species mediated hepatotoxicity can be effectively managed upon administration of such agents possessing anti-oxidants (Attri *et al.*, 2000), free radical scavengers (Sadanobu *et al.*, 1999) and anti-lipid peroxidation (Lim *et al.*, 2000).

Based on a pharmacokinetic model developed by (Paustenbach *et al.*, 1988) about 4% of the carbon tetrachloride that is metabolized is converted to and excreted as carbon dioxide. The remaining metabolic products may bind to proteins, lipids and DNA. The liver and kidney are target organs for carbon tetrachloride toxicity. The severity of the effects on the liver depends on a number of factors such as species susceptibility, route and mode of exposure, diet or co-exposure to other compounds, in particular ethanol. Furthermore, it appears that pretreatment with various compounds, such as phenobarbital and vitamin A, enhances hepatotoxicity, while other compounds, such as vitamin E, reduce the hepatotoxic action of carbon tetrachloride.

REFERENCE DRUG

Silimarin

Silimarin is widely used as a standard hepatoprotective drug in many liver disorders. The main effects of silymarin are the membrane stabilising and antioxidant effects, it is able to help the liver cell regeneration, it can decrease the inflammatory reaction, inhibit the fibro genesis in the liver and the long administration of silymarin significantly increased the survival time of patients with alcohol-induced liver cirrhosis (Fehér and Lengyel, 2008;) Müzes *et al.*, 1990) reported the antioxidant, antiperoxidative effects might be important factors in the mechanism of hepatoprotective action of silymarin.

Silymarin prevents to a considerable degree the increase of the serum enzymes (GOT, GPT, MDH, SDH, ICDH, AP) activity caused by a D-galactosamine injury, enhances the metabolic conversion of the UDP-hexosamine into UDP-acetylhexosamine in the liver and hastens the normalizing of the UDP-glucuronic acid content in the liver of rats (Tyutyulkova *et al.*, 1981). Silymarin corrected the altered immunoreactions and the decreased superoxide-dismutase (SOD) activity of erythrocytes and lymphocytes in patients with alcoholic liver cirrhoses. The scavenger effect of silymarin was demonstrated in the sub cellular fractions of liver cells in animal experiments (Feher *et al.*, 1989).

ECLIPTA ALBA

Eclipta alba Hassk. (Bhringaraja and Fam: Compositae) is a perennial shrub, has a short, flat or round stem, deep brown in color which grows

widely in moist tropical countries. Different uses have been reported for this shrub. It is used as alterative, anthelmintic, expectorant, antipyretic, antiasthmatic, tonic, deobstruent in hepatic and spleen enlargement, in skin diseases and as a substitute for *Taraxacum* (a popular liver tonic). It is good for the diseases of spleen, stomatitis, toothache, hemicrania, fever, pain in liver and cures vertigo (Yunani). Its juice in combination with honey is administered for Catarrh and Jaundice (Chopra *et al.*, 1996). *Eclipta alba* is an indigenous medicinal plant, has a folk (Siddha and Ayurvedha) reputation popularly used for the inflammation, anthelmintic, astringent, deobstruent and hepatoprotective effect (Bhattachary *et al.*, 1997).

Synonyms

- *Eclipta erecta*
- *Eclipta prostrata*
- *Verbesina alba*
- *Verbesina prostrata*



Figure 2. *Eclipta alba*

Kingdom	:	Plantae
Division	:	Magnoliophyta
Class	:	Magnoliopsida
Order	:	Asterales
Family	:	Asteraceae
Genus	:	Eclipta
Species	:	<i>Eclipta alba</i>

Vernacular names

Latin	:	<i>Eclipta alba</i>
Sanskrit	:	Bhringaraja
English	:	Traling Eclipta

Hindi	:	Balari,Bhangra,Bhringraj,Bhengra,Mochkand.
Gujarati	:	Bhangro,Dadhal,Kalobhangro
Canarase	:	Ajagara,Garagadasoppu,Kadigga-garaga
Marathi	:	Bhangra, Maka

Pharmacognostic characteristics

Trailing *Eclipta* is a small, erect or prostrate many branched and a coarsely haired annual herb. It grows upto 20cm in height. It has a round, feeble stem, simple ecliptis hairy leaves, variable in shape, bright green in color with very small flowers, called florets. The leaf epidermis is composed of single layer of parenchymal cells with characteristic non-glandular trichomes, on both surfaces. In transaction, the stem is circular in out line with a ring of collateral endarch vascular bundles of varying sizes and central parenchymatous pith. The root has a diarch structure with normal and secondary growth.

The plant grows all over India, especially, in moist places, upto an elevation of about 800 meters. An erect annual grows 10-15 cm in height, with flat or round, blackish-chocolaty, much branched, pubescent stems. The leaves are opposite, serrate, 3-5 cm long and blackish-green in color. The flowers are small penny-sized, white, on a long stalk. The fruits are many seeded and the seeds are black, resemble cumin seeds. The plant flowers in September and fruits in November. Ayurvedic texts describe three varieties of bhringaraja according to colors of flowers viz. white, yellow and blue. The white variety is commonly used.

Phyto Chemical constituents of *Eclipta alba*

It contains a large amount of resin and an alkaloid principle ecliptine. The presence of reducing sugar and steroids in the seeds has been observed. A number of compounds had been isolated from the plant. Wedelolactone, chemically described as 7-methoxy-5,11,12-trihydroxy-coumestan (Zhang and Guo, 2001) is basically a furanocoumarin, previously reported as responsible for the hepatoprotective activity. Literature survey revealed that HPLC and UV spectrophotometry (Das *et al.*, 1990) methods had been reported for the estimation of wedelolactone in a methanol extract of *Eclipta alba*. Wedelolactone consist of heterocyclic fused ring, which is responsible for fluorescent behavior. Wedelolactone possesses a wide range of biological activities and is used for the treatment of hepatitis and cirrhosis (Wagner *et al.*, 1986).

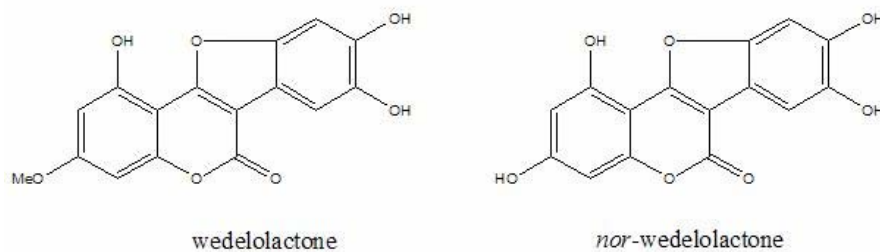


Figure 3. Major chemical constituents of *Eclipta alba*

Medicinal properties and uses

Juice of the leaves is a hepato tonic and deobstruent. Root is a tonic. The herb is used in hepatic and spleen enlargements and in skin diseases. Fresh juice obtained from leaves is given in fever, liver disorders and rheumatism. A paste of the herb mixed with sesame oil is used over glandular swellings, elephantiasis and skin diseases. In Gujarat district and Punjab, it is used externally for ulcers and as an antiseptic for wounds in cattle. Recently Chandra *et al.* (1987) have observed a significant anti-inflammatory activity of the powder in rats. It has been reported to be useful in liver ailments (Handa *et al.*, 1986) and has been shown to possess hepatoprotective activity against carbon-tetrachloride induced liver cell damage in animals. The plant is an active ingredient of many herbal formulations prescribed for liver ailments and shows effect on liver cell generation. There are also reports of clinical improvement in the treatment of infective hepatitis (Dixit and Achar, 1979). *Eclipta alba* leaves showed antihyperglycemic activity (Ananthi *et al.*, 2003). The roots of *Eclipta alba* were found effective in wound healing (Patil *et al.*, 2004). *In vivo* hepatoprotective activity of alcoholic extract (Saxena *et al.*, 1993; Singh *et al.*, 2001) and analgesic study of total alkaloids of *Eclipta alba* were also reported (Sawant *et al.*, 2004).

Ayurvedic Properties

Bhringaraja is bitter in taste, pungent in the post digestive effect and has hot potency (virya). It alleviates kapha and vata dosas. It possesses light (laghu) and dry (roksa) attributes. It is a rejuvenator, tonic and beneficial to eyes, hair and the skin (Kaiyadeva Nighantu)

Piper longum

Piper species are widely distributed in the tropical and subtropical regions of the world and have multiple applications in different folk medicines. In traditional Chinese medicine, many species are used to treat inflammatory diseases. The Chinese Pharmacopoeia contains three monographs: *Piper nigrum* (“Hujiao”), *Piper longum* (“Bibo”) and *Piper kadsura* (“Haifengteng”). In addition, several other *Piper* species are commonly found in China. *Piper longum* fruits are ovoid, yellowish orange, minute, and drupe and are sunk in the fleshy spike. The spikes are red when ripe. Odour is aromatic and the taste is pungent.

Botanical Name	: <i>Piper longum</i>
Common Name	: Long Pepper, Pipli
Part Used	: Fruit, Root, Stem
Habitat	: Most deciduas to evergreen forests
Product offered	: Seeds, Roots, Fruit, Stem
Kingdom	: Plantae
Division	: Magnoliophyta
Class	: Magnoliopsida
Order	: Piperales
Family	: Piperaceae
Genus	: Piper
Species	: <i>P. longum</i>



Figure 4. *Piper longum*

Vernacular names

Hindi , Punjabi	:	Pipul, Pipli, Piplamul
Bengali	:	Pipli, Piplamore (root)
Gujarati	:	Pipli
Kannada	:	Hippali, Hippalibali, Kuna
Malayalam	:	Tippali, Pippali
Marathi	:	Pimpli
Oriya	:	Pippoli
Sanskrit	:	Pippali
Sindhi	:	Pippli
Tamil	:	Thippali
Telugu	:	Tippili, Pippallu
Urdu	:	Pipul, Pipli.

Pharmacognostic characteristics

Pepper long is the dried fruit of *Piper longum*, which is a slender, aromatic plant with creeping jointed stems and perennial woody roots. Leaves numerous, lower one broadly ovate, very cordate with broad rounded lobes at base, upper one oblong–oval, cordate at the base, all sub acute, entire glabrous thin, bullate with reticulate venation.

Phytochemical constituents

The fruits contain 1% volatile oil, resin, alkaloids piperine and piperlonguminine, isobutyldeca-trans-2-trans-4-dienamide and a terpenoid substance. Roots contain piperine, piperiongumine. Dihydrostigmasterol has been isolated. It contains aromatic oil an alkaloid and pipalartine. Besides this it contains sesamin and pipasterol. The root contains pipperin, pippalartin, pipperleguminin, sterols and glycosides.

Medicinal properties and uses

Aromatic, stimulant, carminative, good for constipation, for gonorrhoea, paralysis of the tongue, advised in diarrhoea, cholera, scarlatina, Chronic Malaria and Viral hepatitis. *Piper Longum* is most commonly used to treat respiratory infections such as stomach ache, bronchitis, diseases of the spleen, cough, tumors, and asthma. When applied topically, it soothes and relieves muscular pains and inflammation. In Ayurvedic medicine, it is said to be a good rejuvenator. *Piper longum* helps to stimulate the appetite and it dispels gas from the intestines. An infusion of *Piper longum* root is used after birth to

induce the expulsion of the placenta. It is used as sedative in insomnia and epilepsy. Also as cholagogue in obstruction of bile duct and gall bladder.

Activity-guided fractionation of a methylene chloride soluble extract led to the isolation of three known piperine-related compounds, methylpiperate (1), guineensine (2), and piperlonguminine (3) of these, methylpiperate (1) and guineensine (2) showed significant MAO inhibitory activities (Lee *et al.*, 2008).

Examination on the effects of several extracts of *Piper longum* L. on rabbit platelet function showed Thromboxane A(2) receptor agonist U46619 caused rabbit platelet aggregation, which was potently inhibited by the ethanol or butanol extract of *Piper longum* L. These results suggest that *Piper longum* L. contains a constituent(s) that inhibits platelet aggregation as a non-competitive thromboxane A(2) receptor antagonist (Iwashita *et al.*, 2007). Amides of known structures that contain four subtypes of amides were rapidly determined, and novel amides were also identified for *Piper longum*. Forty-two amides were rapidly identified, of which 22 were found in this plant for the first time and 9 were new compounds (Sun *et al.*, 2007).

Pullela *et al.* (2006) employed a systematic bioassay guided fractionation method and isolated pipataline, pellitorine, sesamin, brachystamide B and guineensine as active principles. A reversed-phase high-performance liquid chromatography method was developed to quantify these active principles in the plant material, which can serve as an effective quality control tool. The extract of *P. longum* at non-toxic concentrations

(10 microg/ml, 5 microg/ml, 1 microg/ml) inhibited the VEGF-induced vessel sprouting in rat aortic ring assay. Moreover, *P. longum* was able to inhibit the VEGF-induced proliferation, cell migration and capillary-like tube formation of primary cultured human endothelial cells. Hence, the observed antiangiogenic activity of the plant *P. longum* is related to the regulation of these cytokines and growth factors in angiogenesis-induced animals (Sunila and Kuttan, 2006).

Piperine has been shown to enhance the bio-availability of structurally and therapeutically diverse drugs, possibly by modulating membrane dynamics, due to its easy partitioning and increasing permeability. Piperine was evaluated and found to exert significant protection against tertiary butyl hydroperoxide and carbon tetrachloride induced hepatotoxicity, by reducing both in vitro and in vivo lipid peroxidation. Methyl piperine significantly inhibited the elevation of total serum cholesterol, and the total cholesterol to HDL-cholesterol ratio, in rats fed with a high cholesterol diet. *Piper longum* Linn, an important medicinal plant belonging to the family piperaceae has been used in traditional medicine by people in Asia and Pacific islands especially in Indian medicine (Guido and David, 1998).

Piper longum is a component of medicines reported as good remedy for treating gonorrhoea, menstrual pain, tuberculosis, sleeping problems, respiratory tract infections, chronic gut related pain and arthritic conditions (Singh *et al.*, 1992).

Piper longum possessed a demonstrable immunostimulatory activity, both specific and nonspecific, as evident from the standard test parameters such as haemagglutination titre (HA), plaque forming cell (PFC) counts, macrophage migration index (MMI) and phagocytic index (Tripathi *et al.*, 1999). Three isolates of black pepper were active against Gram-positive bacteria and moderately active against Gram-negative bacteria. Each isolate was highly active against at least one particular species of bacteria; Piper longuminine (1) against *Bacillus subtilis*, piperine (2) against *Staphylococcus aureus* and pellitorine (3) against *Bacillus sphaericus* (Srinivasa Reddy *et al.*, 2001).

AIMS AND OBJECTIVES

Aim

The present investigation was aimed to determine the hepatoprotective effect of the Biherbal extract (BHE), made up of equal proportions of *Melia azedarach* leaves and *Piper longum* seeds in experimental animals with CCl₄ as hepatotoxic model. The hepatoprotective effect of the Biherbal extract (BHE) was also compared with biological efficacy of individual plant preparations.

Objective

The study was carried out in the following stages:

1. Preparation of the bi herbal ethanolic extract (BHE) of the leaves of *Melia azedarach* and seeds of *Piper longum* and their individual ethanolic extracts.
2. To assess the acute and chronic toxicity of BHE.
3. To determine the effective dose of the test drug which gives optimum therapeutic effect.
4. To compare the hepatoprotective effect of BHE with the standard drug silymarin.
5. To evaluate a tentative mechanism of action of the drug.

The objective also includes the

1. The determination of the major phytochemicals in the test extract and HPTLC finger printing.
2. Quantitative analysis of macronutrients and minerals including protein pattern by SDS-PAGE.
3. *In vitro* antioxidant studies of plant extracts.
4. *In vitro* DNA fragmentation study.

REVIEW OF LITERATURE

Research programs in India and china are active in the investigation of their local medicinal plants, but little ethanopharmacological research has been carried out for the ethnic groups who live in areas of maximum biodiversity, where many plants of interest are likely to occur. Medicinal herbs are significant source of pharmaceutical drugs. Latest trends have shown increasing demand of phytodrugs and some medicinal herbs. The most important classes of herbs for functional liver disease are cholagogues (increase bile flow), cholaretics (increasing bile production) and carminatives (expelling gas and antispasmodic). Cholagogue plants usually have a bitter flavor, and all bitter plants are cholagogic to some degree. Some important traditional cholagogues are dandelion (*Taraxacum officinalis*), greater celandine (*Chelidonium majus*), and wormwood (*Artemesia absinthum*). The most commonly used herbs for liver problems include Phyllanthus, Milk Thistle, Sho-saiko-to, Dandelion Root, Licorice Root, and Chicory root. Phyllanthus amarus in chronic hepatitis B, glycyrrhizin to treat chronic viral hepatitis, and a number of herbal combinations from China and Japan that deserve testing in appropriate studies.

Liver is often abused by environmental toxins, poor eating habits, alcohol and over-the-counter drug use, that damage and weaken the liver leading to important public health problems like hepatitis, cirrhosis and alcoholic liver diseases (Treadway, 1998). These toxins generally lead to pathologically distinct liver diseases in clinical practice, any or all of the three

conditions can occur together at the same time, in the same patient. These three conditions are fatty liver, hepatitis, and Cirrhosis. Paracetamol and CCl₄-induced hepatic injuries are commonly used models for hepatoprotective drug screening (Plaa Hewitt, 1982).

Abstinence from Alcohol

Alcohol consumption has been associated with alcoholic hepatitis, fatty infiltration of the liver, accelerated progression of liver disease, a higher frequency of cirrhosis, a higher incidence of hepatocellular carcinoma, and death. The daily consumption of more than four drinks of alcohol increases the risk of cirrhosis, as well as death from other causes (Thun *et al.*, 1997; Scheig, 1970). Investigators in one study (Corrao and Arico, 1998) found that the effect of alcohol in patients with hepatitis C virus infection is not merely additive but synergistic. The mechanism for the synergistic effect of alcohol and hepatitis C virus is not fully understood, but it has been attributed to the effects of alcohol on viral replication and the immune system, hepatic iron content and hepatic regeneration.

Influence of Iron

As many as 30 percent of patients with liver disease have high serum iron levels, and 10 percent have excessive amounts of iron in their liver tissue (Riggio *et al.*, 1997; Di Bisceglie *et al.*, 1992). The most likely mechanisms of liver injury from excess iron are increased generation of free radicals and increased peroxidation of lipids, which, in turn, lead to mitochondrial dysfunction, lysosomal fragility and cell death. Iron has recently been shown

to influence the natural history of hepatitis C virus infection and the response of chronic hepatitis C to treatment (Olynyk *et al.*, 1995; Van Thiel *et al.*, 1994). To date, no evidence suggest that dietary iron is harmful.

Diet and Exercise

One controlled study (Ueno *et al.*, 199) demonstrated that a weight reduction program (combined diet and exercise) can improve liver function test results and liver histology in patients with nonalcoholic steatohepatitis. With a weight loss of 4.5 to 6.8 kg (10 to 15 lb), liver transaminase levels often return to normal. Investigators in another study (Deems *et al.*, 199) found a correlation between high fat and oil consumption and elevated liver transaminase levels. The findings of these studies suggest that a low-fat diet and exercise could minimize hepatic steatosis.

Liver protecting Herbs

Ayurveda remains one of the most ancient and yet living traditions practiced widely in India, Sri Lanka and other countries and has a sound philosophical and traditional basis (Chopra and Doiphode, 2002). *Atharvaveda* (around 1200 BC), *Charak Samhita* and *Sushrut Samhita*. (1000–500 BC) are the main classics that give detailed descriptions of over 700 herbs. Polyherbal formulations reputed to have hepatoprotective activity that are available on the Indian market comprise about one hundred Indian medicinal plants (Handa and Sharma, 1986).

Andrographis paniculata

For centuries *Andrographis* has been an important herb in the Asian healing systems of Ayurveda, Unani and Traditional Chinese Medicine. Traditionally this herb has been used to potentiate immune system response to inflammation and infections, and as an anti-inflammatory, antipyretic (lowers fevers) and a hepatoprotective (liver protector). Andrographolide, the active constituent isolated from the plant *Andrographis paniculata* showed a significant dose dependent protective activity against paracetamol-induced toxicity on *ex vivo* preparation of isolated rat hepatocytes (Visen *et al.*, 1993).

***Boerhavia diffusa* (Punarnava)**

An alcoholic extract of whole plant *Boerhavia diffusa* given orally exhibited hepatoprotective activity against experimentally induced carbon tetrachloride hepatotoxicity in rats and mice (Ansari *et al.*, 1991).

***Terminalia belerica* (Baheda)**

Compound I isolated from fraction TB5 of *Terminalia belerica* and finally identified as 3,4,5-trihydroxy benzoic acid (gallic acid) was evaluated for its hepatoprotective activity against carbon tetrachloride (CCl₄) - induced physiological and biochemical alterations in the liver. Administration of compound I led to significant reversal of majority of the altered parameters confirming the presence of hepatoprotective activity in Compound I (Anand *et al.*, 1997).

***Picrorhiza kuroa* (Katuki)**

Picrorrhiza kurroa is one of the herbs they recommend to support the liver not only in everyday situations, but in cases where severe viral infections attack. Pre-treatment with picroliv prevented the hepatotoxic effects of paracetamol and galactosamine as evidenced by various biochemical and histopathological observations. Maximum hepatoprotective effect was observed with daily oral doses of 6 and 12 mg/kg for 7 or 8 days. The antihepatotoxic action of picroliv seems likely due to an alteration in the biotransformation of the toxic substances resulting in decreased formation of reactive metabolites (Ansari *et al.*, 1991).

Taraxacum officinale

Traditionally *Taraxacum officinale* has been used as a remedy for jaundice and other disorders of the liver and gallbladder, and as a remedy for counteracting water retention. Generally, the roots of the plant have the most activity regarding the liver and gallbladder. Oral administration of extracts from the roots of *Taraxacum officinale* has been shown to act as a cholagogue, increasing the flow of bile (Vogel, 1977). Bitter constituents like taraxecerin and taraxcin are active constituents of the medicinal herb (Cordatos, 1992).

Solanum nigrum

In Ayurveda, the drug is known as kakamachi. Aromatic water extracted from the drug is widely prescribed by herbal vendors for liver

disorders. Although clinical documentation is scarce as far as hepatoprotective activity is concerned, but some traditional practitioners have reported favorable results with powdered extract of the plant.

Wilkstroemia indica

W. indica is a Chinese herb and has been evaluated in patients suffering from hepatitis B. A dicoumarin, daphnoretin is the active constituent of the herb. The drug has shown to suppress HbsAG in Hep3B cells. It is said to be an activator of protein kinase C (Chen *et al.*, 1996).

Curcuma longa

Like silymarin, turmeric has been found to protect animal livers from a variety of hepatotoxic substances, including carbon tetrachloride (Srinivas and Shalini, 1991), galactosamine, pentobarbitol, 1-chloro-2, 4-dinitrobenzene, 7 4-hydroxy-nonenal (Selvam *et al.*, 1995) and paracetamol. Diarylhepatonoids including Curcumin is the active constituent of the plant.

Milk Thistle (*Silybum marianum*)

The best studied herb for hepatitis and has been shown to help protect and regenerate liver cells. There is no evidence of toxicity related to the pure form of milk thistle, and there is weak evidence of a hepatocyte plasma cell membrane protective effect. For these reasons, it is reasonable not to discourage the use of milk thistle (Flora *et al.*, 1998).

Piper longum

The isolated constituents and n- hexane extracts of *Piper longum* were found to show varying degree of antibacterial activity against all the tested bacteria (Lokhande *et al.*, 2007). Administration of alcoholic extract of *Piper longum* (10 mg/dose/animal) as well as piperine (1.14 mg/dose/animal) could inhibit the solid tumor development in mice induced with DLA cells and increase the life span of mice bearing Ehrlich ascites carcinoma tumor to 37.3 and 58.8%, respectively. Administration of *Piper longum* extract and piperine increased the total WBC count to 142.8 and 138.9%, respectively, in Balb/c mice (Sunila and Kuttan, 2004). Ethanol extract of *Piper longum* fruits and five crude fractions, petroleum ether (40-60), solvent ether, ethyl acetate, butanol and butanone were subjected to preliminary qualitative chemical investigations. The ethanolic extract and all other fractions were screened orally for hepatoprotective activity in adult Wistar rats. The ethanolic extract and butanol fraction have shown significant activity, lowering the serum enzymes glutamic oxaloacetic transaminase and glutamic pyruvic transaminase in rats treated with carbon tetrachloride when compared to control and Liv-52-treated rats (Jalalpure *et al.*, 2003)

Eclipta alba

The drug is traditionally used against Jaundice (Mehra and Handa, 1968). As a reputed herbal medicine in the Ayurvedic and Unani system of medicine, it is incorporated as a major ingredient in a number of Indian antihepatotoxic phytopharmaceutical formulations. The herb of *E. alba*

contains coumestans, i.e. wedelolactone (W) and demethylwedelolactone (DMW) possessing potent antihepatotoxic activity and is recommended for the treatment of hepatitis and cirrhosis. It cure insomnia (Kulkarni, 1990). The drug also showed antiviral activity in mice experimentally infected with Semliki forest encephalitis virus (Singh, 1983). The drug also has been found to be quite beneficial for treatment of jaundice when tested clinically in children (Dixit and Achar, 1981).

SCOPE OF THE PRESENT INVESTIGATION

One of the vital organs of the human body, the liver performs essential functions including detoxification and maintenance of normal immunity by generating protective antibodies. Liver is susceptible to be encountered with abnormal metabolites and toxins which are of endogenous and exogenous in origin. Environmental pollution, drugs, chronic alcohol consumption, pathogens and hereditary factors can be accounted for susceptibility of liver to various diseases and results in functional deficiency.

Many liver protecting agents are consumed by people either to prevent or to cure liver associated disorders. Allopathic medicines are being used to save those patients by relieving them from the hazardous symptoms. But the contraindication and limitations for their use also keep increasing due to age and sex related side effects.

Since the time of its invention various medicinal plants have been traditionally practised to cure liver diseases or to strengthen the vitality of the organ. Many plants such as *Silybum marianum* (milk thistle), *Picrorhiza kurroa* (kutkin), *Curcuma longa* (turmeric), *Camellia sinensis* (green tea) and *Glycyrrhiza glabra* (licorice) have been clinically proved and patented.

India is one of the holistic countries, which believes in traditional system of folklore medicines made up of medicinal plants. Both single plant and formulation consisting of more than one plant are used in ayurvedic system of medicine. Though many medicinal plants are widely used for liver

complaints the ever increasing problem of pollution and alcohol consumption and thereby liver toxicity demands the screening and identification of new plants which can be superior to allopathic medicines due to their less toxic and cost effective nature.

Eclipta alba commonly known as False Daisy and bhringraj, is a plant belonging to the family Asteraceae. Bhrngaraja is equally useful in many diseases both, internally as well as externally. It is one of the best blood purifiers, stimulates the liver and alleviates the general oedema all over the body. *Eclipta alba* also has traditional external uses, like athlete foot, eczema and dermatitis, on the scalp to address hair loss and the leaves have been used in the treatment of scorpion stings. It is used as anti-venom against snakebite in China and Brazil.

Piper longum an important medicinal plant belonging to the family of Piperaceae also known as “Thippali” and used in traditional medicine by many people in Asia and Pacific islands especially in Indian system of medicine. *Piper longum* is a component of medicines reported as good remedy for treating gonorrhoea, menstrual pain, tuberculosis, sleeping problems, respiratory tract infections, chronic gut related pain, and arthritic conditions.

Traditional system of medicine, which is the only accessible health care system for most of the population in rural areas, should be scientifically evaluated so as to improve the clinical efficacy and safety.

Both *E. alba* and *P. longum* have immense therapeutic properties especially for the treatment of liver related disorders. But the practice is only at the traditional level because of the lack of experimental proof to standardise the optimum dosage, efficacy and toxic effects.

The aim of the present study is to provide experimental proof for the hepatoprotective efficacy of the ethanolic extracts of *E. alba*, *P. longum* and the combined biherbal formulation made up of equal concentrations of *E.alba* and *P. longum*. Polyherbal formulations are considered more effective than the single drug and hence the biherbal formulation has been used in the study and compared with the individual plants *E. alba* and *P. longum*.

The present investigation has been divided into two parts

- ❖ The plants have been processed and screened for the phytochemicals and tested for free radical scavenging activities by using standard models *in vitro*.
- ❖ The evaluation of non toxic dosage of test drugs by acute and chronic toxicity in albino Wistar strain rat models according to OECD guidelines. After assessing the dosage by acute and chronic toxicity studies efficacy of the single and the biherbal drugs have been ascertained by using CCl₄ induced hepatotoxicity model in rats. The hepatoprotective nature of the drugs has been assessed by various biochemical estimations and histological observations. Rats treated with

standard drug silymarin have also been utilised in this study to compare the hepatoprotective efficacy of the test drugs.

The results of this study will throw more light on the mechanism of action of the test drugs, which is hidden so far due to the lack of experimental proof. It is sure that the out come of this study will result in the identification of a new biherbal formulation, which can be used to treat liver disorders in future.

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PHYTOCHEMICAL STUDIES ON *ECLIPTA ALBA* AND *PIPER LONGUM*

INTRODUCTION

Plants are considered to be medicinal if they possess pharmacological activities of possible therapeutic use. These activities are often known as a result of millennia of trial and error, but they have to be carefully investigated if we wish to develop new drugs that meet the criteria of modern treatment. Compared with the experience of most modern drugs, the human use and approval of most herbal remedies is awesome.

The requirement by the medical and scientific establishment for research to prove that herbs are effective is not found among the population at large. It is apparent that most ordinary people are content to rely on their impressions of the world to get by it. Judging by the substantial markets for herbal products in the developed world, let alone the vast use in traditional cultures, a great many people have already found herbal medicines useful. The relationship between man and plants has been very close throughout the development of human cultures. It can be recalled that herbal therapy aims to support vital functions of human body.

The role of compounds such as flavonoids and others with activities such as antioxidation and free radical scavenging is largely unknown but could be important in the prevention of chronic inflammatory diseases and cancer (Kinghorn, 1993).

The need to document plant usage and to attempt to confirm their efficacy remains urgent and must be undertaken with same rigour, as would any other scientific study.

Considering the above facts, the present research work has been carried out to evaluate the hepatoprotective action of the combined ethanolic extract of leaves of *Eclipta alba* and the seed of *Piper longum* (BHE)

In Ayurveda the plant *Eclipta alba* is considered a *rasayana* for longevity and rejuvenation. Recent studies have shown that it has a profound antihepatotoxic activity

Piper longum Linn, an important medicinal plant belonging to the family piperaceae has been used in traditional medicine by people in Asia and Pacific islands especially in Indian medicine

Plant Collection and Authentication

The leaves of *Eclipta alba* and seeds of *Piper longum* were collected from the IMPCOPS (Indian Medical practitioners co-operative society), Thiruvannamiyur Chennai, India and were authenticated by Dr.P.T.Kalaichelvan, Professor, Advanced Studies in Botany, University of Madras, Chennai, India. The voucher specimen is available in the herbarium file of the Indian Medical practitioners co-operative society, Thiruvannamiyur, Chennai, India.

EXTRACTION

Preparation of *Eclipta alba* extract (EAE)

The leaves of *Eclipta alba* (1 kg) were shade-dried and pulverized to a coarse powder. The powder was passed through 40-mesh sieve and exhaustively extracted with ethyl acetate in soxhlet apparatus at 60°C. The residue left after ethanol extraction was dried and extracted successively with chloroform and ethanol. The extracts were evaporated under reduced pressure using rota flash evaporator till all the solvent had been removed and extract was stored in refrigerator for further use. All these extracts were subjected to HPTLC finger printing analysis. The ethanolic extract alone was used for phytochemical and pharmacological studies. The ethanolic extract was administered to the animals by dissolving each time with 2% v/v aqueous Tween 80.

Preparation of *Piper longum* extracts (PLE)

The seeds of *Piper longum* (1 kg)) were shade-dried and pulverized to a coarse powder. The powder was passed through 40-mesh sieve and exhaustively extracted with 90% (v/v) ethanol in soxhlet apparatus at 60°C. The residue left after ethanol extraction was dried and extracted successively with ethyl acetate and chloroform (90 %v/v). The extracts were evaporated under reduced pressure using rota flash evaporator till all the solvent had been removed and extract was stored in refrigerator for further use. All these extracts were subjected to HPTLC finger printing analysis. The ethanolic extract alone was used for phytochemical and pharmacological studies. The

ethanolic extract was administered to the animals by dissolving each time with 2% v/v aqueous Tween 80.

Preparation of Biherbal extract (BHE)

The leaves of *Eclipta alba* (1Kg) and seeds of *Piper longum* (1Kg) were shade-dried and pulverized to a coarse powder. Equal quantities of the powder were passed through 40-mesh sieve and exhaustively extracted with 90% (v/v) ethanol in soxhlet apparatus at 60°C. The extract was evaporated under reduced pressure using rota flash evaporator till all the solvent had been removed, the extract was lyophilised and stored in refrigerator for phytochemical and pharmacological studies. The lyophilised material was administered to the animals by dissolving each time with 2% v/v aqueous Tween 80.

Drugs and Chemicals

Sodium dodecyl sulphate (SDS), Ethylene diamine tetra acetic acid (EDTA), Trichloro acetic acid (TCA), Thiobarbituric acid (TBA), Sodium nitroprusside, Sulphanilamide, Phosphoric acid, Naphtyl ethylene diamine dihydrochloride were obtained from Sd Fine Chemicals Ltd.). 1,1-Diphenyl, 2-picryl hydrazyl DPPH), Butylated hydroxytoluene (BHT), Bovine Serum Albumin (BSA), Folin-Ciocalteu reagent, 5,5-dithio bis(2-nitrobenzoic acid) (DTNB), 2,2'-bipyridyl were obtained from Sigma Chemicals Company St. Louis, USA. All other chemicals and reagent used were of analytical grade.

Preliminary Phytochemical Screening

The ethanolic extracts of the EAE, PLE, and BHE were subjected to preliminary phytochemical screening for identification of its active constituents by the method of Kokate *et al.* (1997).

Test for Alkaloids

A small portion of the solvent free extracts were stirred separately with a few drops of dil. Hydrochloric acid and filtered. The filtrate may be carefully tested with various alkaloidal reagents.

- (a) Mayer's reagent - Cream precipitate
- (b) Dragondroff reagent - Orange brown precipitate
- (c) Hager's reagent - Yellow precipitate
- (d) Wagner's reagent - Reddish brown precipitate

Test for Carbohydrates

(a) Molisch's Test

The extracts were treated with 2-3 drops of 1% alcoholic alpha naphthol and 2 ml of conc. Sulphuric acid was added along the sides of the test tube carefully. Formation of violet color ring at the junction of two liquids indicates the presence of carbohydrates.

(b) Fehling's Test

The extracts were treated with Fehling's solution A and B and heated. Appearance of reddish brown color precipitate indicates the presence of reducing sugars.

(c) Benedict's Test

The extracts were treated with Benedict's reagent and heated. Appearance of reddish orange colour precipitate indicates the presence of reducing sugars.

Test for Proteins**(a) Biuret Test**

The extracts were treated with copper sulphate solution, followed by the addition of sodium hydroxide solution appearance of violet colour indicates the presence of proteins.

(b) Millon's Test

When the extracts were treated with Millon's reagent, appearance of pink colour indicates the presence of proteins

Test for Phytosteroids**(a) Libermann Bucharad Test**

When the extracts were treated with con. Sulphuric acid, few drops of glacial acetic acid, followed by the acetic anhydride, there is a formation of violet ring between the two layers, and the appearance of green colour in the aqueous upper layer indicates the presence of steroids.

Test for Phenols

- (a) The different extracts were treated with neutral ferric chloride solution. The appearance of violet color indicates the presence of phenols.
- (b) The different extracts were treated with 10% sodium chloride solution. The appearance of cream color indicates the presence of phenols.

Test for Tannins

- (a) When the extracts were treated with 10% lead acetate solution, appearance of white precipitate indicates the presence of tannins
- (b) When the extracts were treated with aqueous bromine solution, appearance of white precipitate indicates the presence of tannins

Test for Flavonoids

- (a) 5ml of the each extract solution was hydrolyzed with 10% v/v sulphuric acid and cooled. Then it was extracted with diethyl ether and divided into three portions in three separate test tubes. 1ml of diluted sodium carbonate, 1ml of 0.1N sodium hydroxide and 1ml of strong ammonia solution were added to the first, second and third test tubes respectively. In each test tube, development of yellow color demonstrated the presence of flavonoids
- (b) Shinoda's Test The extracts were dissolved in alcohol, to that one piece of magnesium followed by conc. Hydrochloric acid were added drop wise and heated. Appearance of magenta color shows the presence of flavonoids.

Tests for Gums and Mucilage

- (a) About 10ml of various extracts were added separately to 25ml of absolute alcohol with constant stirring and filtered. The precipitate was dried in air and examined for its swelling properties.

Test for Saponins

(a) Foam Test

1ml of the different extracts were diluted with distilled water and shaken well in the test tube. The formation of foam in the upper part of the test tube indicates the presence of saponins.

Test for Glycosides

A pinch of the extracts were dissolved in the glacial acetic acid and a few drops of ferric chloride solution was added, followed by the addition of con. Sulphuric acid, formation of red ring at the junction of two liquids indicates the presence of glycosides.

Tests for fixed oils and fats

- (a) Small quantity of the various extracts were separately pressed between two filter papers. Appearance of oil stain on the paper indicates the presence of fixed oil.

- (b) Few drops of 0.5N alcoholic potassium hydroxide were added to small quantity of various extracts along with a drop of phenolphthalein. The mixture was heated on a water bath for 1-2 hours. Formation of soap or partial neutralization of alkali indicates the presence of fixed oils and fats.

Test for Terpenes

When the extracts were treated with tin and thionyl chloride, appearance of pink colour indicates the presence of terpenes.

Test for Lignin

When the extracts were treated with alcoholic solution of phloroglucinol and con. Hydrochloric acid appearance of red colour shows presence of lignin.

High Performance Thin Layer Liquid Chromatography (HPTLC) finger printing

HPTLC finger printing was performed on CAMAG TLC scanner – 3 instrument, equipped with Linomat IV applicator and CATS 3. 2 software. Precoated aluminium silica gel 60 F₂₅₄ (E. Merck) plates, layer thickness of 0.2 mm were used. Fingerprints were obtained by development in CAMAG twin chamber and were scanned at 254 nm.

ESTIMATION OF MACRONUTRIENTS

Estimation of Total soluble sugars

The estimation of total soluble sugars present in the leaves of *Eclipta alba* and seeds of *Piper longum* were estimated by using anthrone method.

Extraction of sugars

For extracting the sugars 1gm of finely powdered sample was suspended in 40 ml of distilled water and heated in the boiling water bath for 30 min. It was centrifuged for 20 min at 3000rpm. The supernatant was collected and the pellet was suspended in 20 ml of water. The extraction steps were repeated 6 –8 times till the supernatant was free of sugars.

Reagents

1. Anthrone reagent: Dissolved 2 gms of anthrone in 1 litre of con. Sulphuric acid. The reagent was prepared freshly.
2. Stock standard glucose solution: 100mgs of glucose was dissolved in 100ml of distilled water.
3. Working standard glucose solution: Stock solution was diluted 1 in 10 times.

Procedure

From the supernatant 1ml of the solution was taken. To that 4 ml of anthrone reagent was added. Placed the tubes in the boiling water bath for 10 min. Aliquots of standard glucose was also treated in the same way. A blank was set up with 1ml of water. The test tubes were taken out, cooled and the absorbance of the solution was measured at 625 nm using the colorimeter. From the standard graph the amount of carbohydrate present in the sample

was calculated. The sugar contents of the plant were expressed as mgs/100gms of powder.

Estimation of Total Proteins

The estimation of total proteins present in the leaves of *Eclipta alba* and seeds of *Piper longum* were estimated by using Lowry *et al.*, (1951) method.

Extraction of the protein sample

Reagents

1. Ethanol - (80%)
2. Ethanol : Ether mixture (3: 1)
3. Trichloro acetic acid (TCA) -5%

Procedure

The fresh leaves of the *Eclipta alba* and seeds of *piper longum* were extracted in hot 80% ethanol by macerating in a motor and pestle. The homogenate was transferred in a centrifuge tube and centrifuged at 2000rpm for 20 min. The supernatant was discarded. The pellet was suspended in a suitable volume of 5% TCA in an ice bath for 15 min. It was centrifuged and the supernatant was discarded. This process was repeated for twice. The pellet was reextracted once with hot absolute ethanol and twice with ethanol: ether mixture, every time discarding the supernatants after centrifugation. This pellet contains the proteins and nucleic acids.

Estimation of the protein sample (Lowry's method)

Reagents

1. Alkaline copper reagent: 50ml of reagent A (2% Sodium carbonate in 0.1N Sodium hydroxide) was mixed with 1.0ml of reagent B (0.5% copper sulphate in 1% sodium potassium tartarate).

2. Folin's phenol reagent: This was prepared according to the method of Folin and Ciocalteu's. The following were mixed together and refluxed for 10hrs.

Sodium tungstate	-	100mg
Sodium molybdate	-	25g
Water	-	700ml
Phosphoric acid 85%	-	15ml
Con HCl	-	100ml

After refluxing, 150g of Lithium sulphate and 50ml of water were added along with a few drops of bromine. The mixture was boiled for 15min to remove excess of bromine. The contents were cooled, diluted to 1 litre and filtered. This reagent was diluted 1:2 with water before use.

3. Standard protein solution: 10mg of bovine serum albumin dissolved in 100ml of distilled water.

Procedure

Aliquots of the extract were made up to a final volume of 1.0ml with water. A set of standards and blank containing only water were also set up. 5.0ml of alkaline copper reagent was added to all the test tubes, mixed and allowed to stand at room temperature for 10mts. Then 0.5ml of Folin's phenol reagent was added and shaken well. The blue color developed was read at 640nm after 20mts, in the photoelectric colorimeter. The protein contents of the tissues were expressed as mgs/100gms of powder.

Estimation of Total lipid

The estimation of Total lipids present in the leaves of *Eclipta alba* and seeds of *Piper longum* were estimated by using chloroform methanol mixture by the method of Folsch *et al.* (1957).

Reagents

1. Anhydrous sodium sulphate
2. chloroform : Methanol mixture (2:1)
3. sodium chloride - 1%

Procedure

To 1gm of each of the leaves *Eclipta alba* and seeds of *Piper longum*, 5gms of anhydrous sodium sulphate was added and macerated in the mortar and pestle. A small amount of acid washed sand was added as an abrasive since the seed material is tough. To this 20ml of chloroform : methanol

mixture was added and transferred to a tight glass stoppered iodometric flask. The contents of the flask were shaken in a mechanical shaker and filtered through the sintered glass funnel. The extraction was repeated twice and the filtrates were pooled. The solvent present in the residue was removed under vacuum. Since the residue left after drying contain crude lipids it was extracted once again with 10ml of chloroform: methanol mixture containing 1ml of 1% sodium chloride. The pooled sample was transferred to a separating funnel which were shaken thoroughly and allowed to stand for 5 min. The lipids were recovered from the lower layer. The lower layer was drained out and the process was repeated with chloroform: methanol mixture again 3-4 times to extract any residual lipid from it. The lipid containing fractions were collected in a pre weighed beaker. The solvent was evaporated by keeping it in a warm water bath at 50° C. The weight of the beaker with the extract was recorded, and the amount of the crude lipids in the sample was determined by subtracting the weight of the empty beaker. The lipid contents of the tissues were expressed as gms/100gms of fresh tissue

Estimation of Minerals

Preparation of sample for mineral analysis by ashing method

Reagents

1. Desiccator containing fused calcium chloride at the bottom
2. Dilute HCl: Prepared diluting 1ml of HCl with 4ml of water

Procedure

About 5 – 10gms of oven-dried samples were taken in a silica crucible and heated first in the bunsen burner on a low flame till it gets charred. The silica crucible was transferred to a muffle furnace and heated at dull red heat (500-550°C) till it was completely converted into white ash. The ash was kept in the desiccator containing fused calcium chloride at the bottom, till it gets cooled down. The ash was moistened with small amount of distilled water and 5ml of dilute HCl was added to it. The solution was evaporated to dryness on a boiling water bath and this process was repeated twice. To the extract 4ml of dil .HCl was added and warmed in the boiling water bath. The extract was filtered through whatmann filter paper and made up to 100ml in the volumetric flask. It was transferred to preacid washed bottles and stored for mineral analysis.

Estimation of Minerals by Atomic Absorption

The minerals present in the dried powdered plants were analysed quantitatively using atomic absorption spectrophotometer. (AAS400-HGA 900-Perkin Elmer) and expressed as ppm of plant powder.

SDS-PAGE ANALYSIS OF PROTEINS

A SDS-PAGE analysis of proteins was performed with the aqueous extracts of the seeds of *Piper longum* and with the leaves of *Eclipta alba* and the molecular weight of the different proteins present in the plants were identified by comparing with the marker proteins.

Reagents

Stock solutions

1. Acrylamide stock solution: 30% Acryl amide and 0.8% N, N', methylene bis acrylamide was dissolved in 50ml of distilled water.
2. Sodium dodecyl sulphate (SDS)- 10%
3. Ammonium per sulphate (APS)- 10%
4. Tetra ethyl methylene ethylene diamine (TEMED)
5. Tris – HCl buffer: Two bufferts
 - (a) for separating gel- 0.375 M pH 8.8
 - (b) for stalking gel -0.125 M pH 6.8
 1. Preparation of separating gel (6ml)
 2. Tris – HCl buffer pH 8.8 - 1.5ml
 3. Acrylamide - 2.0 ml
 4. Sodium dodecyl sulphate 10% - 0.06 ml
 5. Ammonium per sulphate (APS) 10% - 0.02 ml
 6. TEMED - 0.01 ml
 7. Water - 2.41 ml

Preparation of stalking gel (3ml)

- | | | | |
|----|---------------------------------|---|----------|
| 1. | Tris – HCl buffer pH 6.8 | - | 0.75ml |
| 2. | Acrylamide | - | 0.5 ml |
| 3. | Sodium dodecyl sulphate10% | - | 0.03 ml |
| 4. | Ammonium per sulphate (APS) 10% | | 0.015 ml |

5.	TEMED	-	0.005 ml
6.	Water	-	1.7 ml

Preparation of sample buffer

1.	0.025M Tris PH 6.8	-	0.25
2.	2 mercaptoethanol	-	0.1ml
3.	SDS	-	0.04ml
4.	Glycerol	-	0.2ml
5.	Bromophenol Blue	-	0.001%
6.	Water	-	to make up to 1ml

Preparation of tank buffer PH 8.3

1.	Tris 0.025M	-	1.5g
2.	Glycine 0.192M	-	7.2g
3.	SDS 0.1%	-	0.5g
4.	Water	-	500ml

Protein sample preparation

The protein isolated from the plants as above mentioned procedure was taken and mixed with equal volume of sample buffer in sterilized microfuge tubes and boiled for 3 min in a boiling water bath. It was cooled at room temperature and then used for loading.

Protein Staining Solutions

0.1 gms of coomassie brilliant blue (CBB R 250) was weighed, dissolved completely in 40 ml of methanol, 10 ml of acetic acid was added and made-up to 50 ml using water.

Procedure

1. The polyacrylamide slab was prepared between the two glass plates, a notched upper plate and a lower plate that were separated by the spacers. The sides of the gel plates were sealed using 5% agar solution. The plates were checked for leaks before pouring the separating gel solution.
2. The separating gel was poured in between the plates without any air bubble. 100 μ l of water was laid over the separating gel to get a uniform layer. The separating gel was allowed to get solidify. After the gel gets solidifies the water layer was removed.
3. The stacking gel was added on the top of the separating gel. The comb was introduced on the top without air bubble, and allowed to get solidify.
4. After solidification of the stacking gel the comb was removed , the spacers kept in between the plates were also removed.
5. The plates with the gel were clamped in the electrophoretic apparatus. The top electrolyte compartment was filled with the

running buffer and checked for leaks. If it was perfect the bottom compartment was also filled with the same.

6. About 25 μ l of the processed protein sample was applied on the wells by using the micropipette.
7. Known molecular weight protein markers were also loaded onto the wells.
8. After loading the samples and the markers on to the wells the power pack was “switched on” and a voltage of 15 mA was applied which gives good resolution and avoid heating effects.
9. Once the tracking dye reaches the bottom of the gel the current was “switched off” and the plates were removed and the gel present in between the plates were taken out for staining purposes.
10. The gel was stained for an hour in the freshly prepared staining solution and destained in the destaining solutions till the bands of required intensity was obtained.
11. The proteins present in the sample were studied using the marker proteins.

Molecular weight determination

SDS-PAGE is frequently used to determine the molecular weight of a protein. Since the protein migration is generally proportional to the mass of the protein, a standard curve is generated with proteins of known molecular

weight and the molecular weight of unknown protein can be calculated by extrapolating the graph.

IN- VITRO ANTIOXIDANT STUDIES

1,1-Diphenyl-2 picrylhydrazyl (DPPH) radical scavenging activity

DPPH radical scavenging activity was assessed according to the method of Shimada *et al.* (1992).

Reagents

- | | | | |
|----|-------------------------|---|------------------|
| 1. | Acetate buffer , pH 5.5 | - | 0.1 M |
| 2. | DPPH in ethanol | - | 0.5 mM |
| 3. | L- Ascorbic acid | - | 10mg/100ml water |

Procedure

One ml of crude extracts of EAE, PLE, and BHE at variable concentrations ranging from 100 to 1000 μ g in ethanol were mixed in 1 ml of freshly prepared DPPH solution and 2 ml of 0.1 M acetate buffer at pH 5.5. The resulting solutions were then incubated at 37 °C for 30 min and measured spectrophotometrically at 517nm. Standard antioxidants like L-Ascorbic acid was used as positive control under the same assay conditions. Negative control was without any inhibitor or extract. Lower absorbance at 517 nm represents higher DPPH scavenging activity. DPPH radical scavenging activity of the extracts was calculated accordingly from the decrease in absorbance at 517 nm in comparison with the negative control.

Super oxide scavenging activity

Super oxide anion scavenging activity was measured based on the described method by Robak and Gryglewski (1988).

Reagents

1. Sodium phosphate buffer, pH 7.4 - 100 mM
2. Nitro blue tetrazolium (NBT) - 150 mM
3. NADH - 468mM
4. Phenazine methosulphate (PMS) - 60 mM
5. L-Ascorbic acid - 10mg/100ml water

Procedure

Superoxide radicals were generated in nicotinenamide adenine dinucleotide, phenazine methosulphate (PMS–NADH) system by the oxidation of NADH and assayed by the reduction of nitro blue tetrazolium (NBT). In this experiment, the super oxide radical was generated in 3 ml of sodium phosphate buffer containing 1ml of NBT solution, 1 ml of NADH solution, and different concentrations of the EAE, PLE, and BHE (100 to 1000 μ g) in water. The reaction was started by adding 1 ml of PMS solution to the mixture. The reaction mixture was incubated at 25 °C for 5 min and the absorbance was measured against the corresponding blank solution. L-Ascorbic acid was used as the positive control. The decrease of NBT reduction measured by the absorbance of the reaction mixture correlates with

the super oxide radical scavenging activity of the BHE. The superoxide radical scavenging activity was calculated using the following formula:

$$\text{Superoxide radical scavenging activity (\%)} = [(A_0 - A_1/A_0) \times 100],$$

where A_0 is the absorbance of the control and A_1 is the absorbance of plant extract or the standard sample.

Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity was determined according to the method of Halliwell *et al.* (1987).

Reagents

- | | | | |
|----|-------------------------------|---|------------------|
| 1. | Phosphate buffer pH 7.4 | - | 20 mM |
| 2. | Deoxyribose | - | 8 mM |
| 3. | Ferric chloride | - | 0.1 mM |
| 4. | Ascorbic Acid | - | 0.1mM |
| 5. | EDTA | - | 0.1 mM |
| 6. | H ₂ O ₂ | - | 1 mM |
| 7. | Thiobarbituric acid | - | 1% |
| 8. | Trichloroacetic acid | - | 2% |
| 9. | Standard Mannitol | - | 10mg/100ml water |

Procedure

Briefly for the non site-specific hydroxyl radical system, the reaction mixture containing 0.1 ml deoxyribose, 0.1 ml FeCl₃, 0.1 ml ascorbic acid, 0.1 ml EDTA, and 0.1 ml H₂O₂ were mixed with or without various concentrations of the three extracts in 1ml of final volume made with KH₂PO₄-KOH buffer pH 7.4 and was incubated in a water bath at 37 °C for 1 hr. The extent of deoxyribose degradation was measured by thiobarbituric acid (TBA) method. 1ml of TBA and 1 ml trichloroacetic acid were added to the mixture and heated at 100 °C for 20 min. After cooling to room temperature the absorbance was measured at 532 nm. Mannitol, a classical hydroxyl radical scavenger was used as positive control. The hydroxyl radical scavenging activity was calculated using the following formula:

$$\text{Hydroxyl radical scavenging activity (\%)} = [(A_0 - A_1/A_0) \times 100],$$

where A₀ is the absorbance of the control and A₁ is the absorbance of plant extract or the standard sample.

Nitric oxide scavenging activity

Nitric oxide generated from sodium nitro prusside was measured by the Griess reagent by the method of Marcocci *et al.* (1994).

Reagents

1. Sodium nitroprusside - 5 mM
2. Phosphate buffered saline

3. Griess reagent: 1% sulpha-nilamide, 2% o-phosphoric acid, and 0.1% naphthylethylenediamine dihydrochloride were mixed together.
4. Rutin -10mg/100 ml

Procedure

Various concentrations of the three different extracts were mixed with sodiumnitroprusside in PBS and a final volume of 3 ml was incubated at 25 °C for 150min. After incubation, 0.5ml of sample was removed and diluted with 0.5 ml of Griess reagent. The absorbance of the chromophore formed was read at 546 nm. The inhibition of nitric oxide generation was estimated by comparing the absorbance value of control. Rutin was used as positive control under the same assay conditions.

$$\text{Nitric oxide scavenging activity (\%)} = [(A_0 - A_1 / A_0) \times 100],$$

where A_0 is the absorbance of the control and A_1 is the absorbance of plant extract or the standard sample.

Reducing power assay

The reducing power of the prepared EAE, PLE and BHE was determined according to method of Oyaizu (1986).

Reagents

1. Phosphate buffer pH 6.6 - 0.2 M

2. Potassium ferricyanide solution - 1%
3. TCA - 10%
4. Ferric chloride solution - 0.1%
5. Butylated hydroxyl toluene (BHT)-10mg/100ml water

Briefly, various concentrations of the extracts and the standard compound (BHT) in 1 ml of distilled water were mixed with 2.5 ml of phosphate buffer and 2.5 ml of a potassium ferricyanide solution. The mixture was incubated in a water bath at 50 °C for 20 min. Then 2.5 ml of a 10% TCA solution was added and the mixture was then centrifuged at 3000g for 10 min. 2.5 ml aliquot of the upper layer was mixed with 2.5 ml of distilled water and 0.5 ml of a ferric chloride solution, the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture correlates with greater reducing power.

Hydrogen peroxide scavenging activity

The ability of samples to quench H₂O₂ was determined by Ruch *et al.* (1989).

Reagents

1. Phosphate buffered saline pH 7.4 - 0.1 M
2. H₂O₂ - 2 mM
3. Vitamin E - 10mg/100ml

Procedure

The samples were dissolved in 3.4 ml of phosphate buffered saline (PBS) and mixed with 0.6 ml of 2 mM solution of H₂O₂. Absorbance of H₂O₂ at 230 nm was determined 10 min later in a spectrophotometer. For each concentration, a separated blank sample was used for background subtraction. Vitamin E was used as the standard conditions. The inhibition of H₂O₂ production was calculated as follows: Hydrogen peroxide radical scavenging activity (%) = $[(A_0 - A_1/A_0) \times 100]$,

where A₀ is the absorbance of the control, and A₁ is the absorbance of BHE or the standard sample.

Test for Ferric ion reducing capacity (Fe³⁺ to Fe²⁺)

The Ferric ion reducing capacity was determined according to the method of Wang *et al.* (2003) with minor modifications.

Reagents

1. Phosphate buffer, pH 6.5 - 20mM
2. Ferric chloride - 2mM
3. Potassium thiocyanate - 4mM
4. EDTA - 100µg/ml

Procedure

Here different concentrations of the three extracts (20 μ l to 100 μ l) was mixed with 200 μ l of 20mM phosphate buffer pH 6.5 and 100 μ l of ferric chloride (2mM). The mixture was incubated for 30 min. At the end of the incubation 1ml of potassium thiocyanate (4mM) was added and absorbance of ferric-thiocyanate complex (reddish brown complex) was measured at 460nm using spectrophotometer. The results were compared with standard EDTA which were treated similarly.

Test for ferrous ion chelating activity (binds Fe^{2+})

Metal chelating property for ferrous ion (Fe^{2+}) was estimated according to the method of Tripathi *et al.* (2001).

Reagents

- | | | | |
|----|------------------|---|----------------|
| 1. | Ferrous sulphate | - | 10 μ g. |
| 2. | 2,2'-bipyridyl | - | 1mM |
| 5. | EDTA | - | 100 μ g/ml |

Procedure

Different concentrations of the EAE, PLE, and BHE (10 μ l to 100 μ l) were mixed with a fixed concentration of ferrous sulphate (10 μ g). The mixture was incubated for 30 min. At the end of the incubation, 2ml of 2,2'-bipyridyl (1mM) was added and absorbance of ferrous-bipyridyl complex

(pink colored complex) was measured at 525nm. The results were compared with EDTA which were treated similarly.

Total antioxidant activity

The total antioxidant activity of the extracts were measured by use of a linoleic acid system by the method of Mitsuda *et al.* (1996).

Reagents

1.	Linoleic acid	-	0.2804 g
2.	Tween	-	20
3.	phosphate buffer, pH 7.0	-	0.2 M
4.	Ethanol		
5.	Ammonium thiocyanate	-	30%
6.	Ferrous chloride.	-	20 mM in 3.5% HCl
7.	Butylated hydroxytoluene	-	10mg/100ml

Procedure

The linoleic acid emulsion was prepared by mixing 0.2804 g of linoleic acid in Tween 20 and 50 ml of phosphate buffer (pH 7.0). The mixture was then homogenized. A 0.5 ml of different concentration of the extract and standard sample (in ethanol) was mixed with 2.5 ml of linoleic acid emulsion and 2 ml phosphate buffer. The reaction mixture was incubated at 37⁰C in the dark to accelerate the peroxidation process. The levels of peroxidation were determined according to the thiocyanate method by sequentially adding 5ml

of 75% ethanol, 0.1 ml of ammonium thiocyanate, 0.1 ml sample solution and 0.1 ml ferrous chloride. Butylated hydroxytoluene (BHT) was used as positive control which was treated similarly. After mixing for 3 min, the peroxide values were determined by reading the absorbance at 500 nm.

Determination of Total Phenolic Content

Total phenolic content in the lyophilized extract was determined with the Folin–Ciocalteu’s reagent (FCR) according to a published method of Slinkard and Singleton (1977).

Reagents

1. Folin–Ciocalteu’s reagent: Prepared as previously mentioned.
2. Sodium carbonate solution -7.5%,
3. Gallic acid - 100mg dissolved in 100ml of water.

Procedure

100mg of the sample was dissolved in 0.5ml of water, mixed with 2.5 ml Folin–Ciocalteu’s reagent (diluted 1:10, v/v) followed by 2 ml of sodium carbonate solution. The absorbance was then measured at 765 nm after incubation at 30°C for 90 min. Results were expressed as gallic acid equivalents (mg gallic acid/g dried extract).

Determination of Total Flavonoid Content

The total flavonoid content of the extracts were determined by a colorimetric method as described in the literature of Zhishen *et al.* (1999).

Reagents

1. Sodium nitrite - 15%
2. Aluminium chloride - 10%
3. Sodium hydroxide - 4%
4. Catechin - 100mg dissolved in 100ml of water.

Procedure

An aliquots of sample containing 100mg of test drugs (0.5 ml) was mixed with 2 ml of distilled water and subsequently with 0.15 ml of sodium nitrite solution was added. After 6 min, 0.15 ml of aluminium chloride solution was added and allowed to stand for 6 min, then 2 ml of NaOH solution (4%) was added to the mixture. Immediately, water was added to bring the final volume to 5 ml and the mixture was thoroughly mixed and allowed to stand for another 15 min. Absorbance of the mixture was then determined at 510 nm versus prepared water blank. . Results were expressed as catechin equivalents (mg catechin/g dried extract).

Assay of Protein Oxidation

The effects of the plant extracts on protein oxidation were carried out according to the slightly modified method of Wang *et al.* (2006).

Reagents

1. Potassium phosphate buffer , pH 7.4- 20 mM
2. Bovine serum albumin (BSA) - 4 mg/ml
3. Ferric chloride - 50mM
4. Hydrogen peroxide - 1 mM
5. Ascorbic acid. - 100 mM
6. 2,4-dinitrophenylhydrazine (DNPH)- 10 mM in 2 M HCl
7. TCA - 10%,
8. Ethanol
9. Ethyl acetate
10. Guanidine Hydrochloride, pH 2.3 - 6 M

Procedure

Bovine serum albumin (BSA) was oxidized by a Fenton-type reaction. The reaction mixture (1.2 ml), containing sample extract (100–1000 µg/ml), potassium phosphate buffer, BSA, Ferric chloride hydrogen peroxide and ascorbic acid was incubated for 30 min at 37⁰C. For determination of protein carbonyl content in the samples, 1 ml of 2,4-dinitrophenylhydrazine (DNPH) was added to the reaction mixture. Samples were incubated for 30 min at room temperature. Then, 1 ml of ice cold TCA was added to the mixture and centrifuged at 3000g for 10 min. The protein pellet was washed three times with 2 ml of ethanol/ethyl acetate (1:1 v/v) and dissolved in 1 ml of guanidine hydrochloride. The absorbance of the sample was read at 370 nm. The data were expressed in terms of percentage inhibition, calculated from a control

measurement of the reaction mixture without the test sample. Vitamin E under same assay condition was used as standard.

***In vitro* assay of DNA fragmentation study**

The DNA was subjected to oxidation by the Fenton's reactants and subjected to fragmentation. The protection offered by the plant extract against the DNA fragmentation was studied according to the method of Sultan *et al.* (1995), with minor modifications

Isolation of DNA from Hepatocytes

Reagents

1. Isotonic buffer
 - (a) 0.15mM sodium chloride - 3.0ml
 - (b) 0.1 M Tris-HCl pH 7.5 - 1.0ml
 - (c) 1.5mM Magnesium chloride - 0.15ml

Made up to 100ml with water.
2. Cell lysis buffer
 - (a) 320mM sucrose - 109.6g
 - (b) 1% Triton X-100 - 20ml of 50% Triton X-100
 - (c) 5mM MgCl₂ - 5ml of 1M MgCl₂
 - (d) 10mM Tris-HCl pH 7.5 - 10ml of 1M Tris- HCl
 - (e) Distilled water - to a final volume of 1 liter
3. EDTA-NaCl (100ml)
 - (a) 25mM EDTA PH 8.0 - 5ml of 0.5M EDTA
 - (b) 75mM NaCl - 1.5ml 0.5M NaCl

- (c) Distilled water - 93.5ml
- 4. Proteinase K (10mg/ml)
 - (a) Proteinase K - 10 mg
 - (b) Distilled water - 1mlStored in -20°C
- 5. 5M sodium acetate (100ml)

About 68.5 g of sodium acetate was dissolved in 70 ml of water, pH was adjusted to 5.2 by adding acetic acid. Volume was made up to 100ml with distilled water.
- 6. Phenol- chloroform – Isoamyl alcohol (50 ml)
 - (a) phenol - 25 ml
 - (b) chloroform - 24ml
 - (c) isoamyl alcohol - 1ml
- 7. Tris EDTA Buffer
 - (a) 10 mM Tris HCl pH 8.0 - 1ml from 1M Tris HCl pH 8.0
 - (b) 1mM EDTA pH 8.0 - 0.5ml from 0.5 ml EDTA pH 8.0
 - (c) Distilled water - 98.5ml
- 8. 20 mM potassium phosphate buffer, pH 7.4
- 9. 150mM sodium chloride
- 10. Fenton reaction mixture
 - (a) 100mM ascorbate
 - (b) 100mM FeCl₂
 - (c) 1mM H₂O₂

(d) 104 mM EDTA

The above contents were mixed in 20 mM potassium phosphate buffer, pH 7.4

10. Tris acetate EDTA buffer pH 8

(a) 40 mM Tris

(b) 20mM sodium acetate,

(c) 2mM EDTA,

(d) 18 mM NaCl, pH 8

Procedure

1. About 0.2 gm of liver tissue was homogenised in 2ml of isotonic buffer.
2. The animal cells were collected by centrifugation at 2000rpm for 10 min at 4°C.
3. The cell pellet was resuspended in cold cell lysis buffer.
4. The cells were homogenised in a glass homogeniser with a loose fitting pestle.
5. The above treated homogenate was centrifuged at 4000rpm for 20 min at 4°C to pellet the nuclei.
6. Resuspended the pellet in 8ml of EDTA-NaCl and added 0.8 ml of 10% cell lysis buffer. It was mixed using the vortex mixture.
7. To this mixture 50µl of the proteinase K was added and incubated at 37°C for 3- 5 hrs.

8. Then 0.5ml of sodium acetate buffer and 8 ml of Phenol-chloroform – Isoamyl alcohol were added. The contents were mixed by inverting the tube for one hr.
9. The above treated solution was centrifuged at 12000 rpm for 10 min at 4°C.
10. The upper aqueous layer was collected. Equal volume of Phenol-chloroform – Isoamyl alcohol was added . It was mixed gently by inverting the tube for 1 min.
11. Once again the solution was centrifuged at 12000 rpm for 10 min at 4°C. The upper aqueous layer was collected.
12. About 2 volumes of 100% ethanol was added to the above collected solution to precipitate the DNA.
13. Centrifuged at 5000rpm for 5 min at 4°C. The DNA pellet was washed in 70 % ethanol.
14. The precipitated DNA was dissolved in 20µl of TE buffer.

Assay of DNA fragmentation

1. 1 mg of the extracted DNA was mixed well with in 20 mM potassium phosphate buffer, pH 7.4. To this 1 ml of 150mM sodium chloride was added and left at 4°C for 24 hrs for complete solubilisation

2. 100 μg of the above DNA was treated with 1ml of potassium phosphate buffer, the reaction mixture containing FeCl_3 (100 μM), EDTA (104 μM), H_2O_2 (1 mM).
3. To the above mixture two concentrations of BHE (20 and 40 μg) were added. A standard antioxidant BHT was also treated in the same way. A negative control without the fenton reactants were also used in the assay. A positive control without the extract or standard was also taken.
4. All these reaction mixtures were incubated for 37°C for 30 min, and placed on the ice bath for 10 min to stop the reaction.
5. These were then mixed with the loading buffer (0.5% bromophenol blue and 50% glycerol)
6. Agarose gel electrophoresis was carried out by mixing 1% agarose with ethidium bromide (1 $\mu\text{g}/\text{ml}$)
7. About 10 μl of the treated samples were placed in the wells in the following order. Control, positive control, BHE treated sample (20 and 40 μg), standard BHT.
8. The electrophoresis was conducted in TAE buffer, pH 8 at 60 V for 6hr
9. DNA was visualized under a UV transilluminator (Bio-Rad, Sydney, Australia).

RESULTS

Table I shows the preliminary phytochemical analysis of the various extracts. In the present investigation preliminary phytochemical screening of the EAE, PLE and BHE shows the presence of constituents like alkaloid, carbohydrates, phytosterol, tannins, phenol, flavonoids, glycosides, terpenes, saponins and lignin. In all these extracts proteins, gums and mucilage were found to be absent

Figure I to III shows the present chromatographic pattern of ethyl acetate, chloroform and ethanolic extract of *Eclipta alba*. Ethyl Acetate extract showed 14 peaks at Rf values 0.07, 0.14, 0.18, 0.30, 0.41, 0.49, 0.58, 0.63, 0.72, 0.80 and 0.95. Chloroform extract of *Eclipta alba* showed 11 peaks at Rf values 0.07, 0.14, 0.18, 0.30, 0.41, 0.49, 0.58, 0.63, 0.72, 0.80 and 0.95. The ethanolic extract of *Eclipta alba* showed 12 peaks at Rf values 0.12, 0.19, 0.31, 0.43, 0.51, 0.60, 0.65, 0.73, 0.81, 0.86 and 0.90 at 254nm.

Figure IV to VI shows the HPTLC finger printing of the different extracts of *Piper longum*. The ethyl acetate extract of *Piper longum* showed 11 peaks at Rf values 0.03, 0.08, 0.13, 0.23, 0.30, 0.40, 0.51, 0.61, 0.76, 0.88 and 0.94 whereas the Chloroform extract showed 10 peaks at Rf values 0.09, 0.16, 0.24, 0.32, 0.53, 0.63, 0.77, 0.86 and 0.94. The ethanolic extract of the same plant showed 12 peaks at Rf values 0.03, 0.06, 0.11, 0.19, 0.27, 0.37, 0.47, 0.59, 0.69, 0.80, 0.86 and 0.92 at 254nm.

Figure VII shows the SDS-PAGE electrophoresis results of aqueous extract of *Eclipta alba*. The presence of protein bands with

molecular weight of ranging 7.50 – 123 kDa were seen. The *Piper longum* seeds showed only two protein bands in the molecular weight of 30 kDa and 60 kDa.

The values of the macronutrients like carbohydrates, proteins and lipids present in the leaves of *Eclipta alba* and seeds of the *Piper longum* were shown in the Table II. The carbohydrate content present in the seeds of the *Piper longum* was 15.56mgs / 100gms, where as the leaves of *Eclipta alba* contains 27.78mgs/ 100 gms. The protein content was also found to be high in case of leaves of *Eclipta alba* when compared with the seeds of *Piper longum*. The same was applicable to the lipid content also, in which the leaves of *Eclipta alba* contained 24.94mgs/ 100 gms and *Piper longum* contained 3.89mgs/ 100gms respectively.

Table III shows the concentration of different minerals like Ca, K, Na, Mg, Zn, Si, Fe, Al, Va, Mb, Cu, Ni, Hg, Pb, Ba, Mn, Cr, Co, and Se expressed in ppm. The concentration of these minerals in *Eclipta alba* leaves was found to be in the following order, Ca > Na > K > Mg > Al > Si > Zn > Fe > Va > Cu > Ni > Ba > Mg > Cr > Mb > Co > Hg > Pb > and Se. From the values it was clear that the leaves of *Eclipta alba* has got a high calcium content and has a low content of selenium. At the same time the concentration of these minerals in the seeds of *Piper longum* was found to be in the following order, Ca > Na > K > Mg > Zn > Fe > Al > Va > Si > Mb > Cu > Ba > Hg > Ni > Cr > Mn > Co > Pb > Se showing that the *Piper longum* seeds also contained highest content of calcium and lowest content of selenium.

The hydrogen donating activity was measured by using DPPH test. The DPPH radical scavenging activity of EAE, PLE, BHE and the positive control ascorbic acid were shown in the Figure VIII . These extracts quenched the DPPH radicals in a concentration dependent manner. The BHE has got a maximum DPPH scavenging activity when compared with its individual preparations, EAE and PLE. The BHE contained 252mg ascorbic acid equivalents/g extract of activity, with EC_{50} value 139 μ g/ml. The EC_{50} value of the standard ascorbic acid was found to be 188 μ g/ml.

Table IV depicts Super oxide scavenging activity of different plant extracts. The extracts like EAE, PLE, BHE and the positive control ascorbic acid demonstrated a concentration-dependent scavenging activity of super oxide radicals. The inhibitory activity was minimum in low concentration of plant extract and increases with increase in the concentration. The BHE demonstrated a maximum scavenging activity by neutralizing superoxide radicals with EC_{50} value of 165 μ g/ml. The EC_{50} value of the standard ascorbic acid was found to be 182 μ g/ml.

The plant extracts reduced the hydroxyl radical induced deoxyribose cleavage in a concentration dependent manner. The hydroxyl radical scavenging activity of EAE, BHE, PLE and standard mannitol were shown in the Table V. The hydroxyl radical scavenging activity was more for the BHE when comparable with EAE and PLE. The EC_{50} value of BHE was found to be 288 μ g/ml. With this assay, the BHE was found to contain 696 mg mannitol equivalent/g extract in non site-specific model of hydroxyl radical

scavenging The EC_{50} value of the standard mannitol was found to be $460\mu\text{g/ml}$.

Incubation of solution of sodium nitroprusside in phosphate buffered saline at $25.8\text{ }^{\circ}\text{C}$ for 150 min resulted in generation of nitric oxide. Figure IX shows the Nitric oxide radical scavenging activity of EAE, BHE, PLE and the positive control Rutin. The BHE effectively reduced the generation of nitric oxide radicals, when compared to its individual preparations EAE and PLE. The scavenging of Nitric oxide by BHE was concentration dependent and the EC_{50} value was found to be $431\mu\text{g/ml}$. The BHE was found to contain 415.22 mg/gm equivalent of Rutin. The EC_{50} value of the standard Rutin was found to be $616\text{ }\mu\text{g/ml}$.

Table VI depicts the reducing capability of BHE (measured at 700 nm) relative to BHT, a well-known antioxidant. The reducing potential of the extracts increased in a dose-dependent manner. The absorbance values of the extract at different concentrations were found to be less than that of the reference compound.

In the present investigation the BHE effectively scavenged the H_2O_2 radicals by 78.00% and 82.03% in the concentration of 100 and 200 micrograms where as the vitamin E inhibited only $65.89\pm 0.89\%$ and $75.96\pm 0.56\%$ at the same concentration which was shown in the Table VII. The scavenging potential of EAE was 55.56% and 58.90% at the concentration of 100 and 200 micrograms per ml where as PLE exhibited 63.00% and 64.00% respectively.

Table VIII shows the chelating ability of the BHE for metal transition ions (Fe^{2+} , Fe^{3+}) that increases in a dose dependent manner when compared with control. The metal chelating activities of BHE on Fe^{2+} , Fe^{3+} was 56.08% and 55.19% respectively at 100 μg concentration respectively. EDTA exhibited 78.64% chelation for Fe^{2+} and 85.42% for Fe^{3+} respectively. The phenolic compound may contribute directly to antioxidative action. Based on these results, it might be concluded that BHE is an electron donor capable of neutralizing free radicals.

Table IX shows the changes in the absorbance under the influence of different concentrations of the extract (100-1000 $\mu\text{g}/\text{ml}$) at 37 °C compared to BHT as a positive control. The total antioxidant activities of the plant extract were measured using ferric thiocyanate test, which determines the amount of peroxide produced at the initial stage of lipid peroxidation. According to this the extent of inhibition of lipid oxidation is moderate at low (100 $\mu\text{g}/\text{ml}$) doses of BHE. However, at higher concentrations (800 and 1000 $\mu\text{g}/\text{ml}$), BHE suppressed lipid oxidation to a considerable extent when comparable with its individual preparation EAE and PLE. Lower absorbance indicates a higher level of antioxidant activity.

The protein oxidation was determined in terms of inhibition of protein carbonyl formation (PCO). As shown in Table X, BHE dose-dependently exhibited inhibitory effects of PCO formation. At the same time the individual preparation EAE and PLE exhibited lower inhibitory effects of PCO formation at the same concentration. The oxidative protein damages, provoked by free radicals, have been demonstrated to play a significant role in aging and several pathological events. Radical mediated damages to proteins

might be initiated by electron leakage, Major molecular mechanisms, leading to structural changes in proteins are free-radical mediated protein oxidation characterized by carbonyl formation (PCO).

The antioxidant activity of BHE is probably due to its phenolic contents. Flavonoids are a class of secondary plant phenolics with powerful antioxidant properties. Therefore, it would be valuable to determine the total phenolic and flavonoid content of the plant extracts. The extracts were investigated regarding their composition by different colorimetric techniques, such as the content of total phenolic compounds by the Folin– Ciocalteu's assay and flavonoids by AlCl_3 reagent. The total phenolic and flavonoid contents of the plant extracts, were expressed in terms of gallic acid and catechin equivalents. Total phenolic and Flavonoid contents of each gram of dried BHE were estimated to be equivalent to 73.4 mg gallic acid and 55.4 mg catechin which was shown in Table XI. The antioxidant activity of BHE is probably due to its phenolic content and the secondary plant phenolics the flavonoids.

The inhibitory action of the BHE on DNA fragmentation of the isolated hepatocytes were shown in the Figure X. In the CCl_4 intoxicated group II animals the extent of DNA damage was detected by the increased mobility of the DNA molecule due to the decrease in the molecular weight which was shown in the lane 2. On the other hand in the BHE at the concentration of $20\mu\text{g/ml}$ and $40\mu\text{g/ml}$ protected the DNA from damage which was shown in the lane 3 and 4. This was evidenced by the decreased mobility of the DNA molecule in the agarose gel electrophoresis. The protective effect of BHT the standard antioxidant was shown in lane 5.

DISCUSSION

Reactive oxygen species (ROS) and free transition metal ions cause extensive oxidative damage to cellular biomolecules such as DNA, proteins and lipids. Consequently, they contribute to the pathogenesis of oxidative stress-related diseases (Droge 2002; Hippeli and Elstner, 1999). Although synthetic antioxidants seem to be promising, their toxicity and unwanted side effects rules out their extensive prescription. Hence, there is great interest in the use of naturally occurring antioxidants for treatment or prophylaxis of various oxidative stress-related diseases (Maxwell, 1995). The administration of an antioxidant source comprising of multiple components could offer protection against cancer (Black *et al.*, 1995) and combat oxidative stress-induced physiological malfunctions.

ROS include free radicals such as superoxide $\bullet\text{O}$, hydroxyl radical ($\bullet\text{OH}$) as well as non radical species such as hydrogen peroxide (H_2O_2) (Cerutti, 1991). *In vivo*, some of these ROS play a positive role as energy production, phagocytosis, regulation of cell growth and intracellular signaling (Halliwell and Gutteridge, 1999). On the other hand, ROS are also capable of damaging a wide range of essential biomolecules such as proteins, DNA and lipids (Farber, 1994). ROS are not only strongly associated with lipid peroxidation resulting in deterioration of food materials, but also are involved in development of a variety of diseases including aging, carcinogenesis, coronary heart disease, diabetes and neuro degeneration (Harman, 1980; Moskovitz *et al.*, 2002) Cells have several antioxidant defense mechanisms that help to prevent the destructive effects of ROS. These defense

mechanisms include antioxidative enzymes, such as superoxide dismutase, catalase, and glutathione peroxidase and of small molecules such as glutathione, vitamins C and E (Fridovich, 1999). The efficiency of the antioxidant defense system is altered under pathological conditions and, therefore, the ineffective scavenging and/or overproduction of free radicals may play a crucial role in determining tissue damages (Aruoma, 1994).

Substances termed antioxidants can influence the oxidation process through simple or complex mechanisms including prevention of chain initiation, binding of transitional metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, and radical scavenging (Shahidi and Wanasundara, 1992). Antioxidants are believed to play an important role in preventing or alleviating chronic diseases by reducing the oxidative damage to cellular components caused by ROS (Ceriello, 2003). There is growing interest in natural phenolic antioxidants, present in medicinal and dietary plants, that might help attenuate oxidative damage (Silva *et al.*, 2005). These natural antioxidants not only protect food lipids from oxidation, but may also provide health benefits associated with preventing damages due to biological degeneration.

High consumption of fruits and vegetables is associated with low risk for these diseases, which is attributed to the antioxidant vitamins and other phytochemicals. Polyphenols are especially important antioxidants, because of their high redox potentials, which allow them to act as reducing agents, hydrogen donors and singlet oxygen quenchers (Kahkonen *et al.*, 1999). In addition, they have metal chelating potentials (Rice *et al.*, 1995).

The antioxidant activity of the dietary polyphenolics is considered to be much greater than that of the essential vitamins, therefore contributing significantly to the health benefits of fruits and vegetables. The phytochemical screening of these plant extracts were carried out because compounds in their natural formulations are more active than their isolated preparations.

The phytochemical screening showed, that the leaves of *Eclipta alba* and seeds of *P. longum* were rich in alkaloids, flavonoids, tannins, steroids, terpenoids, glycosides and lignins. The medicinal value of these plants lies in some chemical substances that produce a definite physiological action on the human body. The most important of these bioactive constituents of plants are alkaloids, tannins, flavonoids, and phenolic compounds They were known to show medicinal activity as well as exhibiting physiological activity (Sofowara, 1993). The *in vivo* hepatoprotective activity was mainly due to the presence of these phytochemicals. The medicinal values of plants and vegetables are indicated by their phytochemical and other chemical constituents (Fallah *et al.*, 2005)

Micronutrients consist of vitamins and minerals required by the body in small quantities. They function as essential cofactors in the numerous enzyme catalyzed reactions and their absence can result in impairment of metabolic functions which can lead to serious diseased conditions. Sodium together with potassium assists in the maintenance of the body's electrolyte and water balance. In addition, potassium and sodium plays an important role in nerve conduction, muscle contraction, and the transport of substances across membranes. Supplementation of BHE to CCl₄ rats significantly

maintained acid-base balance by increasing the absorption of electrolytes and minerals from intestine and inhibited electrolytes elimination through urine (Damodara Reddy *et al.*, 2007).

The presence of selenium an important mineral in the BHE is responsible for its antioxidant activity. The main function of selenium is as an antioxidant in the enzyme selenium-glutathione peroxidase. The antioxidant properties of selenoproteins help prevent cellular damage from free radicals, regulate thyroid function and play a role in immune system (Mckenzie *et al.*, 1998; Levander, 1997). Selenium and vitamin E work together synergistically in that they carry out antioxidant and immuno stimulating function better together than individually (Haas, 2001).

Zinc is widely recognized as an essential micronutrient with a catalytic role in over 100 specific metabolic enzymes in human metabolism which plays multiple roles in the perpetuation of genetic materials including transcription of DNA, translation of RNA, and ultimately in cellular division. So supplementation of zinc in the form of plant drug would be helpful in maintaining the normal metabolism of the cells.

Ca salts provide rigidity to the skeleton and calcium ion plays an important role in many metabolic processes. Many neuromuscular and other cellular functions depend on the maintenance of the ionized calcium concentration in the extracellular fluid. Calcium fluxes are important mediators of hormonal effects on target organs through several intracellular signaling pathways (FAO/WHO, 1998) Phosphorous is also important in bone

formation and many essential metabolic activities in the body such as phosphorylation reactions. Mg plays an important role in the metabolism of calcium (Al-Ghamdi *et al.*, 1994). Soft tissue magnesium functions as a cofactor of many enzymes involved in energy metabolism, protein synthesis, RNA and DNA synthesis, and maintenance of the electrical potential of nervous tissue and cell membranes. The presence of these minerals in the plant extract might be responsible for the overall free radical scavenging properties. The important minerals and vitamins found in the plant might also be major contributors to the medicinal value of the plant. Mineral elements may have more roles to play, than presently acknowledged, in the synergy of phytochemicals for the health benefit of man.

In future any one of the protein present in the plants under investigation may be targeted for the production of vaccine against liver diseases, since the leaves of *Eclipta alba* and seeds of *P. longum* showed the presence of many proteins. Plants have been actively targeted for the production of medically important proteins, including vaccine antigens and monoclonal antibodies especially against Hepatitis viruses (Mason *et al.*, 1998).

In traditional medicine these two plants are regularly used for the treatment of liver diseases. To clarify the mechanism of action of particularly with respect to its anti-hepatotoxic effects, the antioxidative and free radical scavenging capabilities of the extract were evaluated.

The DPPH radical is a stable organic free radical with absorption of maximum band around 515–528 nm and thus it is a useful reagent for evaluating antioxidant activity of compounds (Sanchez-Moreno, 2002). The BHE demonstrated a concentration dependent scavenging activity by quenching DPPH radicals. It has been documented that cysteine, glutathione, ascorbic acid, tocopherol, polyhydroxy aromatic compounds (e.g., hydroquinone, pyrogallol, gallic acid), reduce and decolorize 1,1-diphenyl-2-picrylhydrazine by their hydrogen donating capabilities (Blois, 1958). The plant is reported to contain the phytoconstituents eclalbatin, alpha-amyrin, ursolic acid, oleanolic acid (Upadhyay *et al.*, 2001) might be responsible for DPPH scavenging activity of the BHE.

Super oxide anions are the most common free radicals in vivo and are generated in a variety of biological systems and the concentration of super oxide anions increases under conditions of oxidative stress (Lee *et al.*, 2002).

In the NBT the BHE demonstrated a concentration-dependent scavenging activity by neutralizing super oxide radicals. Moreover BHE was found to contain 252mg ascorbic acid equivalents/g extract. The earlier photochemical studies indicate the presence of various long-chain esters and amides, alkaloids, lignans, neolignans, amides, terpenes, steroids, chalcones, flavones and flavanones in *P. longum* extract (Parmar *et al.*, 1999; Stohr *et al.*, 2001) could account for the quenching of super oxide radicals.

BHE neutralized hydroxyl radical induced deoxyribose cleavage in a concentration dependent manner. Hydroxyl radicals are extremely reactive

oxygen species capable of modifying almost every molecule in the living cells and also has the capacity to cause strand damages in DNA leading to carcinogenesis, mutagenesis, and cytotoxicity. These are capable of quickly initiating the lipid peroxidation process as by abstracting hydrogen atoms from unsaturated fatty acids (Aruoma, 1998). These results clearly demonstrated the capacity of BHE to quench hydroxyl radicals and also to chelate the iron metal ions. Several unsaturated amides, aristolactams, lignans, long and short chain esters, terpenes, steroids and alkaloids were already been reported in the plants which were used for the preparation of BHE may be responsible for the hydroxyl radical scavenging activity.

In addition to reactive oxygen species, nitric oxide is also implicated in inflammation, cancer and other pathological conditions (Moncada *et al.*, 1991). A potential mechanism of oxidative damage is the nitration of tyrosine residues of proteins, peroxidation of lipids, degradation of DNA and oligonucleosomal fragments (Hemnani and Parihar, 1998). Nitric oxide or reactive nitrogen species, formed during its reaction with oxygen or with super oxide, such as NO_2 , N_2O_4 , N_3O_4 , nitrate and nitrite are very reactive. These compounds alter the structure and function of many cellular components. The BHE was effective in reducing the generation of nitric oxide radicals in our study. Any compound, natural or synthetic, with antioxidant properties might contribute towards the partial or total alleviation of this damage (Lin *et al.*, 1995).

The reducing potential of BHE increased in a dose-dependent manner. The reducing capacity of compound may serve as a significant

indicator of its potential antioxidant activity (Meir *et al.*, 1995). The absorbance values of the extract at different concentrations were found to be less than that of the reference compound. The phenolic compounds may contribute directly to antioxidative action. Based on these results, it might be concluded that BHE is an electron donor capable of neutralizing free radicals. This would have the effect of converting free radicals to more stable products and thus terminating free radical initiated chain reactions. Yahara *et al.*, 1997 reported the presence of phytoconstituents such as triterpene glycoside, saponins like eclalbasaponin, 3 β , 20 β , 16 β -trihydroxytaraxastane; 3 β , 20 β , 28-trihydroxytaraxastane and sulphated saponins in the leaves of *E.alba*. These phytochemicals might be account for the reduction reactions exhibited by BHE.

Hydrogen peroxide is formed by two-electron reduction of O₃ which is not a free radical, but an oxidizing agent. In the present investigation the BHE effectively scavenged the H₂O₂ radicals. Inhibition of free radical induced damage by supplementation of antioxidants has become an attractive therapeutic strategy for reducing the risk of diseases (Brash and Harve, 2002). H₂O₂ can easily cross the cell membrane and exerts an injurious effect on tissues through a number of different mechanisms such as, perturbing intracellular Ca²⁺ homeostasis, increasing intracellular ATP, inducing DNA damage, and cell apoptosis (Mallakckakron *et al.*, 2004). The compounds piperlonguminine, piperine, apigenin, dimethyl ether and β sitosterol were reported by Parmar *et al.*, in the *P. longum* species which was used in the preparation of BHE could be responsible for its H₂O₂ scavenging activity.

A significant result were obtained from BHE on the metal chelating activities of Fe^{2+} and Fe^{3+} . The phenolic compound may contribute directly to antioxidative action. Based on these results, it might be concluded that BHE is an electron donor capable of neutralizing free radicals. The extracts of spices and herbs may well act as electron donors and they can react with free radicals to convert them into more stable products and terminate radical chain reactions. Phenolic compounds in plants act as antioxidants by chelating metal ions, preventing radical formation, and improving the antioxidant endogenous system (Tanaka *et al.*, 1998). The antioxidant activity of BHE is probably due to its phenolic contents. Polyphenolics is a highly inclusive term that covers many different subgroups of phenolic acids and flavonoids. More than 5000 polyphenolics, including over 2000 flavonoids have been identified, and the number is still growing (Harborne, 1996). BHE contained considerable amount of the flavonoid and phenolic compounds. The phenolic compounds exhibit considerable free radical scavenging activities, through their reactivity as hydrogen- or electron-donating agents, and metal ion chelating properties (Rice *et al.*, 1996). According to Pietta, 2000 flavonoids are a class of secondary plant phenolics with powerful antioxidant properties. So the presence of considerable amount of these compounds could be accounted for the free radical scavenging activities.

The total antioxidant activity of BHE was measured using ferric thiocyanate test which determines the amount of peroxide produced at the initial stage of lipid peroxidation. Lower absorbance indicates a higher level of antioxidant activity. Flavonoids exert a protective effect against lipid per

oxidation *in vitro* as free radical scavengers and metal-chelating agents (Afanasev *et al.*, 1989). They generally occur as *O*-glycosides in which one or more of the hydroxyl groups are bound to sugars. This glycosylation renders flavonoids more water soluble, making them store readily in the cell vacuole where they are commonly found (Robards and Antolovich, 1997). The above-mentioned effects might be expected for the BHE in reducing the lipid peroxidation.

The oxidative protein damages, provoked by free radicals, have been demonstrated to play a significant role in ageing and several pathological events (Stadtman and Levin, 2000). Major molecular mechanisms, leading to structural changes in proteins are free-radical mediated protein oxidation characterized by carbonyl formation (PCO). The test drug BHE was effective in reducing the PCO formation. This might be due to the cumulative effect of the phytochemicals present in it.

Oxidative DNA damage has been implicated to be involved in various degenerative diseases (Jenner, 1991) including Alzheimer's disease, Parkinson's disease, Hodgkin's disease and Bloom's syndrome (Imlay and Linn, 1988). The decrease in the Fenton's reaction-mediated degradation of DNA by the presence of BHE suggested that these extracts have compounds which may combat against free radical-mediated degradation to the deoxyribose sugar moiety of DNA.

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HEPATOPROTECTIVE STUDIES

Early tests for pharmacological activity were done with animal models, but numerous methods have not been introduced for testing of biological activity. A related area of interest is the scientific study of “Nutraceuticals” i.e., plants used for food but also taken regularly as a preventive measure against disease.

Worldwide several people are suffering from hepatic damage induced by alcohol and drug abuse. The liver plays a major role in the digestion, metabolism and storage of nutrients. Today an increasing impact of liver disease and liver injury is being recognized. Especially liver injury due to pharmacological treatment plays a significant role. (Gerbes *et al.*, 2006) reported that during recent years new insights have been brought into the pathomechanisms of liver injury. In certain cases this provides the basis for novel therapeutic strategies.

Carbon tetrachloride (CCl₄) is a toxic substance that is used to induce liver damage in rats, and according to (Ohta *et al.*, 1998). CCl₄ by itself does not have cytotoxic effects on the liver but its metabolic products are responsible for the toxicity. CCl₄ can damage a number of tissues particularly the liver and kidney of many species (Drill, 1952). Administration of CCl₄ can cause cirrhosis (Cameron and Karunaratne, 1936) and ultimately lead to hepatic carcinoma (Reuber and Glover, 1970).

It has been reported that CCl₄ intoxication results in the peroxidation of lipids and lipid membranes of rats. Ohta *et al.* (1997) observed an increase in lipid peroxidation (LPO) as a result of CCl₄ treatment. Therefore CCl₄, a hepatotoxin for evaluating hepatoprotective agents, is commonly used to induce liver damage by producing free radical intermediates.

Studies on hepatotoxicity induced by CCl₄ indicated that hepatic damage can probably be prevented by some herbal extracts. Herbs are also known to play a vital role in the management of various hepatic disorders Venkateswaran *et al.*, 1998).

Hepatoprotective studies by Mitra *et al.* (1998) showed that plants have active ingredients that are capable of free radical scavenging in living systems. The dependence of humankind on plants is as ancient as evolutionary history. Plants play a significant role in maintaining human health and improving the quality of human life. Many synthetic antioxidants, such as Butylated hydroxyanisole, butylated hydroxytoluene, t-butylhydroquinone and propyl gallate, are used to retard lipid peroxidation (Wanita and Lorenz, 1996). However, the use of synthetic antioxidants is under strict regulation due to the potential health hazards caused by such compound (Park *et al.*, 2001).

It was therefore decided to investigate the *in vivo*, hepatoprotective efficacy of the ethanolic extracts of *E. alba* and *P. longum* and the combined biherbal formulation made up of equal concentrations of *E. alba* and *P. longum* a traditional Indian medicinal plant, on CCl₄-induced

hepatotoxicity and to elucidate the mechanism underlying these protective effects in rats.

It was also found important to determine the acute toxicity value (LD_{50}) of the Biherbal extract in mice and to determine histologically the chronic toxicity effects of Biherbal extract on the internal organs of rats.

Animals

Adult albino male rats of Wister strain (150-175g) and mice (27-35g) obtained from animal house in Madras Medical College, Chennai, India were used in the pharmacological and toxicological studies. The animals were maintained in well - ventilated room temperature with natural 12 ± 1 hr day-night cycle in the propylene cages. They were fed with balanced rodent pellet diet from Poultry Research Station, Nandanam, Chennai, India and tap water *ad libitum* was provided throughout the experimental period. The animals were sheltered for one week prior to the experiment for getting acclimatized to laboratory temperature. The protocol has got the ethical committee clearance from IAEC (Institute Animal Ethical Committee) of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals).

Chemicals

Ammonium thiocyanate, Ferric chloride, Ferrous sulphate ($FeSO_4$), Potassium ferricyanide [$K_3 Fe(CN)_6$], Potassium thiocyanate, Sodium carbonate, Sodium dodecyl sulphate (SDS), Ethylene diamine tetra acetic acid

(EDTA), Trichloro acetic acid (TCA), Thiobarbituric acid (TAB), Sodium nitroprusside, Sulphanilamide, Phosphoric acid, Naphtyl ethylene diamine dihydrochloride, Reduced Glutathione (GSH), 1-chloro-2,4-dinitrobenzene (CDNB), Glutathione oxidised (GSSG), Epinephrine, Nicotinamide Adenine Dinucleotide Phosphate (NADPH) were obtained from Sd Fine Chemicals Ltd., India. Bovine Serum Albumin (BSA), Folin-Ciocalteu reagent, 5,5-dithio bis(2-nitrobenzoic acid) (DTNB), 2,2'-bipyridyl] were obtained from Sigma Chemical Company, St. Louis, USA. All other chemicals and reagent used were of analytical grade.

Acute toxicity

Biherbal extract (100mg/ml), will be used as a stock solution in the entire study. A stock solution will be prepared by dissolving 100mg in 10 ml of 2% Tween 80 as a vehicle (Adjuvant). Either sex of albino mice, weighing 27 – 35 g will be used in both acute toxicity experiments.

An initial test will be carried out to determine the approximate lethal and non-lethal doses of the Biherbal extract according to R. A. Turner (1965)

1. Acute toxicity test was performed according to the World Health Organization (WHO) guideline (WHO, 2000) and the Organization of Economic Co-operation and Development (OECD) guideline for testing of chemicals (OECD, 2001). An initial test will be carried out to determine the approximate lethal and non-lethal doses of the Biherbal extract according to Turner, 1965.

2. Eight groups of eight mice each will be used in the experiments. The Biherbal extract, in doses of 0.05, 0.1, 0.25, 0.5, 1.0, 2.0, 4.0 and 5.0g/kg body weight respectively will be administered orally, using intragastric tubes, to the animals as a single dose. The control group will be given an equal volume of 2% Tween 80. All the animals will be observed at the first, second, fourth and sixth hours and thereafter once daily over 14 days for (Rhodes, 1999), clinical signs of toxicity such as respiratory pattern, colour of body surfaces, frequency and nature of movement, marked involuntary contraction or seizures of contraction of voluntary muscle, and loss of reflex etc, and the number of dead mice will be recorded and used in the calculation of the acute toxicity value (LD_{50}). The mice will also be observed for other signs of toxicity, such as, excitation, tremors, twitches, motor coordination, righting reflex and respiratory changes. Twenty-four hours after the oral dosing the animals were lightly anaesthetized with ether and blood was withdrawn from the orbital plexus. They were then killed by cervical dislocation and vital organs were dissected out. Organ to body weight ratio, various haematological and biochemical variables were studied. Tissues of vital organs *viz.*, lung, liver, kidney, spleen, heart and testis or ovaries were fixed in 10% buffered formalin for microscopic examination. Standard procedures were used for the analysis of haematological, biochemical and histological parameters.

Chronic toxicity study

Male and female Wistar rats were kept in polypropylene cages with maximum of four animals per cage at constant conditions. Temperature was

set to $23 \pm 2^\circ \text{C}$, relative humidity to 60 [+ or -] 10%. The animals were kept under artificial illumination with a photoperiod of 12 h. Water was available *ad libitum*. Each cage was labelled with the allocation of the animals to the respective trial group, and the diet to be used. Whenever more than one animal was kept in a cage, the animals obtained colour markings for differentiation. The cages were tended at regular intervals. The care and treatment of the rats were in accordance with the institutional ethical committee guidelines. All the animal experimentation was carried out with the prior approval from CPCSEA. Basic diet was dry pellet feed (Sai Meera Foods, Bangalore).

Thirty six weanling rats (18 female, 18 male) with an average initial body weight of 100 (female rats) and 110g (male rats) were allocated to three groups. Each group consisted of six male and female randomly allocated animals. The first group served as control and obtained standard diet. The second group obtained diet with Biherbal extract 25mg/kg, the third group obtained diet with Biherbal extract 50mg/kg. After 3 months, four male and four female animals of each group were sacrificed for toxicity testing. The remaining two male and female animals were set to standard diet and observed for additional 4 weeks in order to detect deviations in spontaneous behaviour or growth parameters prior to sacrificing and submission to the same toxicological examination.

Twenty-four hours before the animals were sacrificed, blood samples were taken for the examination of haematological and biochemical parameters. Laboratory parameters such as blood glucose, total blood protein,

ALT, AST and total cholesterol, red and white blood cell count and differential leucocyte count were carried out according to standard procedures. The animals were killed by cervical decapitation under ether anaesthesia. The major organs (liver, lungs, kidneys, heart, testis and ovaries) were removed and macroscopically examined for physiological abnormalities. The organs were then submitted to histological examination.

HEPATOPROTECTIVE EFFICACY STUDIES

Experimental protocol

The rats were divided into seven groups of six animals each and were given dose schedule as given below:

- Group 1 : Animals were given a single administration of 0.5 ml vehicle 2% v/v aqueous Tween-80 p.o daily for 14 days. This group served as normal control.
- Group 2 : Animals were given a single dose of 2ml/kg, p.o CCl₄ in 2% v/v aqueous Tween-80 daily for 7 days.
- Group 3 : Animals were pre-treated with 50 mg/kg, p.o of BHE in 2% v/v aqueous Tween-80 for 14 days intoxicated with CCl₄ on days 7 to 14.
- Group 4 : Animals were pre-treated with 50 mg/kg, p.o of MAE in 2% v/v aqueous Tween-80 for 14 days intoxicated with CCl₄ on days 7 to 14.

- Group 5 : Animals were pre-treated with 50 mg/kg, p.o of PLE in 2% v/v aqueous Tween-80 for 14 days intoxicated with CCl₄ on days 7 to 14.
- Group 6 : Animals received 50 mg/kg, p.o Silymarin in 2% v/v aqueous Tween-80 daily for 14 days and administered with CCl₄ on days 7 to 14. This group served as positive control.
- Group 7 : Animals received only 50 mg/kg, p.o of BHE in 2% v/v aqueous Tween-80 daily for 14 days.

On the 15th day after the experimental period the body weight of the animals were recorded. The animals were sacrificed after 12 hrs fasting under mild pentobarbitone anaesthesia. Blood was collected and the serum was separated by centrifuging at 3,000 rpm for 10 min. The serum was used for analysing various biochemical parameters. A portion of blood was used for certain biochemical estimation. Liver was excised from the animals, washed in ice-cold saline, and dried gently on the filter paper. The weight of the liver was taken. A 10% liver homogenate was prepared in Tris HCl buffer [0.1M pH 7.4]. The homogenate was centrifuged and the supernatant was used for the assay of various parameters.

Biochemical analysis - Blood and Serum

Estimation of Glucose

The blood glucose was estimated by Orthotoluidine method by Hyvarimen and Nikkila (1962).

Reagents

1. 10% TCA
2. Orthotoluidine reagent: 12.5gms of thiourea and 12.0 gms of boric acid were dissolved in 50ml of distilled water by heating. 75ml of Orthotoluidine (redistilled) and 375 ml of glacial acetic acid were mixed separately. These two solutions were mixed and the total volume was made up to 500ml with distilled water. The reagent was left overnight in the refrigerator and filtered.
3. Glucose standard: 100mg of pure glucose was dissolved in 100ml-distilled water containing 0.01% benzoic acid.

Procedure

To 0.1ml whole blood 1.0 ml of 10% TCA was added. The above mixture was centrifuged to precipitate the proteins. To 1ml of the above supernatant 4.0 ml of orthotoluidine reagent was added. Aliquots of standard containing different concentrations of glucose were made up to 1.0ml with water and 4.0 ml of orthotoluidine reagent was also added. Mixed well and kept in boiling water bath for 8mts. Cooled and read at 610nm against the blank which contained 1ml of water and 4.0 ml of orthotoluidine reagent. The values were expressed as mg/dl blood.

Estimation of Urea

Blood urea was determined by the method of Bousquet *et al.* (1971).

Reagents

1. 10% Sodium tungstate
2. 2/3 N Sulphuric acid
3. DAM-TSC (Diacetyl monoxime –Thiosemicarbazide) reagent: 36mM Diacetyl monoxime and 61.7mM thiosemicarbazide were dissolved in 100 ml of 2% glacial acetic acid.
4. Acid ferric reagent: 3.6ml sulphuric acid, 0.12mg of ferric chloride and 38.6ml Orthophosphoric acid were mixed and made upto 100ml with distilled water.
5. Standard Urea: 10mg of urea was dissolved in 100ml of distilled water.

Procedure

In a test tube 0.1ml of blood was taken. To that 3.3 ml of water, 0.3ml of 10% sodium tungstate and 0.3ml of 2/3N sulphuric acid were added to precipitate the proteins. The mixture was centrifuged and to 1ml of the supernatant fluid 1 ml of water, 0.4 ml of DAM reagent and 1.6ml of acid ferric reagent were added and placed in a boiling water bath for 30 minutes. Aliquots of the standard urea and blank containing 2.0ml of water were also treated in the similar manner. After cooling the colour developed was read at 520nm using spectrophotometer.

The values were expressed as mg/dl.

Estimation of Bilirubin

Serum bilirubin was estimated by the method of Malloy and Evelyn (1937).

Reagents

1. Absolute methanol
2. 1.5% HCl
3. Diazo reagent

Solution A: Dissolve 1gm of sulphanilic acid in 15ml conc. HCl and made up to 1 litre with distilled water.

Solution B: 0.5gm of sodium nitrite dissolved in 100ml distilled water.

Solution C: Mixed 10ml of solution A and 0.3ml of solution B.

4. Stock Standard: 10 mg of bilirubin was dissolved in 100ml of chloroform.
5. Working standard: Stock solution was diluted 1 in 5 times.

Procedure

In a test tube 0.2 ml of serum was taken, and 1.8ml of distilled water was added. A blank was set up by adding 0.2ml of 1.5% hydrochloric acid. To all the test tubes 1.0 ml of diazo reagent was added. Finally to all the test tubes 2.5 ml of methanol, was added and allowed to stand at room temperature for 30 mts in dark and read at 540nm. For the standard curve, pipetted out various concentrations of bilirubin in to a series of test tubes, made up the volume in all the tubes with 2.5ml of methanol and added 1ml of diazo reagent was added and read colorimetrically.

Estimation of Total Protein

Estimation of total protein was carried out by the method of Gornall (1949).

Reagents

1. Biuret reagent. Weighed 1.50 gm of cupric sulphate and 6.0 gm of sodium potassium tartrate transferred to a dry 1 litre volumetric flask, and dissolved it in about 500 ml of water with constant swirling. Added 300 ml of 10 per cent sodium hydroxide. Finally the volume was made up to 1 litre with water, mixed, and stored in a cool dry place.
2. 22.6% sodium sulphate.
3. Std Protein solution: 400mgs of BSA was dissolved in 100 ml of distilled water.

Procedure

In to the test tube marked as “B” pipetted 2.0 ml of sodium sulphate solution. In to the “t” test tube, 0.5 ml. of serum and 9.5 ml of sodium sulphate were taken. Stopped the tube and mixed thoroughly by inversion. From that mixture 2 ml was transferred to another test tube. Standard protein solution was also treated in the same way. Now into each of the three test tubes pipetted 8.0 ml of biuret reagent and mix thoroughly. Allowed these to stand for 30 minutes at room temperature. Using a photoelectric colorimeter, or spectrophotometer, the colour intensity was measured at 540 nm.

The amount of serum protein was expressed as g / dl.

Estimation of Albumin

The albumin present in the serum was estimated by the method of Reinhold (1953).

Reagents

1. Biuret reagent – Prepared as mentioned earlier
2. Ether
3. Sulphate – Sulphite Reagent: About 20.8g of sodium sulphate and 7.0g of sodium sulphite were dissolved in about 90ml water, with constant stirring. Then 0.02ml of conc. H_2SO_4 was added and made up to 100ml with distilled water.

Procedure

To 0.4ml of serum, 5.6ml of sodium sulphate-sulphite reagent and 3ml ether was added, the test tubes were stoppered and shaken well. Care should be taken not to shake more vigorously, otherwise the albumin may denature. The tubes were capped and centrifuged for 5mts. The pipette was inserted into the clear solution carefully below the globulin layer, and 3ml of solution was taken, 5ml of biuret reagent was added and incubated for 30 min at room temperature. The violet color was read at 540nm.

The amount of serum albumin was expressed as g/dl.

Estimation of Globulin

Albumin was subtracted from the total protein to obtain the amount of globulin. The amount of serum globulin was expressed as g/dl.

Estimation of Cholesterol

Estimation of serum cholesterol was carried out by the method of Wybenga *et al.* (1970).

Reagents

1. Cholesterol reagent. Dissolved 520 mg of Ferric per chlorate in 600 ml ethyl acetate, contained in a 1-liter Erlenmeyer flask. Placed the flask in an ice bath and cooled the contents to 4°C added gradually 400 ml of cold concentrated sulphuric acid. in small portions, mixed after each portion is added, and not allowed the temperature to exceed 45°C.
2. Cholesterol standard: 200 mgs of cholesterol per 100 ml of glacial acetic acid.

Procedure

Added 50 µl of cholesterol standard and 50µl of serum was taken to the vials marked “Standard” and “Test,” respectively, and added 5 ml of cholesterol reagent mixed the contents of each vial thoroughly for at least 10 seconds. Cooled and the absorbance is read in the spectrophotometer at

560 nm against the blank, which contained 50 μ l of glacial acetic acid and 5 ml of cholesterol reagent.

Cholesterol concentration was expressed as mg/ dl of serum.

Estimation of Triglycerides (TGL)

The triglycerides were estimated by the method of based on the method of Rice and Vanhandle (1970).

Reagents

1. Chloroform: methanol mixture 2:1 (v/v)
2. Saturated sodium chloride
3. Activated silicic acid: Silicic acid was washed with 4N HCl and then distilled water until the pH was neutral. After drying, ether was added in sufficient amount, stirred well and the supernatant was decanted. Silicic acid was then dried at 60°C and activated at 100°C over night prior to use.
4. 0.4% potassium hydroxide in ethanol.
5. Sodium meta periodate 0.1 M: 2.149 g of sodium meta periodate was dissolved in 100 ml of water.
6. Sodium meta arsenate 0.5M: 6.496 g sodium meta arsenate was dissolved in 100 ml of water.

7. Chromotropic acid : 1.14 g of chromotropic acid was dissolved in 100 ml of distilled water and stored as a stock solution in a brown bottle. Before use this solution was mixed with 45ml of sulphuric acid – water mixture in the ratio of 2:1.
8. 0.2N sulphuric acid
9. Tripalmitin standard : 100 mg of Tripalmitin was dissolved in 100 ml of chloroform in a standard flask. The stock solution was diluted to 1 in 10 times with chloroform.

Procedure

About 0.2ml of serum was mixed with 9.8 ml of Chloroform:methanol mixture and left for 30 min. It was centrifuged and 4ml of the lipid extract was added to the tubes containing 8ml of saturated sodium chloride and shaken vigorously. The contents were allowed to settle for an hour and centrifuged. The supernatant containing saline – methanol phase was discarded. The washed chloroform phase was filtered in to a dry tube. 200mg of silicic acid was added to chloroform phase shaken vigorously and allowed to stand for 30 min. It was centrifuged. 0.5 ml of supernatant was taken, to which 0.5 ml of potassium hydroxide solution was added and the mixture was saponified in a water bath at 60°C for 20 min, to the above mixture, 0.5ml of 0.2 N sulphuric acid was added and kept in a boiling water bath for 10 min. After cooling the tubes 0.1ml of sodium meta periodate was added and allowed to stand for 10 min. The excess sodium meta periodate was reduced

by the addition of 0.1ml sodium meta arsenate, finally 0.5 ml of Chromotropic acid reagent was added, mixed thoroughly and kept in a boiling water bath for 30 min. The colour developed was estimated at 540nm.

Triglycerides concentration was expressed as mg/ dl of serum.

Estimation of Phospholipids (PL)

The phospholipids content in serum was determined by the method of Zilversmith and Davis (1950).

Reagents

1. Ethanol- ether 3:1(V/V mixture)
2. 3% Ammonium molybdate
3. 3% Ascorbic acid
4. 70% Perchloric acid
5. Standard Phosphate: 35.1 mg of potassium dihydrogen phosphate was dissolved in 100ml of water to give a concentration of 80 µg/ml.
6. Working Standard solution: A concentration of 8 µg/ml was prepared by diluting the stock solution in the ratio of 1: 10 with distilled water.

Procedure

To 1.0 ml of serum, 3.0 ml of the ethanol- ether mixture was added and mixed well. The protein precipitate was separated by centrifugation and the supernatant solvent was transferred to another tube. 0.1ml of the lipid extract was dissolved in 1ml of perchloric acid and digested on a sand bath till the solution becomes colourless. After cooling the solution was made up to 5.0ml with double distilled water. In the standard 1ml of working standard solution was taken. Blank contained 1ml of water. To all the tubes 0.5ml of ammonium molybdate and ascorbic acid were added and the mixture was kept in a boiling water bath for 6 mts. The blue color developed was read at 710 nm using a spectrophotometer. The values were expressed as mg/dl of serum after multiplication by factor 25.

Serum Lipoproteins

Fractional precipitation of lipoproteins

Lipoproteins were fractionated by a dual precipitation technique of Wilson and Spiger (1973).

High-density lipoprotein (HDL)

Reagents

1. Heparin –manganese chloride reagent: 3.167 g of manganese chloride was added to 1ml solution of heparin containing 20,000 units. This mixture was made up to 8ml with distilled water.

Procedure

About 2ml serum was added to 0.18ml heparin- manganese chloride reagent and mixed well. The solution was allowed to stand at 4°C for 30minutes and then centrifuged at 2000 rpm and maintained at 10°C for 30 minutes. The supernatant contained the HDL fraction. 1ml of this was used for the estimation of cholesterol by the method of Wybenga as described previously.

HDL- Cholesterol concentration was expressed as mg/ dl of serum.

Low- density lipoprotein (LDL)**Reagents**

1. Sodium- dodecyl sulphate (SDS): 10% solution of SDS was prepared in 0.15 M sodium chloride and the pH was adjusted to 9 with sodium hydroxide.

Procedure

To 2ml of serum was added to 0.15ml of sodium-dodecyl sulphate. The contents were mixed well and incubated at 37°C for 2 hrs. The contents were centrifuged in a refrigerated centrifuge at 10,000 rpm for 15 mts. The VLDL aggregated as pelleted. The supernatant contained the HDL and LDL fractions. 1ml of this was used for the estimation of cholesterol by the method of Wybenga as described previously.

LDL-Cholesterol concentration was expressed as mg/ dl of serum.

Very low-density lipoprotein (VLDL)

The pelleted portion contains the VLDL cholesterol. 1ml of this was used for the estimation of cholesterol by the method of Wybenga as described previously.

VLDL- cholesterol was expressed as mg/ dl.

Assay of serum Aspartate amino transferase (AST) (Glutamate oxaloacetate transaminase, E.C.2.6.1.1)

Assay of serum aspartate amino transferase was carried out by the method of Reitman and Frankel (1957).

Reagents

- a. Phosphate buffer - 0.1M, pH 7.4.
- b. Substrate: 2.66 gms of DL aspartate and 38 mg of α -keto glutartate were dissolved in 20.5ml of 0.1N NaOH with gentle heating. This was made up to 100 ml with buffer.
- c. 2,4-Dinitrophenyl hydrazine reagent (DNPH): 1.0mM DNPH in 2.0 N HCl.
- d. 0.4 N NaOH
- e. Standard Pyruvate: 11 mg of sodium pyruvate was dissolved in 100ml of phosphate buffer. This contained 1 μ mole pyruvate/ml.

Procedure

In different tubes 1.0 ml of the buffered substrate was added. To one tube 0.1ml of serum was added and incubated at 37° C for 1 hr. Then 1ml of DNPH reagent was added to arrest the reaction. To the 'Blank 'tube 0.1ml of serum was added only after the addition of DNPH reagent. The tubes were kept aside for 15 minutes, and then 0.4 N NaOH was added and read at 520 using the spectrophotometer. Aliquots of standard were also treated in the same manner.

Enzyme activity was expressed as U / Litre.

Assay of serum Alanine amino transferase (ALT) (Glutamate pyruvate transaminase, E.C.2.6.1.2)

The reagents and method used were same as those used for the assay of aspartate amino transferase except for the substrate solution and incubation time was reduced for 30 minutes.

Reagents

1. Substrate: 1.78 gms of DL alanine, 38 mg of α -ketoglutarate were dissolved in phosphate buffer, and 0.5ml of 0.1 N NaOH was added and the volume was made up to 100ml with buffer.

Enzyme activity was expressed as U / Liter.

Estimation of Alkaline Phosphatase (ALP) (ortho-phosphoric monoester phosphohydrolase, E.C. 3.1.3.1)

The serum alkaline phosphatase was assayed by the method of Kind and King (1954).

Reagents

1. Carbonate – bicarbonate buffer 0.1M, pH 10.0: 6.36gm of sodium carbonate and 3.36gm of sodium bicarbonate were dissolved in 1000 ml of distilled water.
2. Substrate 0.01M: 254mg of disodium phenyl phosphate was dissolved in 100 ml of water.
3. 0.5 N Sodium bicarbonate
4. 0.5N NaOH
5. 4-Amino-antipyrine (0.6%): 0.6 g was dissolved in water and made to 100 ml.
6. Potassium Ferricyanide (2.4%): 2.4 g was dissolved in water and made to 100 ml.
7. Stock Phenol Standard (1 mg/ ml): Pure crystalline phenol, 1 g was dissolved in and made to 1 liter with 0.1 N HCl.
8. Working Phenol Standards (0.01 mg/ml) 1 ml, stock standard was diluted to 100 ml with 0.1 N HCl.

Procedure

For the enzyme estimation four test tubes were taken and they were marked as “B” (Blank), “T” (Test), “C” (Control) and “S” (Standard). In the “T” test tube, 1 ml. buffer were added to 1 ml of substrate and warmed at 37°C for three min. Then 0.1 ml of serum were added and mixed. The solution was incubated at 37° C for 15 min. To the incubated solution 0.8 ml of Sodium hydroxide and 1.2 ml of sodium bicarbonate were added. Then 1 ml 0.6% 4-Amino-antipyrine was added and mixed. Finally 1 ml Potassium Ferricyanide was added and mixed. The Control was treated same as test, except that serum was added after the NaOH addition. Aliquots of standard was also treated in the similar manner. Blank was set up same as standard, but water is substituted for phenol. The colour developed was measured at 620 nm using the colorimeter.

The enzyme activity was expressed as IU/litre.

Estimation of Acid Phosphatase (ACP) (ortho-phosphoric monoester hydrolase, E.C.3.1.3.2)

The serum acid phosphatase was assayed by the method of Kind and King (1954).

Reagents

1. Citrate Buffer 0.1M, pH 4.8.

2. Substrate 0.01M: 254mg of disodium phenyl phosphate was dissolved in 100 ml of water.
3. 0.5 N Sodium bicarbonate
4. 0.5N NaOH
5. 4-Amino-antipyrine (0.6%): 0.6 g was dissolved in water and made to 100 ml.
6. Potassium Ferricyanide (2.4%): 2.4 g was dissolved in water and made to 100 ml.
7. Stock Phenol Standard (1 mg/ ml): Pure crystalline phenol, 1 g was dissolved in and made to 1 liter with 0.1 N HCl
8. Working Phenol Standards (0.01 mg/ml): 1 ml, stock standard, diluted to 100 ml with 0.1 N HCl.

Procedure

Four test tubes were taken and they were marked as “B” (Blank), “T” (test), “C” (Control) and “S” (Standard). In the “T” test tube, 1 ml of buffer was added to 1 ml of substrate warmed at 37° C for three min. Then 0.1 ml of serum was added and mixed. The solution was incubated at 37°C for one hour. To the incubated solution 1.0 ml of Sodium hydroxide and 1.0 ml of sodium bicarbonate, 1 ml 0.6% 4-Amino-antipyrine was added and mixed. Finally 1 ml Potassium Ferricyanide was added and mixed. The Control was treated same as test, except that serum was added after the NaOH. Aliquots of standard were made up of 1.1 ml in alkaline buffer and treated similarly.

Blank was set up same as standard, but water was substituted for phenol. The colour developed was measured at 620 nm using the colorimeter.

The enzyme activity was expressed as K.A units.

Estimation of Lactate dehydrogenase (LDH) (L-lactate:NAD oxido-reductase, E.C.1.1.1.27)

The Lactate dehydrogenase present in the serum was assayed by the method of King (1965).

Reagents

1. Glycine buffer 0.1M, pH 8.5: 750.5mg of glycine and 585mg of NaCl were dissolved in 100ml of distilled water.
2. Buffered Substrate: 125ml of glycine buffer, 75ml of 0.1N NaOH and 4.0g of lithium lactate were added, mixed well and kept in cold room.
3. Nicotinamide adenine dinucleotide (NAD⁺): 10mg of NAD⁺ was dissolved in 2.0ml of water.
4. 2, 4- Dinitrophenyl hydrazine (DNPH) 0.02%: 20mg of DNPH was dissolved in 100ml of 1N HCl.
5. 0.4N NaOH
6. Standard: 11mg of sodium pyruvate was dissolved in 100ml of buffer. This contained 1 μ mole of pyruvate/ ml.

Procedure

Pipetted out 1.0ml of the buffered substrate and 0.1ml of serum into two tubes and 0.2ml of distilled water was added to the blank, to the test, 0.2ml of NAD^+ solution was added and shaken well. The tubes were incubated at 37°C for 15mts. Exactly after that time the reaction was stopped by adding 1.0ml of DNPH solution. NAD^+ was added to the control tubes. It was left at 37°C for another 15mts. About 5.0ml of 0.4N NaOH was added and the color developed was read at 420nm within 5mts. A set of standards were also treated in a similar manner.

The enzyme activity was expressed as U/L.

Estimation of Acetyl cholineesterase (ACE) (Acetylcholine acetylhydrolase, E.C.3.1.1.7)

Determination of Acetylcholine esterase was carried out by the method of Biggs *et al.* (1958).

Reagents

1. Stock buffer; Dissolved 12.37 gms of Sodium barbitone, 1.361 gms of Potassium dihydrogen phosphate and 175.35 gms of sodium chloride in water and made to a litre
2. Stock–buffer indicator solution: Dissolve 100mg of bromothymol blue in 2 ml of 2 N NaOH and washed in to the 1 litre flask with 150 ml of stock buffer and diluted to 950 ml

with water. Adjust the p H to 8.0 by adding 16 ml of HCl and then diluted to 1 litre with water.

3. Working–buffer indicator solution: Diluted 476.2ml of the Stock–buffer indicator solution to 1 litre with water.
4. Standard acetyl choline bromide solution: (15 %) 15 gms acetyl choline bromide dissolved in 100ml of water.
5. Acetic acid, 0.15 N: Diluted 1 in 10 for use.

Procedure

Measured 4.2 ml of Working –buffer indicator solution in to a suitable tube and added 0.1 ml of serum and 0.2 ml of substrate. Mixed thoroughly and read the absorbance at 620 nm. Incubated at 37° C for 30 minutes and read again. Units of enzyme activity were expressed as micromoles of acetic acid liberated from acetylcholine by 1 ml of serum in 30 minutes. To obtain the calibration curve a series of dilutions of acetic acid to water was prepared which corresponds to 0 –150 units of enzyme activity in steps of 10 units. A series of these tubes containing 2ml of stock –buffer indicator solution, 1.4ml of water, 0.1ml of normal serum and 1ml of above series of diluted acetic acid was added and treated as the same way as test. A standard curve was plotted from which units of activity was calculated.

Enzyme activity expressed as U/l.

Estimation of Gamma glutamyl transferase (γ GT) (5 Glutamyl peptide amino acid 5 glutamyl transferase, E.C. 2.3.2.2)

The assay of γ -Glutamyl transferase was carried out by the method of Szasz (1969).

Reagents

1. Substrate; 30.4 mg of L- γ glutamyl-p-nitro anilide was added to 10 ml of water, heated to dissolve at 50- 60° C.
2. Tris-HCl buffer : 0.1M , pH 8.5.
3. Glycyl-glycine; 13.2 mg was dissolved in 10 ml of water
4. Standard: 13. 8 mg of p-nitro anilide was dissolved in 100ml of distilled water.
5. 10 % acetic acid

Procedure

Into three test tubes marked as “B”, “C” and “T” 1.0 ml of buffer and 2.2 ml of glycyl glycine was added. 0.5ml of substrate solution was added in the “B” and “T” test tubes. About 0.2ml of enzyme was added to “C” and “T” test tubes. Incubated at 37°C for 30 minutes and 1.0 ml of 10% acetic acid was added to all the test tubes. 0.5ml of substrate solution was added in to the “C” tube and the absorbance was measured at 410 nm.

Enzyme activity was expressed as U/Liter.

Estimation of 5'-Nucleotidase (5'-NT) (5'-Ribonucleotide phosphohydrolase, E.C. 1.1.1.42)

The 5'-Nucleotidase was assayed by the method of Luly *et al.* (1972), with slight modification.

Reagents

1. Tris HCl buffer: 184 mM pH 7.5
2. Magnesium sulphate : 50mM
3. Potassium chloride: 650mM
4. EDTA :1mM
5. 2.5% Ammonium molybdate
6. 5' Adenosine monophosphate (5'AMP) 40mM
7. ANSA - 500mg of Amino naphthol Sulphonic acid was dissolved in 195ml of 15% sodium meta bisulphate and 5ml of 20% sodium sulphite was added to it. The solution was stored in the brown bottle.
8. Stock Standard Phosphorous: 35.1 mg potassium dihydrogen phosphate in 100ml of water.
9. Working Standard Phosphorous: Dilute the stock standard 1 in 10 times which contain 8mcg/1ml.

Procedure

The assay medium contained 1.0ml Tris HCl buffer, 0.1ml of Magnesium sulphate, 0.1ml potassium chloride, 0.1ml of EDTA and 0.1ml of 5'AMP. The reaction was initiated by addition of 0.2ml of enzyme preparation and incubated at 37.C for 15mts. The reaction was terminated by the addition of 2.0 ml of 10% TCA. After centrifugation the phosphorus in the supernatant was estimated as discussed earlier in the phospholipids.

Enzyme activity was expressed in U/ litre.

Assay of Isocitrate Dehydrogenase (ICD) (Isocitrate NADP⁺ oxidoreductase, E.C. 1.1.1.42)

Assay of Iso citrate dehydrogenase was carried out by the method of Belljoice and Baron (1960).

Reagents

1. 0.15 M Sodium chloride
2. Tris-HCl buffer 0.1 M in 0.15 M Sodium chloride pH 7.5.
3. Buffered substrate: Dissolved 1.845 g of trisodium isocitrate in 100 ml of Tris buffer.
4. NADP - 10 mg /ml in 0.15 M Sodium chloride
5. Reduced NADP - 10 mg /ml in 0.15 M Sodium chloride
6. Manganous chloride - 0.03 M in 0.15 M Sodium chloride

7. Standard α -oxoglutaric acid - Stock containing 70 mg/ 100ml water. Diluted this 1 in 10 for use.
8. EDTA-Dissolved 5.6 gms in 100ml of water by adjusting the pH 8.0 using NaOH pellets.
9. 2,4-Dinitrophenyl hydrazine - Dissolved 19.8 mg 2,4-Dinitrophenyl hydrazine in 1 N HCl and made upto 100ml with water.
10. 0.4 N NaOH.

Procedure

The assay medium contained 0.5ml α -oxoglutaric acid, 0.1ml of MnCl_2 solution and 0.1ml of NADP to all the three test tubes marked as “B”, “C” and “ T”. To the control tube 0.033ml of NADPH_2 was added. About 0.3ml of 0.15 M NaCl_2 was added to all the tubes. The reaction was initiated by addition of 0.2ml of enzyme preparation and incubated at 37°C for 1hr. At the end of incubation period added 1.0 ml of EDTA to the blank and standard test tubes and 1ml of DNPH to all the test tubes immediately. Allowed to stand for 20 minutes, and then added 10 ml of 0.4n sodium hydroxide. Stood for 15 minutes and read against the reagent blank at 390nm.

Enzyme activity was expressed as IU / L.

Biochemical analysis - Liver tissues

Estimation of Total lipid

Total lipids were extracted from the liver tissue according to the method of Folch *et al.* (1957).

Procedure

The tissues were washed with saline and dried with a filter paper. A weighed amount of tissue (500mg) was homogenized with 7.0 ml of chloroform: methanol (1:2) mixture in a potter Elvehjem homogeniser and filtered through a whatman No 1 filter paper into a conical flask. The residue after filtration was scrapped and homogenized with 10ml chloroform-methanol mixture 2:1 v/v and the resulting filtrate was evaporated to dryness. The weight of the flask with and without the dried lipid was recorded and the differences in weight gave the total lipid content of the tissues.

The total lipids were expressed as mg/ gm of wet tissue.

“Folch” wash

Reagents

1. 0.1N potassium chloride
2. Folch's reagent: 0.1N HCl: Methanol: Chloroform (10:10:1)

The lipid extract from above procedure was redissolved in 2ml of Folch's reagent and 1.0ml of 0.1N potassium chloride was added and the

contents were shaken well. The upper aqueous phase containing ganglioside and other water soluble compounds were separated. The lower phase containing neutral and phospholipids in chloroform was again washed 3 times with 2.0ml of Folch's reagent and the upper aqueous phase was aspirated. The lower chloroform phase was made up to known volume. Aliquots were taken for analysis of cholesterol, triglycerides and phospholipids.

Estimation of Cholesterol

The total cholesterol present in the tissue homogenate after Folch's wash was estimated by the method of Wybenga *et al.* (1970), as mentioned earlier.

The total cholesterol was expressed as mg/ gm of wet tissue.

Estimation of Triglycerides (TGL)

The triglycerides present in the tissue homogenate after Folch's wash was estimated by the method of Rice and Vanhandle (1970) as mentioned earlier.

The triglycerides was expressed as mg/ gm of wet tissue.

Estimation of Phospholipids

The tissue phospholipids were estimated by the method of Rouser *et al.* (1970).

Reagents

1. 3% Ammonium molybdate
2. 3% Ascorbic acid
3. 70% Perchloric acid
4. Standard Phosphate: 35.1 mg of potassium dihydrogen phosphate was dissolved in 100ml of water to give a concentration of 80 $\mu\text{g/ml}$
5. Working Standard solution: A concentration of 8 $\mu\text{g/ml}$ was prepared by diluting the stock solution from 1 to 10ml-distilled water.

Procedure

0.1ml of the lipid extract was dissolved in 1ml of perchloric acid and digested on a sand bath till the solution becomes colorless. After cooling the solution was made up to 5.0ml with double distilled water. In the standard 1ml of working standard solution was taken. Blank contained 1ml of water. To all the tubes 0.5ml of ammonium molybdate and ascorbic acid were added and the mixture was kept in a boiling water bath for 6 mts. The blue color developed was read at 710 using a photochem colorimeter. Expressed as mg/gm of wet tissue after multiplication by factor 25.

Estimation of Glycogen

The liver tissue glycogen was extracted and estimated by the method of Morales *et al.* (1973).

Reagents

1. 30% potassium hydroxide solution
2. Absolute alcohol
3. Anthrone reagent: 0.2% anthrone in con. Sulphuric acid was prepared just before use.
4. 1.0M Ammonium acetate solution
5. Saturated ammonium chloride
6. Glucose standard: 100mg of pure glucose was dissolved in 100ml-distilled water containing 0.01% benzoic acid.

Procedure

The alkali extract of the tissue was prepared by digesting 50mg of fresh tissue with 3.0 ml of 30% potassium hydroxide solution in boiling water bath for 15 min. The tubes were cooled and mixed with 5.0ml of absolute alcohol and a drop of 1.0M ammonium acetate to precipitate glycogen and left in the freezer overnight for complete precipitation. Glycogen was collected by centrifugation at 2000g for 20 mts. The precipitate was dissolved in water with the aid of heating in a boiling water bath for 5 mts. Aliquots of glycogen solution were taken up after suitable dilution and 4.0ml of anthrone

reagent was added heated in the boiling water bath cooled the tubes in an ice bath. The tubes were shaken well, covered with marble caps and heated in a boiling water bath for 20mts. After cooling the absorbance was read at 640nm in spectrophotometer against a water blank treated in a similar manner. Standard glucose solutions were also treated similarly.

The glycogen content was calculated from the amount of glucose present in the sample and was expressed as mg/g fresh tissue.

Estimation of Protein

The liver tissue protein was estimated by the method Lowry *et al.* (1951) as mentioned earlier in Chapter I.

The protein contents of the tissues were expressed as mg/g of fresh tissue.

Estimation of Aspartate Aminotransferase (AST) Glutamate oxaloacetate transaminase, E.C.2.6.1.1)

The Aspartate Aminotransferase in the tissue homogenate was estimated by the method of Reitman and Frankel (1957) as mentioned earlier.

The enzyme activity is expressed as μ moles of pyruvate liberated/min/mg protein.

Estimation of Alanine Aminotransferase (ALT) Glutamate pyruvate transaminase, E.C.2.6.1.2)

The alanine aminotransferase in the tissue homogenate was estimated was estimated by the method of Reitman and Frankel (1957) as mentioned earlier.

The enzyme activity is expressed as μ moles pyruvate liberated/min/mg protein.

Estimation of Alkaline Phosphatase (ALP) ortho-phosphoric monoester phosphohydrolase, E.C. 3.1.3.1)

The Alkaline Phosphatase present in the tissue homogenate was estimated by the method of Kind and King (1954) as mentioned earlier.

The results were expressed as μ - moles of phenol liberated /min/ mg protein.

Estimation of Acid Phosphatase (ACP) (ortho-phosphoric monoester hydrolase, E.C.3.1.3.2)

The Acid Phosphatase present in the tissue homogenate was estimated by the method of Kind and King (1954) as mentioned earlier.

The results were expressed as μ - moles of phenol liberated /min/ mg protein.

Estimation of Lactate Dehydrogenase (L-lactate: NAD oxido-reductase E.C.1.1.1.27)

The liver tissue Lactate dehydrogenase was estimated by the method of King (1965) as mentioned earlier.

The enzyme activity is expressed as μ moles of pyruvate liberated /min/ mg protein.

Estimation of Gamma Glutamyl Transferase(5 Glutamyl peptide aminoacid 5 glutamyl transferase, E.C. 2.3.2.2)

The Gamma Glutamyl transferase in the liver tissue was assayed by the method of Szaz (1969) as mentioned earlier.

One unit of enzyme activity was defined as the amount of enzyme required to release 1 micro* mole of p- nitroanilide/minute under incubation conditions.

Estimation of 5'-Nucleotidase (5'-Ribonucleotide phosphohydrolase, E.C. 1.1.1.42)

The 5'-Nucleotidase present in the tissue homogenate was estimated by the method of Luly *et al.* (1972) as mentioned earlier.

The results were expressed as μ moles of phosphorous liberated/minute mg of protein.

Estimation of Xanthine Oxidase (xanthine:NAD⁺ oxidoreductase, E.C. 1.17.32)

Estimation of Xanthine oxidase was carried out by the method of Fried and Fried (1957).

Reagents

1. Phosphate buffer- 0.1 M, pH 8.2
2. EDTA - 10 mM
3. Gelatin -1 %
4. Phenazine metho sulphate (PMS): The solution of PMS in the phosphate buffer at the concentration of 0.2 mg / ml was prepared at the time of the assay and was protected from light
5. Nitroblue tetrazolium salt (NBT):NBT solution containing 5 mg/ ml was prepared in phosphate buffer just before use and was protected from light
6. 1mM Xanthine solution: prepared in phosphate buffer just before use.

Procedure

To 0.6ml of buffer , 0.4ml of EDTA , 0.4 ml of gelatin , 0.1ml of PMS and 0.3ml of NBT were added in a test tube . To this 0.2ml of enzyme was added and incubated at room temperature for 5 minutes. Then 0.5ml of

buffer and 0.5ml of substrate were added and the increase in the optical density was measured in 532 nm at 2 minutes interval for 10 minutes.

The activity of enzyme was expressed as Unit / mg of protein. One unit corresponds to the amount of the enzyme required to bring about change in optical density 0.01/ min.

Estimation of Lipid peroxidation

Basal

Lipid peroxidation in the liver homogenate was assayed by the method of Ohkawa and Yagi (1979).

Reagents

1. 8.1% Sodium dodecyl sulphate (SDS)
2. 0.8% Thiobarbituric acid (TBA)
3. 20% Acetic acid
4. 15:1 v/v n- Butanol: Pyridine mixture

Procedure

The reaction mixture contained 0.2 ml of liver homogenate, 1.5 ml of TBA, 0.2ml SDS, 1.5ml of acetic acid and 0.8ml of distilled water. The above solution was kept in the boiling water bath at 90°C for 1 hr and cooled in tap water. After cooling 1ml of distilled water and 5ml of mixture of n-butanol: pyridine (15:1 v/v) were added and shaken vigorously. After centrifugation at

4000 rpm for 10 min, the organic layer was taken and its absorbance was read at 532 nm.

The lipid peroxide concentration was expressed as nano moles of MDA liberated / min / mg of liver homogenate.

Hydrogen Peroxide induced Lipid peroxidation

Hydrogen Peroxide induced Lipid peroxidation was assayed by the method of Devasagayam and Tarachand (1987).

Reagents

1. 0.15M Tris-HCl buffer , pH 7.4
2. 10mM KH_2PO_4
3. 10% Trichloroacetic acid (TCA)
4. 10mM Hydrogen peroxide
5. 1% Thiobarbituric acid (TBA)

Procedure

The peroxidation system consists of 1.4 ml of Tris buffer, 0.2ml of H_2O_2 , 0.2ml of KH_2PO_4 and 0.2ml of homogenate. The tubes were incubated at 37°C with constant shaking for 20 minutes. The reaction was stopped by the addition of 1.0 ml of 10 % TCA. Then 1.5ml of TBA reagent was added and heated in the boiling water bath for 20 minutes. The tubes were centrifuged and the colour developed in the supernatant was read at 532 nm using spectrophotometer.

The MDA content of the sample after peroxide stress was expressed as nano moles of MDA liberated / min / mg of liver homogenate.

Ascorbate induced Lipid peroxidation

Ascorbate induced Lipid peroxidation was assayed by the method of Devasagayam and Tarachand (1987).

Reagents

1. 0.15M Tris-HCl Buffer, pH 7.4
2. 10 mM KH_2PO_4
3. 10% Trichloroacetic acid (TCA)
4. 1mM Ascorbic acid
5. 1% Thiobarbituric acid (TBA)

Procedure

The peroxidation system consisted of 1.4 ml of Tris buffer, 0.2ml of ascorbate 0.2ml of KH_2PO_4 and 0.2ml of homogenate. The tubes were incubated at 37 ° C with constant shaking for 20 minutes. The reaction was arrested by the addition of 1.0 ml of 10 % TCA. Then 1.5ml of TBA reagent was added and heated in the boiling water bath for 20 minutes. The tubes were centrifuged and the colour developed in the supernatant was read at 532 nm using spectrophotometer.

The MDA content of the sample after peroxide stress was expressed as nano moles of MDA liberated / min / mg of liver homogenate.

FeSO₄ induced Lipid peroxidation

FeSO₄ induced Lipid peroxidation was assayed by the method of Devasagayam and Tarachand (1987).

Reagents

1. 0.15M Tris-HCl Buffer, pH 7.4
2. 10 mM KH₂ PO₄
3. 10% Trichloroacetic acid (TCA)
4. 10mM ferrous sulphate
5. 1% Thiobarbituric acid (TBA)

Procedure

The peroxidation system consists of 1.4 ml of Tris buffer, 0.2ml of FeSO₄, 0.2ml of KH₂ PO₄ and 0.2ml of homogenate. The tubes were incubated at 37°C with constant shaking for 20 minutes. The reaction was arrested by the addition of 1.0 ml of 10 % TCA. Then 1.5ml of TBA reagent was added and heated in the boiling water bath for 20 minutes. The tubes were centrifuged and the colour developed in the supernatant was read at 532 nm using spectrophotometer.

The MDA content of the sample after peroxide stress was expressed as nano moles of MDA liberated / min / mg of liver homogenate.

Lipid peroxidation products

Estimation of Conjugated dienes (CD)

Dienes conjugates were estimated by the method of Klein and Klein (1983).

Extraction of Lipids

Lipids were extracted from the liver homogenate with chloroform/methanol (2:1) according to the method described by Folch *et al.* (1957) with the modification.

Procedure

The lipid in chloroform was isolated and dried. Aliquots of lipid extracts were evaporated to dryness. The lipid residue was suspended in methanol and the absorbance at 213 nm and 233nm were measured against the blank. The extent of peroxidation is determined by the measurement of conjugated diene content which is arrived by computing the ratio of absorbance at wave lengths 233 nm and 213nm.

Units expressed as Δ^{233} /mg of protein.

Estimation of Lipid hydro peroxide (LOOH)

Lipid hydro peroxide (LOOH) assay was performed essentially as described by Nourooz-Zadeh *et al.* (1996) with minor modifications.

Reagents

1. Triphenylphosphine (10mMol/L)
2. 90% Methanol
3. FOX2-reagent: FOX2-reagent was prepared by dissolving 38 mg Xylenol Orange 440 mg of Butylated hydroxy toluene in 450 ml HPLC-grade methanol with stirring. 49 mg Ammonium ferrous sulphate dissolved in 50 ml of 250-mmol/l of sulphuric acid was added to the methanol solution.

Procedure

Aliquots of sample were incubated for 30 minutes, at 20-25°C in 10 μ L of triphenylphosphine to remove hydroperoxides and to generate a blank, and 90 μ L of tissue samples were incubated in 10 μ l methanol to generate a test sample. Both the blank and the test sample were mixed with 900 μ L FOX2 reagent and incubated for 30 minutes, at 20-25°C. After centrifugation at 12000 rpm for 10 min, the absorbance of the supernatants was monitored at 560 nm The hydroperoxide concentration of each sample was calculated from the difference between the absorbance of the blank and test samples.

The LOOH content of the sample was expressed as μ moles of LOOH liberated / min / mg of Protein.

Assay of Nitric oxide end products (NO)

Nitric oxide end products like Nitrite and Nitrate determination was carried out by the method of Miranda *et al.* (2001).

Reagents

1. 10 % TCA
2. Griess reagent: 1% sulfanilamide in 1N HCl, 15% N-1-naphtylethylenediamine dichloride
3. 50 mM Potassium phosphate buffer
4. Vanadium (III) chloride (8 mg/ml)

Procedure

Approximately 4.0 ml of tissue homogenate was treated with 2.5 ml of 10% TCA solution, and centrifuged at 3000 rpm for 30 min. After centrifugation at room temperature, 100µl of supernatant was applied to a clean tube, 100 µL vanadium (III) chloride was added to each tube (for reduction of nitrate to nitrite) and this was followed by addition of 450µl of Griess reagent. After mixing well, all tubes were left in a dark place for 30 minutes at room temperature. At the end of the reaction time, the absorbance was measured on a spectrophotometer at a wavelength of 550nm. A blank was prepared in the same way but 150µl potassium phosphate buffer (50mM) was used instead of supernatant. Aliquots of sodium nitrite were also treated in a similarly for standard calibration.

Nitric oxides of the sample were expressed as nmol/mg of protein.

Determination of Enzymic antioxidant systems

Assay of Catalase (CAT) (H_2O_2 Oxidoreductase, E.C. 1.11.16)

The antioxidant enzyme Catalase was assayed by the method of Sinha (1972).

Reagents

1. 0.01M Phosphate buffer, pH 7.00
2. Dichromate acetic acid reagent: This reagent was prepared by mixing 5% solution of potassium dichromate with glacial acetic acid in the ratio of 1:3.
3. 0.2M Hydrogen peroxide

Procedure

To 0.1ml of the homogenate was added to 1.0ml of phosphate buffer. To this 0.5ml of hydrogen peroxide was added. The reaction was stopped at "0", "30" and "60" seconds by the addition of 2.0 ml of the dichromate acetic acid reagent. The tubes were boiled for 10 minutes, cooled and read at 620nm . For standards different amounts of hydrogen peroxide, ranging from 20-100 μmoles were taken and processed as above.

Enzyme activity were expressed as n moles of H_2O_2 decomposed/ min/mg protein.

Estimation of Superoxide dismutase (SOD) (Superoxidase dismutase; Copper-zinc superoxide dismutase, E.C. No. 1.15.1.1)

The Super oxide dismutase was estimated according to the method of Misra and Fridovich (1972).

Reagents

1. 0.1 M Carbonate – bicarbonate buffer, pH 10.2 containing 5.7 mg EDTA / 100ml
2. 3mM Epinephrine
3. Absolute ethanol
4. Chloroform

Procedure

In the test tube labelled as “Test” 0.5ml of liver homogenate was taken and to this 0.25 ml of cold ethanol and 0.15 ml of chloroform was added and kept for 15 minutes in a shaker and centrifuged. To 0.5ml of the supernatant 2.0 ml, of the buffer was added. The reaction was initiated by the addition of 0.4ml of epinephrine and change in the optical density per min was measured at 480 nm. 100% auto oxidation of epinephrine to adrenochrome was performed in a control tube without the enzyme.

The enzyme unit was defined as the enzyme required to give 50 % inhibition of epinephrine auto oxidation.

Assay of Glutathione-S-Transferase (GST) (RX: Glutathione R-transferase, E.C.2.5.1.18)

The enzyme Glutathione-S-Transferase was estimated by Habig *et al.* (1974).

Reagents

1. 0.5 M Phosphate buffer pH 6.5
2. 25 mM 1-Chloro 2,4 dinitrobenzene (CDNB)
3. 30 mM Reduced Glutathione

Procedure

To 1.0 ml of phosphate buffer, 0.1ml of 1-Chloro 2,4 dinitrobenzene, 1.7 ml of water and 0.1ml of liver homogenate were added. After 5 minutes of incubation at 37°C, 0.1 ml of reduced glutathione was added and the change in the optical density was measured for 3 minutes for 30 secs interval. Complete assay mixture without enzyme was used as control. Optical density was measured at 340nm.

Enzyme activity was expressed as n moles of CDNB conjugate formed/ min/ mg of protein.

Assay of Glutathione Reductase (GR) (Glutathione: NADP+ oxidoreductase, E.C.1.8.1.7.)

The enzyme Glutathione reductase activity was carried out by the method of Dobler (1981).

Reagents

1. 0.3 M Phosphate buffer, pH 6.8
2. 25 mM EDTA
3. 12.5mM Glutathione oxidised (GSSG)
4. 3mM Nicotineamide adenine dinucleotide phosphate (NADPH)

Procedure

The reaction mixture containing 1.5 ml of buffer, 0.5ml of EDTA, and 0.2 ml of GSSG was incubated at 37°C for 10 minutes. To the incubated mixture 0.1ml of the homogenate and 0.1 ml of NADPH solution was added. The change in the optical density was monitored at 340 nm at 37°C for 3 minutes at 30 secs interval.

The enzyme activity was expressed as n moles of GSSG utilized / min/ mg protein of liver homogenate.

Assay of Glutathione Peroxidase (GPx) (Glutathione: hydrogen-peroxide oxidoreductase, E.C.1.11.1.9.)

Glutathione Peroxidase was estimated by the method of Necheles *et al.* (1968).

Reagents

1. 0.4 M Phosphate buffer, pH 7.0
2. 10.mM Sodium azide

3. 4.0 mM Reduced glutathione
4. 2.5 mM Hydrogen peroxide
5. 10% TCA
6. 0.3 M Phosphate solution
7. 0.4 mM EDTA
8. 1.0 mM 5,5'-Dithio-bis-2 Nitro benzoic acid

Procedure

The incubation mixture consisted of 0.2ml of liver homogenate, 0.4 ml of buffer, 0.1ml of sodium azide, 0.2ml of reduced glutathione, and 0.2ml of EDTA, mixed well. To this mixture 0.1ml of hydrogen peroxide was added and incubated at “0”, “1.5” and “3.0” minutes. Then 1.0 ml of TCA was added to arrest the reaction. It was centrifuged and to 1.0 ml of the supernatant 4.0ml of phosphate solution and 0.5ml of DTNB were added and the residual glutathione was measured at 412 nm .Non enzymic oxidation of glutathione was measured in the blank containing all the reagents with buffer substituted for the enzyme source.

The activity of glutathione peroxidase was expressed as n moles of GSH oxidised / min/ mg protein liver homogenate.

Determination of non Enzymic antioxidant systems

Estimation of liver ascorbic acid

Estimation of liver ascorbic acid was carried out according to the method of Omaye *et al.* (1971).

Reagents

1. 10% TCA
2. Dinitrophenyl- Thiourea- copper sulphate reagent: 3 gms of 2,4-Dinitrophenyl hydrazine, 0.4gms of thiourea and 0.05 gms of copper sulphate were dissolved in 100ml of 9N H₂SO₄
3. 3. 65% H₂ SO₄

Procedure

To 0.5ml of the homogenate, 0.5 ml of water , 1ml of TCA, are added, mixed and centrifuged. To 1ml of the supernatant, 0.2ml of DTC reagent was added and incubated at 37° C for 3hrs. Then 1.5 ml of ice cold H₂SO₄, was added, mixed well and the solution was allowed to stand at room temperature for another 30 minutes. The colour developed was read at 520 nm. Standards of ascorbic acid were treated similarly.

The level of ascorbic acid was expressed as mg/g wet tissue.

Estimation of Vitamin E

Estimation of Vitamin E was carried out according to the method of Desai (1984).

Reagents

1. Ethanol
2. Petroleum ether

3. Batho phenanthroline reagent: 0.2% 4,6-diphenyl 1,10-phenanthroline in ethanol.
4. 0.001 M ferric chloride in ethanol
5. 0.001M O-Phosphoric acid in ethanol.

Procedure

To 1ml of the tissue homogenate, 1ml of ethanol was added and thoroughly mixed. Then 3ml of petroleum ether was added, shaken rapidly and centrifuged. 2ml of the supernatant was taken and evaporated to dryness. To this 0.2 ml of diphenyl 1,10-phenanthroline was added. The assay mixture was protected from light and 0.2ml of ferric chloride was added followed by 0.2ml of O-phosphoric acid. Total volume was made up to 3ml with ethanol. The colour developed was read at 530 nm. Standards were treated similarly.

The level of Vitamin E was expressed as mg/g wet tissue.

Estimation of Vitamin A

Estimation of Vitamin A was carried out by the method of Kaser and Stekol (1943).

Reagents

1. Absolute ethanol
2. Light petroleum ether
3. Chloroform
4. Acetic anhydride

5. Carr-price reagent: This contains 25% solution of antimony trichloride in chloroform. Kept it in the room temperature in a tightly stoppered brown bottle.

Procedure

To 3ml of homogenate in a stoppered flask added 3ml of ethanol, slowly drop by drop with shaking, to precipitate the protein. Added 6ml of light petroleum and shake vigorously for ten minutes. Poured the emulsion into a centrifuge tube, cork and spin at low speed for about one minute. The petroleum layer gets separated. To 4ml of the petroleum layer taken in the colorimeter tube evaporate off the solvent by placing in a water bath at 40-60°C. Dissolve the residue in 0.5ml of chloroform and added a drop of acetic anhydride to remove the water present. With colorimeter adjusted with the chloroform blank added quickly 3ml of Carr-price reagent and read the colour developed at 620 nm.

The level of serum Vitamin A was expressed as mg/g of wet tissue.

Determination of Ceruloplasmin activity

Ceruloplasmin activity was determined according to the method of Raven (1961).

Reagents

1. p-Phenylene diamine hydrochloride: 0.5% for purification of Phenylene diamine hydrochloride was dissolved in minimum volume of hot distilled water, decolourised with charcoal ,

filtered hot and allowed to crystallize. The crystals were stored over calcium chloride.

2. 0.04M Acetate buffer: pH 5.3
3. 0.5% Sodium azide

Procedure

0.1ml of fresh serum was taken into 15ml test tube. 1.0 ml of 0.5% sodium azide was added to the control. Then 8.0 ml of acetate buffer was added to each tube, followed by 1.0ml of the p-phenylene diamine hydrochloride. The solution was mixed and placed in the water bath at 37°C for one hour. After incubation, the tubes were removed and added 1.0 ml of sodium azide to each of the tubes. The contents were mixed and cooled at 4-10°C for 30 minutes. The colour intensity was measured at 530 nm against reagent blank.

The level of serum Ceruloplasmin was expressed as mg / dl.

Determination of serum Uric acid

Determination of serum uric acid was carried out according to the method of Caraway (1963).

Reagents

1. Colouring reagent: 50 gms of sodium tungstate was dissolved in 400 ml of distilled water, to that 40 ml of phosphoric acid was added and refluxed for 2hrs. A drop of bromine was added, cooled and diluted to 500ml with water.

2. 20% sodium carbonate
3. Standard Uric acid: 100mgms of uric acid was dissolved in 150 ml of water containing 60 mg of lithium carbonate by heating at 60 ° C, the solution was cooled at room temperature and added 2ml of formaldehyde diluted to about 500ml.
4. Working standard: 1.0 ml of the stock standard and 2.0 ml of 300 mg/ 1ml BSA were diluted to 10 ml with water. The working standard was prepared fresh. Albumin was added to account for the positive error induced by co precipitation of uric acid and proteins.

Procedure

To 0.6ml of serum 5.4 ml of diluted tungstic acid was added and centrifuged. Into three test tubes 3ml of each supernatant, standard, and water were taken and labelled as “T”, “S” and “B”. 0.6ml of sodium carbonate and 0.6ml of phospho tungstic acid reagent were added, mixed and placed in a water bath at 25°C for 10 minutes. The blue colour developed was read at 700 nm.

The level of serum Uric acid was expressed as mg / dl.

Assay of liver Glutathione

Assay of liver Glutathione was carried out by the method of Ellman (1959).

Reagents

1. 0.3M Disodium hydrogen phosphate
2. 5,5'-Dithiobis-2-nitrobenzoic acid reagent (DTNB): 40 mg of DTNB was dissolved in 100ml of 1% sodium citrate.

Procedure

To the test tube marked as "T" 1.0 ml of the homogenate was taken and 1 ml of water is added. To that 1ml of 10 % TCA was added. The blank test tube contained 1ml of TCA. Both the test tubes were centrifuged. To 1ml of the supernatant 4.0ml of phosphate reagent and 0.5ml of DTNB solution was added and the color developed was read at 412 nm.

Liver glutathione was expressed as micromole GSH/ mg of protein.

Assay of Total thiols

Assay of total thiols was carried out by the modified method of Sedlak and Lindsay (1968).

Reagents

1. 0.2M Tris-HCl buffer in EDTA, pH 8.2
2. 0.01M 5,5'-dithiobis-2-nitrobenzoic acid, reagent (DTNB)
3. Methanol
4. 0.02 M EDTA

Procedure

100 mg of tissue was homogenized in 4ml of 0.02M EDTA. To 1ml of the homogenate 1.5 ml of 0.2 M Tris buffer and 0.1 of 0.01 M DTNB (5,5'-dithiobis- 2-nitrobenzoic acid) were added the mixture was brought to 6.5 ml with methanol. The test tubes were capped and left to stand for 20 min, then centrifuged at 3000 rpm/min at room temperature for 10 min. The absorbance of the clear supernatant was read at 412 nm.

Liver total thiols were expressed as μ moles of GSH mg/g of protein.

Estimation of Iron in liver tissue

Estimation of Iron in the liver tissue was carried out by the method of Ramsay (1957).

Reagents

1. Stock ferric chloride solution : 145 mg of ferric chloride in 100ml of 0.5N HCl
2. Working ferric chloride solution : Dilute 1 in 100 of the stock ferric chloride solution
3. Sodium sulphite: 2.25 gms of anhydrous sodium sulphite in 100 ml of water.
4. 0.2% 2-2 Dipyridyl in 3% glacial acetic acid

5. Stock standard 100 μ gm/ ml: Dissolve 0.48 gms of ferrous sulphate in water and 1ml of conc H₂SO₄ and made up to 1 litre in water.
6. Working standard 5 μ gm/ ml: 5 ml of the stock diluted to 100ml with distilled water.
7. Chloroform

Procedure

About 1.0 ml of the tissue homogenate was taken and 0.5ml of bipyridyl solution and 0.5ml of sodium sulphite were added blank contained 1.0 ml of distilled water. Mixed well and kept in boiling water bath for 5 minutes cooled and then added chloroform. Mixed well and centrifuge and read supernatant at 520 nm in spectrophotometer against blank. Aliquots of standard were also treated in the similar manner.

Iron in the liver tissue was expressed as mg/ g wet tissue.

Determination of membrane bound enzymes

Liver membrane was prepared by the method of Song *et al.* (1969).

Frozen rat liver samples of about 2 g were homogenized in 2 volumes each of ice-cold 1 mM sodium carbonate, pH 7.50, using 20 strokes of a loose-fitting homogeniser. The homogenates were diluted and filtered through 3 layers of surgical gauze. After centrifugation at 1500 g for 10 min, the pellets were resuspended in buffer and 5.5 volumes of 70.7% sucrose were

added, mixed, and then distributed into centrifuge tubes. 8 ml of 48.2% and 4 ml of 42.5% sucrose were layered over this suspension. After centrifuging for 60 min at 12,000 rpm, the material which accumulated around the interface of 42.5% and 48.2% sucrose was collected, diluted, and washed. The final pellet was resuspended in 0.25 M sucrose, 30 mM histidine, 1 mM EDTA (pH 6.8), and stored at -80°C .

Estimation of Total Adenosine tri phosphatase (ATP Phosphohydrolase E.C. No. 3.6.1.4)

Total adenosine tri phosphatase activity was estimated by the method of Evan (1969).

Reagents

1. 0.1M Tris-HCl buffer pH 7.0
2. 0.1M Magnesium chloride
3. 0.1M Potassium chloride
4. 0.1M Sodium chloride
5. 0.1M calcium chloride ATP
6. 0.01M ATP
7. 10% TCA
8. Ammonium molybdate - 2.5 gm of ammonium molybdate was dissolved in 100ml of 5 N Sulphuric acid
9. ANSA - 500mg of Amino naphthol Sulphonic acid was dissolved in 195ml of 15% sodium meta bisulphate and 5ml of 20% sodium sulphite was added to it. The solution was stored in the brown bottle.

Procedure

The incubation mixture contained 1ml of Tris-HCl buffer, 0.2ml each of magnesium chloride, calcium chloride, potassium chloride, sodium chloride, ATP and homogenate. The mixture was incubated at 37°C for 15 minutes the reaction was arrested by the addition of 1ml of 10% TCA, mixed well and centrifuged. The phosphorous content of the supernatant was estimated.

The enzyme activity is expressed as μ moles of phosphorous liberated/ min/ mg of protein.

Estimation of Na⁺/ K⁺ ATPase (Adenosine tri phosphatase EC 3.6.1.37)

Estimation of Na⁺, K⁺ ATPase was estimated by the method of Bonting (1970).

Reagents

1. 0.09M Tris-HCl buffer - pH 7.5
2. Sodium chloride - 0.60M
3. EDTA - 0.001M
4. ATP - 0.04M
5. 10% TCA
6. Ammonium molybdate - 2.5 gm of ammonium molybdate was dissolved in 100ml of 3 N Sulphuric acid.

7. ANSA - 500mg of amino naphthol Sulphonic acid was dissolved in 195ml of 15% sodium bisulphate and 5ml of 20% sodium sulphite was added to it. The solution was stored in the brown bottle.

Procedure

The incubation mixture contained 1ml of Tris-HCl buffer, 0.2ml each of potassium chloride, sodium chloride, EDTA, ATP and homogenate. The mixture was incubated at 37°C for 15 minutes. The reaction was arrested by the addition of 1ml of 10% TCA, mixed well and centrifuged. The phosphorous content of the supernatant was estimated.

The enzyme activity is expressed as μ moles of phosphorous liberated/ min/ mg of protein.

Estimation of Ca^+ -ATPase (ATP phosphohydrolase EC 3.1.3.1)

The activity of Ca^+ - ATP ase was assayed according to the method of Hjerten and Pan (1983).

Reagents

1. Tris-HCl buffer – 125mM, pH 8.0
2. Calcium chloride - 50mM
3. ATP - 10mM
4. 10% TCA

5. Ammonium molybdate – 2.5 gm of ammonium molybdate was dissolved in 100ml of 3 N Sulphuric acid
6. ANSA - 500mg of amino naphthol Sulphonic acid was dissolved in 195ml of 15% sodium bisulphate and 5ml of 20% sodium sulphite was added to it. The solution was stored in the brown bottle.

Procedure

The incubation mixture contained 0.1ml of each of Tris- HCl buffer, calcium chloride, ATP and enzyme preparation. After incubation at 37°C for 15 minutes the reaction was arrested by the addition of 1ml of 10% TCA, mixed well and centrifuged. The phosphorous content of the supernatant was estimated.

The enzyme activity is expressed as μ moles of phosphorous liberated/ min/ mg of protein.

4.2.9.4 Estimation of Mg^{2+} -ATPase (ATP phosphohydrolase EC 3.6.1.3)

The activity of Mg^{2+} -ATPase was assayed by the method of Ohnishi *et al.* (1982).

Reagents

1. Tris-HCl buffer – 375mM, pH 7.6
2. Magnesium chloride- 25mM
3. ATP - 10mM

4. 10% TCA
5. Ammonium molybdate – 2.5 gm of ammonium molybdate was dissolved in 100ml of 3 N Sulphuric acid
6. ANSA - 500mg of amino naphthol Sulphonic acid was dissolved in 195ml of 15% sodium bisulphate and 5ml of 20% sodium sulphite was added to it. The solution was stored in the brown bottle.

Procedure

The incubation mixture contained 0.1ml each of Tris-HCl buffer, Magnesium chloride, ATP and enzyme preparation. After incubation at 37°C for 15 minutes the reaction was arrested by the addition of 1ml of 10% TCA, mixed well and centrifuged. The phosphorous content of the supernatant was estimated

The enzyme activity is expressed as μ moles of phosphorous liberated/ min/ mg of protein.

Estimation of Glycolytic enzymes

Estimation of Hexokinase (ATP: D-Hexose-6-Phosphotransferase E.C.2.7.1.1)

Hexokinase was analysed by the method of Branstrup (1957).

Reagents

1. 0.01M Tris-HCl buffer, pH 8.0
2. Substrate 0.005M Glucose
3. ATP – 0.072M
4. 0.05M Magnesium chloride
5. 0.01M Potassium Chloride
6. 0.5M sodium fluoride
7. 0.0125M Potassium hydrogen phosphate
8. 10% TCA
9. Ortho toluidine reagent - 940 ml of acetic acid was dissolved in 60 ml of Ortho toluidine, to this added 3g of thiourea
10. Glucose standard: 100mg of pure glucose was dissolved in 100ml-distilled water containing 0.01% benzoic acid.

Procedure

The incubation mixture was taken in two tubes marked as “0” minute and “30” minute which contained 2.5ml buffer, 1ml substrate, 0.5ml ATP, 0.1ml magnesium chloride, 0.1ml sodium fluoride, 0.4ml potassium hydrogen phosphate and 0.4ml of potassium chloride were pre incubated at 37°C for 5 minutes. 0.5ml of enzyme was added in both. To the” 0” minute tube added 1ml of the 10 % TCA immediately to stop the reaction. To the “30” minute tube 10 % TCA was added after 30 minutes. The two tubes were incubated at 37°C for 30 minutes. Both the tubes were centrifuged. Taken 1ml of the two supernatant separately and 1ml of water as blank, 4ml of ortho toluidine

reagent was added in all three tubes kept in boiling water bath for 8 minutes, cooled and read at 620nm. Standard containing varying concentrations of glucose and reagents were also similarly treated.

The enzyme activity is expressed as nmoles of Glucose utilized /min /mg of protein.

Estimation of Phospho gluco isomerase (n-glucose-g-phosphate ketol-isomerase, EC 5.3.1.9)

The enzyme was assayed by the method of Horrocks *et al.* (1963). The assay is based on the estimation of fructose using fructose thiourea reagent.

Reagents

1. 0.1M Borate buffer pH 7.8.
2. Buffered substrate- 3mg disodium glucose-6-phosphate was dissolved in 1ml buffer. This was prepared fresh before use.
3. 30% HCl
4. Resorcinol – thiourea reagent: 100mg of resorcinol and 250mg of thiourea were dissolved in 100ml of glacial acetic acid. This was stored in brown bottle.
5. Colour reagent: 30% HCl, resorcinol- thiourea reagent, and H₂O were mixed in the proportion 7:1:1. The solution was used on the same day as prepared.

6. Standard solution of fructose: 54 mg of fructose was dissolved in 100 ml of 0.25% benzoic acid.

Procedure

In to each of the tubes labelled “test” and “blank”, 1ml of buffered substrate was added. A suitable amount of the enzyme extract was added in to the test and the tubes were incubated at 37°C for 30 minutes. After the period of incubation enzyme was added to the blank tube, and 9ml of colouring reagent was immediately added to all tubes. The tubes were heated in the boiling water bath maintained at 70°C for 15 minutes. Standards containing varying concentrations of fructose and a reagent blank were similarly treated. The tubes were cooled in running water and the colour was read at 410 nm.

The enzyme activity is expressed as n moles of fructose utilized /min /mg of protein.

Estimation of Aldolase (Ketose-1-phosphate aldehyde lyase E.C.4.1.2.7)

Aldolase activity was estimated by the method of King (1965).

Reagents

1. 0.1M Tris-HCl buffer: pH 8.6
2. Fructose 1,6 diphosphate: 8.33 mg dissolved in 5ml of 0.05M in buffer, prepared just before use.
3. Hydrazine sulphate ; 0.56 N, pH 8.6
4. 0.1% DNPH in 2N HCl

5. 0.75N Sodium hydroxide
6. 10 % TCA
7. Standard DL glyceraldehyde: 123 mg was dissolved in 1.0 litre of water. This was left at room temperature for 2-4 days to permit polymerization.

Procedure

To 2.0 ml of fructose-1,6 diphosphate, 0.25 ml of hydrazine sulphate and 1.0 ml of the buffer was added to 1ml of enzyme extract and incubated at 37°C for 15 minutes and the reaction was stopped by addition of 1.0 ml of 10% TCA. 0.1ml of enzyme was added to the blank tubes and were centrifuged. 1.0 ml of the supernatant was transferred to the tubes containing 1.0 ml of the 0.75 N sodium hydroxide and left in the room temperature for 10 minutes . 1ml of the colour reagent was added in the test and the blank tube and incubated at 37°C for 1 hr. The colour developed by the addition of 0.75 N sodium hydroxide was read at 540 nm in UV spectrophotometer.

The enzyme activity was expressed as n moles of glyceraldehyde formed /min/ mg protein

Determination of Glyconeogenic enzymes

Estimation of Glucose-6-phosphatase (D-glucose-6-phosphate: NADP+ 1-oxidoreductase, E.C. 3.1.3.9)

Estimation of Glucose-6-phosphatase was carried out by the method of King (1965).

Reagents

1. Citrate buffer- 0.1M, pH 6.5
2. Glucose-6 Phosphate : 0.01M
3. Ammonium molybdate – 2.5 gm of ammonium molybdate was dissolved in 100ml of 3 N Sulphuric acid
4. ANSA - 500mg of amino naphthol Sulphonic acid was dissolved in 195ml of 15% sodium bisulphate and 5ml of 20% sodium sulphite was added to it. The solution was stored in the brown bottle.
5. 10 % TCA.

Procedure

The incubation mixture in a total volume of 1.0 ml contained 0.5ml of substrate and 0.2ml of the enzyme. Incubation was carried out at 37° C for 60 minutes. The reaction was arrested by the addition of 1.0 ml of TCA and centrifuged. The phosphorous content of the supernatant was estimated.

Enzyme activity was expressed as n moles of phosphorous liberated / mg of protein / min.

Estimation of Fructose 1,6-diphosphate (D-fructose-1,6-bisphosphate D-glyceraldehyde-3-phosphate-lyase, E.C. No. I. I. I .37)

Estimation of Fructose 1,6-diphosphate was carried out by the method of Gancedo and Gancedo (1971).

Reagents

1. Tris-HCl Buffer : 1 M, pH 7.0
2. Fructose 1, 6 diphosphate 0.05 M
3. Magnesium chloride: 0.1 M
4. Potassium chloride: 0.1 M
5. EDTA : 0.001 M
6. TCA 10 %
7. Ammonium molybdate – 2.5 gm of ammonium molybdate was dissolved in 100ml of 3 N Sulphuric acid
8. ANSA - 500mg of amino naphthol Sulphonic acid was dissolved in 195ml of 15% sodium bisulphate and 5ml of 20% sodium sulphite was added to it. The solution was stored in the brown bottle.

Procedure

The assay medium in the final volume of 2.0 ml contained 1.2 ml of buffer, 0.1ml of substrate solution, 0.25 ml of magnesium chloride, 0.1ml of potassium chloride, 0.25 ml of EDTA and 1ml of enzyme source. The incubation was carried out at 37°C for 15 minutes. The reaction was terminated by the addition of 1.0 ml of TCA. The suspension was centrifuged and phosphorus content was estimated.

The enzyme activity was expressed as n moles of phosphorous liberated / mg of protein / min.

Protein bound carbohydrate complexes

Isolation of Glycoprotein from liver tissues

About 500mg of the liver tissue was homogenised in 7.0 ml of methanol, filtered and scraped from the filter paper. To that material 10ml of the chloroform was added and it was filtered. To the residue 4.0 ml of the chloroform was added and once again it was filtered. To the residue 7.0 ml of Chloroform: Methanol (2:1) mixture was added, homogenised and filtered. The defatted tissue was taken for the estimation of glycoprotein.

Estimation of Hexose

Hexose was estimated by the method of Niebes (1972).

Reagents

1. Orcinol – Sulphuric acid reagent:
Solution A: 60ml of concentrated sulphuric acid was mixed with 40ml of distilled water.
Solution B: 1.6g of orcinol (recrystallized from benzene) was dissolved in 100ml of distilled water. 7.4ml of solution A was mixed with 1ml of solution B just before use.
2. Standard: 50mg of each of galactose and mannose were dissolved in 100ml of distilled water. This solution is diluted to a proportion of 1:10 to give concentration of hexose 100micrograms/ ml.

Procedure

For hydrolysis 25 mg of the defatted tissue was mixed with 2ml of 3N HCl and hydrolysed at 100°C for 4 hrs. The hydrolysate was neutralized with sodium hydroxide. From the hydrolysed sample 0.5ml of the neutralized solution was made up to 1.0 ml with distilled water and 8.5ml of ice-cold orcinol reagent was added very slowly. The mixture was heated at 80°C for 15mts, cooled and left in the dark for 25mts for color development. Standard solutions containing 25-100µgms were treated in the similarly. Then the absorbance was read at 540nm.

The hexose content is expressed as mg/100mg of defatted tissue.

Estimation of Hexosamine

Hexosamine was estimated by the method of Wagner (1979).

Reagents

1. Acetyl acetone reagent:
Solution A - Trisodium phosphate 0.1M: 4.1g of trisodium phosphate was dissolved in 25ml of distilled water.
Solution B - Potassium tetraborate 0.5N: 305.5 mg of potassium tetraborate was dissolved in 2ml of distilled water.
3.5ml of acetyl acetone was added to the mixture of solution A and solution B in the ratio of 98:2 (v/v).

2. Ehrlich's reagent: 320mg of p-dimethyl amino benzaldehyde was dissolved in 21ml of isopropanol and 3ml of concentrated HCl was added to it.
3. Standard: 100 mgs galactosamine was prepared in 100ml of water. This solution was diluted to a proportion of 1:10 to give concentration of hexosamine 100micrograms/ ml in distilled water.

Procedure

For hydrolysis 25 mg of the defatted tissue was mixed with 2ml of 3N HCl and hydrolysed at 100°C for 4 hrs. The hydrolysate was neutralized with sodium hydroxide.

0.5ml of the neutralized sample was made up to 1ml with distilled water. 0.6ml of acetyl acetone reagent was added to all the tubes and heated in a boiling water bath for 30mts. After cooling, 2ml of Ehrlich's reagent was added and the contents were shaken well. The pink color developed was measured at 540nm against the reagent blank. Standard solution containing 10-40 µg of galactosamine were also treated in the similar manner.

The content of hexosamine in tissues is expressed as mg/100ml of defatted tissue.

Estimation of Sialic acid

Sialic acid was determined by the method of Warren (1959).

Reagents

1. Periodic acid 0.25 M: 14g of sodium periodate was dissolved in 100ml of 0.1N sulphuric acid.
2. Sodium meta arsenite 4%: 4gm of sodium meta arsenite was dissolved in 100ml of 0.5N hydrochloric acid.
3. Thiobarbituric acid: 144mg of thiobarbituric acid was dissolved in 20ml of distilled water. The pH of the solution was adjusted to 9 with 6 N Sodium hydroxide. This reagent was prepared just before use.
4. Acidified butanol: 5ml of concentrated hydrochloric acid was added to 95ml of n- butanol.
5. Standard: 10mg of N-acetyl neuraminic acid was dissolved in 100ml of distilled water.

Procedure

About 25 mg of defatted tissue was mixed with 0.5ml of 0.1N Sulphuric acid and hydrolysed at 80°C for 1hr. 0.5ml of the neutralized samples were taken along with standards (in the range of 10-50µg). Blank contained 0.5 ml of 0.1N sulphuric acid, 0.25ml of periodate was added to all tubes and incubated at 37°C. After 30mts, 0.25ml of arsenite solution was added to inhibit the reaction. Contents were mixed and 2ml of thiobarbituric acid was added and the tubes were heated in a boiling water bath for 6mts. After cooling, pink color developed was extracted into 5ml of acidified butanol phase, and was measured at 540nm against a reagent blank.

The sialic acid content is expressed in tissues as mg/100mg of defatted tissue.

Histopathological Investigations

The rats were sacrificed liver, kidney, intestine, and heart were dissected out and cleaned well with cold physiological saline to remove blood and adhering tissues. The samples were then fixed in 10% formalin- saline and embedded in paraffin. Serial sections were cut at 5µm and stained with haematoxylin and eosin. The sections were examined under light microscope and photographs were taken.

Statistical Analysis

Results will be expressed as mean \pm standard error of mean (S.E.M.). Statistical significance is determined by one-way analysis of variance (ANOVA). The data obtained from toxicity studies will be analyzed using Dunnet's 't' test P values less than 0.05 will be considered significant.

RESULTS

Acute toxicity

Death was recorded during the treatment period in treated groups given 500mg/kg of *Biherbal extract* orally. Table I depicts the changes of animals in general behaviour, other physiological activities like giddiness, sniffing, aggressiveness, tachypnoea, and convulsion finally at the dose level of 500mg/kg. From Tables II and III, it was observed that there was a significant difference in the organs like lung, liver (**P<0.01) and heamatological parameters like Hb (*P<0.05) and W.B.C. (**P<0.01). Table IV shows that there was a remarkable alterations in biochemical parameters like Glucose, Sodium, AST and ALP (**P<0.01). Pathological examinations of the tissues on a gross and macroscopic basis indicated that there were no detectable abnormalities. Hence, it can be concluded that the BHE is practically toxic or lethal after an acute exposure at the dose range of 500mg/kg. Thus the test limit (ED₅₀) from this acute oral toxicity studies was fixed as 50 mg/kg body weight.

Chronic toxicity

There were no significant differences in food consumption and variation in the body weight was negligible. In the 3 month experiment, no mortality was observed in any of the treatment group. From Tables V,VI andVII it was evident that there was no relevant difference in body weight development, hematological or biochemical parameters and organ weights for all groups.

For the rats of the Biherbal extract treated group, values of AST, ALT and cholesterol were slightly elevated as compared to control but not statistically significant as observed from TableVII. They were, however, still in the normal range. No macroscopical abnormalities were detected in the examined organs. Histologically, there were only minor pathological findings of inflammatory or degenerative origin which is evident from Figures I to X. Inflammatory processes, especially in the respiratory system, showed the same frequency and extent in the Biherbal extract treated groups as in control groups. A harmful effect of the Biherbal extract to the organs could be ruled out. In particular, no histological changes were found for all groups in hearts, testis or ovaries. Slight inflammatory infiltrates or signs of bronchopneumonia with diffuse foci were found in the lungs of single animals of both, control and treatment groups.

Similarly, slight inflammatory infiltrates in the connective tissue or the glomeruli of the kidneys were observed in single animals, as well as mild turbid swellings in the liver lobules. In all cases, the degree of inflammatory or degenerative processes was only minor, and the phenomena occurred in all treatment groups with the same frequency. It can therefore be assumed that the observations were unrelated to the ingestion of Biherbal extract. The withdrawal of the Biherbal extract ingestion after 3 months did not induce any detectable change in behavioural patterns, nor were there any differences to controls for the hematological, biochemical, anatomical or histological parameters.

Table VIII depicts the effect of BHE on body weight, liver weight, blood glucose, urea and serum bilirubin in various experimental rats. A significant ($p > 0.01$) reduction in the liver weight was observed in group III BHE pre-treated animals when compared to that of group II rats intoxicated with CCl_4 . BHE supplementation reverted the decreased level of blood glucose in CCl_4 intoxicated rats. A significant increase in the Serum urea ($p > 0.01$) and bilirubin ($p > 0.001$) levels in CCl_4 group II rats were found to be restored to normal levels on BHE pretreated group III animals.

Figure XI shows the effect of the plant extract on serum total proteins, albumin and globulin levels. The serum levels of total proteins, albumin and globulin were significantly decreased ($p > 0.001$) in CCl_4 intoxicated group II animals. There was a prominent increase in the total protein levels of BHE pretreated CCl_4 intoxicated group III animals showing the regeneration of hepatic cells, when compared with the group IV and V, which received EAE and PLE respectively.

Table IX depicts the change in serum levels of the liver marker enzymes like AST, ALT, ALP, ACP, ICD and LDH. The significant increase in the serum transaminases AST and ALT levels was seen in the group II CCl_4 intoxicated animals. These enzymes were brought back to near normal levels in BHE pretreated group III animals ($P < 0.001$). Similarly the elevated ALP, ACP and LDH enzyme levels in group II CCl_4 intoxicated animals were also significantly decreased in the group III BHE pretreated CCl_4 intoxicated animals ($p < 0.01$, $P < 0.001$). Comparison of Group I control rats with that of group VII which received only BHE showed no significant variation in the

marker enzyme levels suggests that there was no adverse side effects due to the administration of Tween –80 and BHE alone.

From Figure XII, it was evident that BHE plays a protective role on the serum enzyme levels of ACE, γ GT and 5'-Nucleotidase. The enzymes like γ -GT ($p \leq 0.001$) and 5'-Nucleotidase ($p < 0.05$) were significantly increased in group II CCl₄ intoxicated animals. These increased levels were brought back to near normal levels in BHE treated group III animals. The probable mechanism by which the BHE extract exerts its protective action against CCl₄ induced hepatocellular metabolic alterations could be by the stimulation of hepatic regeneration. Serum acetyl choline esterase levels in the present study decreased in CCl₄ treated group II animals. BHE treatment reverted the decreased levels to near normalcy ($p < 0.001$) which was comparable to that of standard silymarin drug. The BHE was effective in correcting these marker enzyme levels when compared with its individual preparations like EAE and PLE extracts. Comparison of group I and VII shows no significant variation in these enzyme levels indicates no appreciable adverse side effects due to the administration of Tween–80 and BHE only.

Table X shows the changes in liver tissue levels of ALT, AST, LDH, ALP and ACP in different groups of experimental animals. All the marker enzyme levels were significantly ($p < 0.001$) increased in CCl₄ intoxicated rats. Being cytoplasmic in location the marker enzymes AST, ALT, ALP and LDH are increased when hepatocytes get damaged as in the case of CCl₄ damage. The stabilization of AST, ALT, LDH, and ALP levels by BHE is a clear indication of the improvement of the functional status of the liver cells.

Comparison of group I and VII shows no significant variation in these enzyme levels indicates no appreciable adverse side effects due to the administration of Tween-80 and BHE only.

Table XI shows the changes in liver tissue levels of γ GT, 5' Nucleotidase and Xanthine oxidase in different groups of experimental animals. All these enzymatic parameters were significantly ($p < 0.001$) increased in the group II CCl_4 intoxicated animals. In the present study, the elevated levels of XO, γ GT, 5'Nucleotidase activity in the CCl_4 administered group was shown to be effectively counteracted by the administration of BHE.

Moreover, the hepatoprotective activity of BHE was much stronger than that of the reference drug silymarin, administered at the same concentrations.

The BHE was effective in correcting these marker enzyme levels when compared with its individual preparations like EAE and PLE extracts. Comparison group I and VII shows no significant variation in these enzyme levels indicates no appreciable adverse side effects due to the administration of Tween-80 and BHE only.

Figure XIII shows the serum lipid profile of the control and the various experimental animals. There was a significant increase ($p \leq 0.001$) in the levels of triglycerides and cholesterol in CCl_4 intoxicated Group II animals. This showed that a block in the secretion of hepatic triglycerides and cholesterol into the plasma, a major mechanism underlying the fatty liver

induced by CCl_4 and other toxins in the rats. The concentration of phospholipids was significantly ($p \leq 0.001$) increased in CCl_4 intoxicated Group II animals. The decreased levels of cholesterol, triglycerides and increased level of phospholipids were brought back to near normal by the treatment of BHE. This observed restoration of the CCl_4 evoked changes in the serum lipid profile explains the protective nature of BHE.

Table XII depicts the serum lipoprotein profile of the control and the experimental animals. Increased levels of LDL and VLDL cholesterol ($p < 0.01$) and decreased level of HDL in CCl_4 intoxicated rats were restored to near normal levels on BHE treatment. The recovery towards normalization of serum lipoproteins caused by BHE is almost similar to that caused by silymarin, in the present study which was used as a positive control.

The BHE was effective in correcting these serum lipid and lipoprotein profile when compared with its individual preparations like EAE and PLE extracts. Comparison of group I and VII shows no significant variation in these parameters indicating that no appreciable adverse side effects due to the administration of Tween -80 and BHE alone.

Table XIII depicts the concentration of total lipids, cholesterol, phospholipids and triglycerides in liver tissue. Significant enhancement in the concentrations of total lipids, cholesterol, triglycerides and phospholipids were observed in the tissues of group II rats which received CCl_4 alone. The increased lipid profile parameters in the liver tissue were significantly brought towards normalization on treatment with BHE. Moreover it was

observed that the combined preparation of BHE was found to be more effective, when compared with its individual preparation that was given to the group IV and group V animals respectively.

Figure XIV shows the values of liver glycogen and protein in different experimental rats. In the present study a significant ($p < 0.001$) decrease in the liver protein and glycogen ($p < 0.001$) were observed in CCl_4 intoxicated group II rats. It has been observed that there was a significant hypoglycemia with a drop in hepatic glycogen content. Acute liver failure due to CCl_4 intoxication was characterized by a disturbed protein synthesis. These protein and glycogen levels were returned to near normal in the BHE treated groups, which shows the protective nature of the drug against the CCl_4 damage.

Comparison of group I and VII shows no significant variation in these parameters which indicates no appreciable adverse side effects due to the administration of Tween -80 and BHE only.

From Table XIV was evident that the levels of lipid peroxidation LPO products malanaldehyde, conjugated dienes, nitric oxide and hydro peroxides were significantly increased [$p < 0.01$] in the liver tissues of CCl_4 intoxicated group II rats. Increased production of Reactive Oxygen Species [ROS] due to oxidative stress plays an important role in liver diseases. CCl_4 has been reported to induce lipid peroxidation and alter the antioxidant defence system through formation of free radicals, which in turn causes damage, and degeneration of hepatic tissues. These values were returned to normal levels

in BHE treated group III animals showing the protective nature of the drug by scavenging the free radicals.

Figure XV shows the changes in the *in-vitro* lipid peroxidation in liver cells in the presence of ascorbate, FeSO₄ and H₂O₂ induced in different groups of experimental animals. The hepatic tissue of untreated CCl₄ induced rats showed a 2-fold rise in basal lipid per oxidation levels as well as a 1.3-fold, 1.8-fold and 1.54 fold increase in MDA in the presence of inducers such as ascorbate, ferrous sulphate and hydrogen peroxide respectively.

Table XV shows the level of various antioxidant enzymes of liver in various experimental group of rats. The enzyme activities like SOD, CAT, GPX, GST, and GR were significantly decreased [$p < 0.01$] in CCl₄ intoxicated group II rats when compared to those of normal control rats. These antioxidant enzyme levels were brought back to near normal levels on treatment with BHE group III animals.

Table XVI and Figure XVI shows the levels of non-enzymic parameters in normal, CCl₄ intoxicated and plant drug treated groups of different experimental animals. The decreased levels of vitamin E, Vitamin A, total thiols, glutathione, uric acid, and cereloplasmin were observed in CCl₄ treated group II rats. These values were restored to near normal levels significantly ($p < 0.01$) in group III CCl₄ intoxicated BHE pretreated group III animals

Thus the free radical scavenging property of BHE could have maintained the near normal levels of non –enzymic antioxidants in-group III

animals. Decreased activities of non-enzymic antioxidants in CCl₄ treated group II rats may increase their susceptibility to oxidative injury. Elevated levels of these non-enzymic antioxidants in BHE treated group III animals offer protection against the oxidative injury caused by the free radicals.

Table XVII shows the activities of membrane bound ATP ases in the liver of various experimental animals. The levels of membrane bound enzymes were significantly decreased in the liver tissues of CCl₄ intoxicated group II rats. The Na⁺/K⁺-ATPase, Mg²⁺-ATP ase and Ca²⁺-ATP ase are the membrane bound enzymes which are responsible for the transport of these ions respectively across the cell membrane at the expense of ATP. The decreased level of these enzymes in CCl₄ intoxicated rats may be due to the alteration of membrane lipid composition due to the release of free radicals. The rats which received BHE, EAE, and PLE restored the enzyme levels to near normal levels which could be due to the ability of plant extracts to protect the membranes from oxidative damage through inhibition of lipid per oxidation.

Table XVIII depicts the effect of BHE on the glycolytic enzymes in the various experimental group animals. A significant decrease in the activities of glycolytic enzymes, in the liver of CCl₄ intoxicated rats were restored to near normal levels in BHE pretreated group III rats.

Table XIX shows the effect of BHE in the gluconeogenic enzymes in the various experimental group animals. The activities of gluconeogenic enzymes like, glucose-6-phosphatase and fructose-1, 6-diphosphatase in liver

were found to be significantly decreased in CCl₄ intoxicated rats. The BHE restored the normal levels of these enzymes, which shows the protective nature of the drug on the mitochondria from damage, there by normalizing these gluconeogenic enzymes and keeping the blood glucose in normal levels.

Figure XVII shows the average values of liver glycoprotein in different experimental rats. Glycoprotein are linear polymers of amino acids with branching chain of carbohydrates that may include hexose, hexoseamine and sialic acid. There was a significant decrease in the levels of Glycoprotein ($p < 0.001$) was observed in the group II CCl₄ treated animals due to the increased load of the toxic metabolites. The increase in glycoprotein content of BHE treated group II animals suggested the cytoprotective nature of the formulation.

The Figures XVIII to XXI depicts the histopathological changes in the liver, kidney, heart and intestinal tissues. CCl₄ selectively damages the liver which is shown in Figure XIX(b) by the formation of centrilobular necrosis. The BHE treated liver cells showed near normal architecture. The histopathological changes were negligible as far as kidney heart and intestinal tissues are concerned.

DISCUSSION

Liver plays a major role in detoxification and excretion of many endogenous and exogenous compounds, any injury to it or impairment of its functions may lead to many implications on one's health. Management of liver diseases is still a challenge to the modern medicine (Reddy *et al.*, 1993; Handa, 1991) The modern medicine have little to offer for alleviation of hepatic ailments, where as most important representatives are of phytoconstituents (Handa and Kapoor, 1999). Liver is the most important organ concerned with metabolic activities of the human body. It has tremendous capacity to detoxify toxic principles and synthesize useful principles. Therefore damage to the liver inflicted by hepato-toxic agents is of grave consequences. There is an over increasing need for an agent which could protect it from such damage. A great deal of research has been carried out to evaluate scientific basis for the claimed hepatoprotective activity of herbal agents as a single agent or in formulation.

Liver detoxifies and excretes destructive agents in many toxication cases. The toxins are converted into the intermediate reactive radicals, prior to their hepatotoxic effects, followed by degenerative necrotic and atrophic liver parenchyma cells with interstitial connective tissue (Robins and Kumar, 1987). CCl₄-induced hepatotoxicity in rats represents an adequate experimental model of cirrhosis in man and it is used for the screening of hepatoprotective drugs (Al-Shabanah *et al.*, 2000) It is well established that CCl₄ induces hepatotoxicity by metabolic activation, therefore it selectively causes toxicity in liver cells maintaining semi-normal metabolic function.

CCl_4 is bio-transformed by the cytochrome *P450* system in the endoplasmic reticulum to produce trichloromethyl free radical ($\bullet\text{CCl}_3$). Trichloromethyl free radical then combines with cellular lipids and proteins in the presence of oxygen to form a trichloromethyl peroxy radical, which may attack lipids on the membrane of endoplasmic reticulum faster than trichloromethyl free radical. Thus, trichloromethylperoxy free radical leads to elicit lipid peroxidation, the destruction of Ca^{2+} homeostasis, and finally, results in cell death. A number of investigators have utilized this chemical to produce liver cirrhosis in experimental animals (Parola *et al.*, 1992).

The ancient traditional medicine - Ayurveda: The origin of Ayurveda has been lost in prehistoric antiquity, but their characteristic concepts appear to have been nurtured between 2500 and 500 BC in India (Mukherjee, 2001) Herbal drugs constitute a major part in all traditional systems of medicine. Herbal medicine is a triumph of popular therapeutic diversity. Plants above all other agents have been used for medicine from time immemorial because they have fitted the immediate personal need, are easily accessible and inexpensive. There are approximately 1250 Indian medicinal plants, which are used in formulating therapeutic preparation according to ayurveda and other traditional system of medicine (Mills and Kerry, 2000).

Therefore we have undertaken this study to evaluate the efficacy of BHE which was made up of equal quantities of *E. alba* and *P. longum* in CCl_4 induced hepatotoxic rats. The BHE showed significant protection as assessed by biochemical, pharmacological and histological parameters. Probably hepato- protective effect is a combined action of all ingredients.

The hepatoprotective nature of any formulation was evaluated after fixing the LD₅₀ value by acute toxicity studies. In addition to acute toxicity studies chronic toxicity effects of the BHE was evaluated according to OECD (2001) guidelines. In acute toxicity about 50% animal death was recorded during the treatment period in treated groups given in 500 mg/kg of BHE orally. Hence it can be concluded that BHE is practically toxic or lethal after an acute exposure at the dose range of 500 mg/kg. This test limit for acute oral toxicity is generally considered to be 5.0 g/kg body weight. The LD₅₀ value of BHE was 500 mg/kg body weight, one tenth of the LD₅₀ value was considered as ED₅₀ value for any pharmacological studies. So for hepatoprotective efficacy studies 50 mg/kg body weight BHE was used which was considered as its ED₅₀ value.

Significant difference in the organ weight of lung and liver was observed in the test animals which received the lethal dose drugs. The histological parameters such as Hb, WBC and biochemical parameters like glucose, Na, AST and ALP also showed significant difference. The similar results were shown by Caisey and King (1980).

There were no significant differences in food consumption and variation in the body weight was negligible. In the 3 month experiment, no mortality was observed in any of the treatment group. There was no relevant difference in body weight development, hematological or biochemical parameters as mentioned by Carol, 1995 and organ weights for all groups.

For the male rats of the Biherbal extract treated group, values of AST, ALT and cholesterol were slightly elevated as compared to control but not statistically significant (Barry, 1995).

The 3 month daily oral application of Biherbal extract to rats yielded no signs of toxicity. In addition, no behavioural or physiological changes were observed on discontinuation of Biherbal extract feeding after 3 months treatment. In rats, the extract tested was proven non-toxic under the experimental conditions. The dosage range and study duration applied in this study are relevant for long-term human use, and represent dosage schemes by far exceeding the normal human application. The results of this study do not allow the conclusion of any toxicity of Biherbal extract, particularly to the liver.

In the present investigation a significant ($p < 0.01$) increase in the liver weight was shown in Group II CCl_4 intoxicated animals, when compared to that of group III BHE pretreated animals. According to Saxena (1979) this is due to the accumulation of lipids largely the triglycerides in the CCl_4 rats. This was reduced in the BHE treated rats leading to the decreased weight of the liver. The decrease in glucose level was significant in CCl_4 treated groups. This might be due to the glucose 6 phosphatase deficiency in the CCl_4 administered rats. CCl_4 elevated the serum levels of urea and bilirubin due to its enzymatic activation of $\bullet\text{CCl}_3$ free radical, which in turn alters the structure and function of liver cells. According to Rao (1973) there is a defective excretion of bile by the liver due to hepatotoxin injury which is reflected in their increased levels in the serum. Hyperbilirubinaemia is a very

sensitive test to substantiate the functional integrity of the liver and severity of necrosis, of hepatocytes (Singh et al., 1998). In BHE pretreated group III animals these biochemical parameters were decreased ($p < 0.001$), when compared with the CCl_4 intoxicated group II animals. The combinational preparation (BHE) was found to be effective, when compared with the drugs given individually to the group IV and group V animals.

Similar to our results Dubey *et al.* (1994) reported a depression in total protein level of serum due to the defect in protein biosynthesis. The serum levels of total proteins, albumin and globulin were significantly decreased in CCl_4 intoxicated rats when compared to control animals. This is due to the disruption and disassociation of polyribosomes from endoplasmic reticulum following CCl_4 administration according to Clawson (1989). The decrease in serum albumin might be due to a reduction in hepatic albumin biosynthesis (Kheir *et al.*, 1992). The total protein levels were increased in BHE pretreated, CCl_4 intoxicated group III animals showing the regeneration of hepatic cells. This may be due to the promotion of the assembly of ribosome on endoplasmic reticulum to facilitate uninterrupted protein biosynthesis. Stabilization of serum bilirubin and urea levels through the administration of the plant extract is further a clear indication of the improvement of the functional status of the liver (Anupam Bishayee *et al.*, 1995). The reason for this improvement might be that *P. longum* Linn. contains flavonoids, which might have scavenged the free radical offering hepatoprotection. This increase in the protein levels are more pronounced in BHE treated group III animals, when compared with the group IV and V, which received these drugs

individually. Comparison between Group I and VII shows no significant variation in protein levels indicates no appreciable adverse side effects due to the administration of Tween - 80 and BHE only. Group comparison between group III and group VI shows no significant variation in these parameters indicating that the BHE has got the same effect as that of silymarin which was considered as the positive control.

The plasma levels of the liver marker enzymes like AST, ALT, ALP, ACP, γ GT, 5'-nucleotidase, ICD and LDH were significantly ($p < 0.001$) increased in CCl₄ intoxicated group II animals. According to Brent and Rumack (1993); Fehèr and Prònai (1993) hepatotoxic compounds such as CCl₄ are known to cause remarkable increase in serum transaminases and induce liver injury through lipid peroxidation by free radical derivatives of the compound indicating the cellular leakage and loss of the functional integrity of cell membranes in liver as observed in our results. Acid phosphatases are frequently employed as the marker enzymes to assess the lysosomal changes as reported by Tanaka and Iizuka (1968). The rise in serum levels of γ GT, 5'-nucleotidase and ICD has been attributed to the damaged structural integrity of the liver. Moreover according to Rao (1973) in liver injury due to hepatotoxin, there is a defective excretion of bile by the liver which is reflected in their increased levels in serum Oral administration of BHE at a dose of 50 mg/kg body weight to rats caused a decrease in the activity of the above enzymes, which may be a consequence of the stabilization of plasma membrane as well as repair of hepatic tissue damage caused by CCl₄. This is supported by the view by Thabrewet *et al.* (1987) that serum levels of

transaminases return to normal with the healing of hepatic parenchyma and regeneration of hepatocytes. The preliminary phytochemical screening showed the presence of many compounds which could account for the regeneration of hepatocytes. According to Khopde *et al.* (2001) compounds in their natural formulations are more active than their isolated form. Anupam Bishayee (1995) reported similar studies using carrot extracts.

Serum acetyl Choline esterase levels in the present study decreased abruptly in CCl₄ treated group II animals. Zakut *et al.* (1988) reported that Pseudocholinesterase is low also in some instances of liver disease, including decompensated cirrhosis, hepatitis, metastatic carcinoma, chemical toxicity, and in malnutrition. BHE treatment reverted the decreased levels to near normalcy ($p < 0.001$) Lipid peroxidation, is accepted to be one of the principal causes of CCl₄ induced liver injury, and is mediated by the production of free radical derivatives of CCl₄. Antioxidant principles from herbal resources are multifaceted in their effects and provide enormous scope in correcting the imbalance through regular intake of a proper diet. According to Vitaglione *et al.* (2004) natural antioxidants have been proposed and utilized as therapeutic agents to counteract liver damage. Similar hepatoprotective activity could be expected by our BHE also.

Hepatic cells participate in a variety of metabolic activities and contain a host of enzymes. As reported by Wells (1988) liver tissues, contain higher concentrations of AST and ALT in cytoplasm and AST in particular also exists in mitochondria. In liver injury, the transport function of the hepatocytes is disturbed, resulting in the leakage of plasma membrane

(Zimmerman and Seef, 1970). Being cytoplasmic in location the marker enzymes AST, ALT and LDH are increased when hepatocytes get damaged as in the case of CCl₄ damage. Apart from that, γ GT, 5'Nucleotidase and xanthine oxidase were also elevated in the CCl₄ intoxicated group II animals. It is well documented by Krauskopf *et al.* (2005) that XO is an important prerequisite factor in the process of O₂ generation in CCl₄ toxicity and this observation concurred with our finding where a significant rise in XO was noticed in CCl₄ administrated rats followed by a significant increase in O₂ generation leading to oxidative stress. According to Kiso *et al.* (1984) oxidative stress is considered to play a prominent causative role in many diseases including liver damage. Cellular γ GT has a central role in glutathione homeostasis by initiating the breakdown of extracellular glutathione (GSH), the critical antioxidant defence for the cell as reported by Kugelman *et al.* (1994). So an increase in the γ GT enzyme level indicates the increased breakdown of glutathione in the CCl₄ intoxicated rats and due to the increased deprivation of glutathione which cannot counter act with the synthesis of the same altering antioxidant defense mechanism leading to hepatotoxicity. In this study, the elevated level of XO, γ GT, 5'Nucleotidase activity in the CCl₄ administered group was shown to be effectively counteracted by the administration of BHE. The stabilization of AST, ALT, ACP, and ALP levels by BHE is a clear indication of the improvement of the functional status of the liver cells. It has been shown by Yasuda *et al.* (1980) that protective agents exert their action against CCl₄ induced liver injury by impairment of CCl₄ mediated lipid peroxidation, either through decreased production of free radical derivatives or due to the antioxidant activity of the

protective agent itself. The preliminary phytochemical screening of these extracts showed the presence of phenolic compounds and flavonoids which may be effective in protecting the rats from CCl₄ injury. The compounds Piperonaline, Pellitorine, Piperine, Piperanine and Piperlonguminine are isolated from *Piper longum* may be responsible for the protective activity (Stohr *et al.*, 2001).

The plasma and liver tissue lipid profile of the control and the experimental animals were discussed below. In the present investigation there was a accumulation of triglycerides and cholesterol in the hepatic cells which was paralleled by a decrease in the concentration of plasma lipids and lipoproteins as mentioned in the report of Littleton and John (1979). Several investigators showed that a block in the secretion of hepatic triglycerides into the plasma is the major mechanism underlying the fatty liver induced by CCl₄ and other toxins in the rats. Torres-Durán *et al.*, 1998; Devarshi *et al.*, 1986 reported that fat from the peripheral adipose tissue is translocated to the liver and kidney leading to its accumulation during toxicity. Moreover the hepatic triglycerides is not released as such, but it is combined with lipoprotein . Due to the interference with the synthesis of protein moiety by CCl₄ the synthesis of lipoprotein also, gets affected leading to the decreased levels of cholesterol in the serum. Remarkable increase in the concentration of phospholipids was noticed both in the serum and the liver of group II rats. According to Weissberger (1940) hepatotoxic treatment produces an increase in the level of phospholipids in serum which may be due to the decrease in mitochondrial fat oxidation. The altered biochemical parameters in the liver tissue were

significantly brought towards normalization by co-administration of BHE. The recovery towards normalization of these lipid profile caused by BHE is almost similar to that caused by silymarin, in the present study. Similar results have been reported by Morazzoni and Bombardelli (1995). The presence of phytoconstituents such as triterpene glycoside, saponins like eclalbasaponin, 3β , 20β , 16β -trihydroxytaraxastane; 3β , 20β , 28-trihydroxytaraxastane and sulphated saponins in the leaves of *E.alba* might be account for the hepatoprotective activity exhibited by BHE

In accordance to the present study, it has been observed that there was a significant hypoglycemia with a drop in hepatic glycogen content. This might be due to the peripheral uptake and utilization of glucose that is consequently increased. Acute liver failure due to CCl_4 intoxication was characterized by a disturbed protein synthesis. In the present study also there is significant $p < 0.001$ decrease in the liver protein was observed in CCl_4 intoxicated group II rats. This may be due to the ROS generated as a result of $\bullet\text{CCl}_3$ radical which in turn affect the amino acids histidine, methionine and tyrosine. Oxidation of protein sulphahydril groups have been significantly contributed to the damage, depending on the protein affected. These protein and glycogen level were returned to near normal levels in the BHE treated groups, which shows the protective nature of the drug against the CCl_4 damage. The compounds piperlonguminine, piperine, apigenin, dimethyl ether and β sitosterol were reported by Parmar et al 1993 in *P. longum* might have been accounted for the hepatoprotective activity.

The levels of Lipid peroxidation products, Malanoaldehyde, Conjugated dienes, Iron, Nitric oxides and Hydroperoxides were significantly increased in the liver tissues of CCl₄ intoxicated group II rats. Serious attention is now paid to the cytotoxicity of active oxygen: free radicals as the cause of various pathological conditions. Lipid peroxidation, is accepted to be one of the principal causes of carbon tetrachloride-induced liver injury, and is mediated by the production of free radical derivatives of carbon tetrachloride. According to Niki (1995) lipid peroxides produced from unsaturated fatty acids via radicals, cause histotoxicity and promote the formation of additional free radicals in a chain reaction-type manner. According to Floyd *et al.*, (1984) if the *in vivo* activity of enzymes or scavengers is not high enough to inhibit these radicals, various diseases such as arteriosclerosis, liver disease, diabetes, inflammation, renal failure or accelerated aging may result.

The measurement of lipid peroxidation (LPO) is one of the most commonly used assays for radical induced damage (Svingen *et al.*, 1979; Sevanian and Hochstein, 1985). LPO is a chain reaction which can occur by enzymatic or non-enzymatic reactions, in most cases catalyzed by transition metals, where active oxidants cause the breakdown of polyunsaturated fatty acids in membrane phospholipids.

There are several assays for the determination of LPO, but two of the more frequent techniques to measure this process are to quantify the presence of Conjugated dienes (CD) and to determine thiobarbituric acid-reactive substances (TBARS). Conjugated dienes are formed by the rearrangement of double bonds of the PUFAs during the peroxidative process, and they are

considered an estimation of “*in vivo*” LPO, whereas the TBARS assay relies on the adducts formed between thiobarbituric acid and the carbonyl end products of lipid peroxidation progression, mainly malondialdehyde.

Non-enzymatic *in vitro* lipid peroxidation and formation of lipidperoxides can be initiated by adding ascorbate in the presence of oxygen and Fe^{3+} or Fe^{2+} ions to various tissue preparations. It was reported by Rosa and Catala, 1998 that Fe^{2+} and ascorbic acid stimulated lipid peroxidation in rat liver microsomes and mitochondria. Ascorbic acid is a critical antioxidant that acts as a free radical scavenger and may regenerate other antioxidants, including vitamin E (Chan, 1993). However, the reducing capacity of ascorbic acid can potentially lead to redox cycling of transition metals, which in turn can generate hydroxyl radicals in the presence of hydrogen peroxide. The addition of ascorbic acid to iron in the form of FeSO_4 greatly increases oxidative damage *in vitro*. Markers of lipid peroxidation, protein modification and DNA damage are all enhanced by ascorbic acid. In addition, ascorbic acid has been suggested to directly produce genotoxic lipid hydroperoxides in the absence of metals. Thus, metal coordination by polyphenolics may be most effective in antioxidant action. In order to clarify the mechanism of action of these drugs, *in vitro* experiments were undertaken. The hepatic tissue of untreated CCl_4 induced rats showed a 2 fold rise in basal lipid peroxidation levels as well as 1.3, 1.8 and 1.5-fold increase in LPO in the presence of inducers such as ferrous sulphate, ascorbate and H_2O_2 respectively. According to the results obtained, BHE inhibited lipid peroxidation in liver homogenate, which is shown by the decreased levels of MDA, produced. Recent studies by

Miller *et al.*, (1996) showed that iron-chelating activity of some flavonoids is closely related to their antioxidant action.

The *in vivo* oxidants such as CD, LOOH, iron and nitric oxide metabolites were also found to be increased in CCl₄ treated rats (Geller *et al.*, 1993) suggested that increased NO production and plasma nitrite/nitrate levels are also found during chronic hepatic inflammation, suggesting a role for NO in the hepatic response to inflammatory stimuli. It has been demonstrated by Beckman *et al.*, 1990 that nitric oxide (NO) reacts with O₂ in pathological states to produce peroxynitrite, a potent oxidizing agent. Peroxynitrite can initiate intracellular LPO formation and NP-SH oxidation, resulting in producing an extreme cellular membrane damage. The increase in the LPO substances in the liver of CCl₄ intoxicated rats indicates enhanced lipid peroxidation leading to tissue injury and failure of defence mechanisms to prevent the formation of excess free radicals. In the present study, BHE extract was effective in reducing the production of TBARS indicating the formation of decreased MDA. Amalaraj and Ignacimuthu (2001) reported that the hepatoprotective effect is associated with antioxidant rich plant extracts. The possible action of the BHE in decreasing the LPO reaction may be correlated with its antioxidant chemicals that affects oxygen radical-dependent damage *in vivo* by blocking radical formation, that is, by removing its precursors, superoxide, hydrogen peroxide and then transition metals. Quantitative analysis of the BHE showed the presence of considerable amount of Polyphenols in the form of flavonoids, could account for the prevention of LPO both in *in vivo* and *in vitro* experiments.

The levels of various antioxidant enzymes like SOD, CAT, GPx, GST, and GR activities were significantly decreased [$p < 0.01$] in CCl₄ intoxicated group II rats when compared to BHE treated rats. This indicates the antilipid peroxidative nature of the system against CCl₄ treatment is enhanced by BHE. Glutathione [GSH] constitutes the first line of defence against the free radical. In states of excessive oxidative stress, GSH is converted into GSSG and depleted leading to lipid peroxidation. According to Reckengel *et al.*, 1991 it is very important to maintain the level of GSH to prevent lipid peroxidation. GSSG is reduced to GSH by GR, which is NADPH dependent enzyme. It plays a role in maintaining adequate amounts of GSH. So, the reduction of GR results in decreased level of GSH. Reduction in GR and GSH was observed in CCl₄ treated group II rats indicates damage to the liver cells. The decreased levels of GSH in Group II animals may be due to the increased utilization or lower expression of GSH. GST is a soluble protein which is located in cytosol, plays an important role in the detoxification and excretion of xenobiotics. GST catalyzes the conjugation of the thiol functional groups of glutathione to electrophilic xenobiotics and results in increasing solubility. Since GST increases solubility of hydrophobic substances, it plays an important role in excretion of xenobiotics. GP_x a selenium-containing enzyme which is believed to reduce hydrogen peroxide and various hydroperoxides using glutathione as a reducing agent to form water and corresponding alcohols, respectively. Cellular hydroperoxides can otherwise serve as substrates for the metal mediated Fenton reaction to generate the highly reactive hydroxyl radical. According to Halliwell and Gutteridge (2000) the unavailability of GSH reduces the activities of GR, GP_x and GST.

Reconstitution of the levels of GSH, GP_x and GST activity in the rats treated with BHE confirms the protective and antioxidant efficiency of the BHE.

The enzymes like SOD and CAT plays an important role in the elimination of ROS derived from the peroxidative process in liver tissues. SOD removes superoxide by converting it to H₂O₂, which can be rapidly converted to water by CAT. As stated by Halliwell *et al.*, (1992) the antioxidant or free radical generation inhibition is important in protection against CCl₄ induced liver lesions. Potential antioxidant therapy should therefore include either natural free radical scavenging antioxidant enzymes or agent, which are capable of augmenting the activity of these enzymes, which include SOD, CAT and GPx (Bhattacharya *et al.*, 1997). The components in BHE contain active principles like piperine, and piperidine which are known for hepato protective and antioxidant activity. It is reported that piperine and piperidine could reduce the tert-butyl hydroperoxide and CCl₄ induced lipid peroxidation both *in vitro* and *in vivo* resulting in significant hepatoprotection in rats (Koul and Kapil, 1993; Khajuria *et al.*, 1998; Surh, 1999).

The free radical scavenging property of BHE could have maintained the near normal levels of non - enzymic antioxidants in-group III animals. Several experimental studies investigated the role of antioxidative vitamins, minerals, drugs and plant-derived compounds in the prevention and therapy of liver fibrosis. Decreased activities of non-enzymic antioxidants like vitamin C, vitamin E, Vitamin A, thiols, uric acid, and ceruloplasmin levels in CCl₄ treated group II rats may increase their susceptibility to oxidative injury.

Elevated levels of these non-enzymic antioxidants in BHE treated group III animals offer protection against the oxidative injury caused by the free radicals. Parola, 1993 stated that Vitamin E inhibited m-RNA expression of tumour growth factor- β and $\alpha 2$ procollagen in CCl_4 -induced animal fibrosis. Beta-carotene treatment also decreased liver hydroxyproline level during CCl_4 administration and reduced liver fibrosis by inhibition of lipid peroxidation in animal studies as stated by Seifert *et al.*, 1995.

Recently it has been shown by Abul *et al.*, (2002) that uric acid can be used as a scavenger of peroxynitrate (ONOO), a toxic product of the free radicals nitric oxide and superoxide. The production of (ONOO) has been implicated in the pathogenesis of central nervous system inflammatory diseases, including multiple sclerosis and its animal correlate experimental autoimmune encephalomyelitis. The mechanism of uric acid action as an antioxidant may relate to the formation of a urate-free radical after oxidant exposure. This urate-free radical is being scavenged by ascorbate. Thus it is suggested that urate and ascorbate interact as plasma antioxidants. According to Denkop (1979). Ceruloplasmin is produced in the liver in response to tissue injury and released into the circulation. Ceruloplasmin is considered a preventive plasma antioxidant because it sequesters transition metals, thereby preventing them from participating in free radical reactions as reported by Frei *et al.*, 1988.

Plasma total sulfhydryl groups have also been suggested by Sedlak and Lindsa (1968) to contribute significantly to the antioxidant capacity of plasma. The Phytoconstituents as reported by Upadhyay *et al.*, (2001) such as

eclalbatin, alpha-amyrin, ursolic acid, oleanolic acid, ecliptasaponin, daucosterol, stigmasterol-3-*O*-glucoside and coumestans were also present in BHE as main active principles for its hepatoprotective activity.

The levels of membrane bound enzymes were significantly decreased in the liver tissues of CCl₄ intoxicated group II rats. The Na⁺, K⁺ ATPase, Mg²⁺ ATPase and Ca²⁺ ATPase are the membrane bound enzymes which are responsible for the transport of these ions respectively across the cell membrane at the expense of ATP. According to (Stehoven and Bonting (1981) activated oxygen species which induce lipid peroxidation and damage various cell functions is known to inactivate many enzymes, both cytosolic and membrane-bound. Marin *et al.* (1992) reported that during lipid peroxidation process the activity of different membrane-bound enzymes are changed. This ultimately leads to changes in membrane permeability or to the destruction of cells or whole cell systems. According to Frank and Massaro (1980); Tirmenstein and Nelson (1990); Vermeulen *et al.*, (1992) have stated that the decreased level of these enzymes in CCl₄ rats may be due to alteration in membrane lipid composition and/or content, lipid peroxidation, disturbance in calcium homeostasis and oxidation and alkylation of thiol groups of glutathione and proteins. The rats which received BHE, EAE, and PLE retained the levels of TBA reactive substances and the activities of these enzymes were also restored to the near normal levels which could be due to the ability of plant extracts to protect the SH group from oxidative damage through inhibition of lipid peroxidation. In this work, the effect of CCl₄ on drug oxidizing system may be a significant factor in the alteration of

phospholipid and cholesterol were observed experimentally. The mitochondrial ATPase requires tyrosine and glutamate residues respectively for its hydrolytic and proton channel activity. Therefore the possible hepatoprotective effect of BHE on the chemical-induced liver injuries may be due to: (1) inhibiting Cytochrome P-450 activity, (2) preventing the process of lipid peroxidation, (3) stabilizing the hepatocellular membrane and (4) enhancing protein and glycoprotein biosynthesis.

Liver is the candidate organ involved in glucose homeostasis. It is the main site for glycolysis, a process where glucose is degraded and gluconeogenesis, where glucose is synthesized from lactate, amino acids and glycerol. According to Bhavapriya and Govidasamy (2000) these are the two important complementary events that balance the glucose load in our body. The activity of gluconeogenic enzymes like glucose-6-phosphatase and fructose-1,6-diphosphatase were found to be significantly decreased in CCl₄ intoxicated rats. This might be due to the glucose 6 po4 deficiency in CCl₄ administered rats. The BHE restored the normal levels of these enzymes which protects the mitochondria from damage thereby protecting these gluconeogenic enzymes .

Activity of hexokinase and phosphofructokinase enzymes which are ATP dependent are reported to be under regulation by citrate (Goyal *et al.*, 1990) which is a TCA cycle intermediate. Since in CCl₄ damage ATP depletion of the same was also considered as an important factor in the decreased activity of these enzymes. According to Arathi and Sachdanandam (2003) decrease in activity of phosphoglucoisomerase might be expected to

inhibit the proportion of glucose 6-phosphate metabolized via the glycolytic pathway. Aldolase, another key enzyme in the glycolytic path way, decreases in liver diseases and this may be due to cell impairment and necrosis. Our results revealed the decrease in the activities of these microsomal marker enzyme due to the increased formation of malondialdehyde following CCl₄ treatment BHE treatment restored these enzyme levels to near normal showing the regenerating ability of the plant extracts.

Glycoproteins are linear polymers of aminoacids with branching chain of carbohydrates that may include hexose, hexoseamine and sialic acid. Elevated glycoprotein may be due to the tissue necrosis, rapidly metabolizing tumor cell destruction from normal connective tissue and non specific stimulus in the process similar to that observed in many infectious diseases. According to Kishore (1983) the main change observed during the necrotic stage of CCl₄ poisoning was a highly significant reduction in the sialyl transferase activity followed by a considerable decrease in the sialic acid content. The similar effect was observed in our study also. According to Robinson *et al.*, (1964) the microsomal portion only is responsible for the glycoprotein synthesis and the increased load of the toxic metabolites in the system might affect the microsomes which may further be responsible for the decreased glycoprotein components in the CCl₄ treated rats. The increase in glycoprotein content of BHE treated group II animals suggested the cytoprotective nature of the formulation.

Histopathological examination of the livers provided supportive evidence for this study. Liver of rats administered with CCl₄ showed

centrilobular necrosis with mononuclear infiltration in the portal area, fatty deposition and loss of cell boundaries. In animals treated with the BHE, there was much lesser hepatocellular necrosis, mononuclear infiltration and loss of cell architecture in comparison to livers from control animals.

Histopathological observations showed faster regeneration of the hepatic cells in rats treated with BHE seems to suggest the possibility of BHE being able to condition the hepatic cells towards accelerated regeneration. Similar histopathological observations observed with silymarin seem to suggest that the ability to cause accelerated regeneration may be a feature common to certain medicinal plants to protect against liver dysfunction.

The mechanism by which BHE exerts its more protective action against CCl_4 induced alterations might be due to the synergistic activity of the plants. However, the fact is that BHE when given prior to CCl_4 administration can produce more rapid recovery of the liver when compared with those exposed to CCl_4 only, which indicates that the protective action may be due to the antioxidant property as in the case of other agents known to oppose the hepatotoxic effects of CCl_4 .

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SUMMARY

Hepatoprotective efficacy of BHE on CCl₄ intoxicated rats was performed to propose a new insight to the pharmacological action of BHE, which is a combined biherbal ethanolic extract made up of equal quantities of leaves of *Eclipta alba* and the seeds of *Piper longum*. Since polyherbal formulations are generally considered to be more effective due to the synergistic activity than a single herbal drug, the study has been undertaken to evaluate the protective effect of biherbal extract in comparison with its individual plants *Eclipta alba* and *Piper longum*.

The present investigation has been divided into two parts. In one part of the investigation the plants have been processed, screened for the phytochemicals and tested for free radical scavenging activities by using standard models in vitro.

The phytochemical investigation of the plants revealed the following results

In the present investigation, preliminary phytochemical screening of EAE, PLE and BHE showed the presence of constituents like alkaloid, carbohydrates, phytosterol, tannins, phenol, flavonoids, glycosides, terpene, saponins and lignin. In all these extracts proteins, gums and mucilage were found to be absent.

The presence of phytochemicals was confirmed by HPTLC finger printing of individual preparations of different extracts of *Eclipta alba* and

piper longum. The estimation of the macronutrients like carbohydrates, proteins, lipids and micronutrients present in the leaves of *Eclipta alba* and seeds of the *piper longum* showed the nutritive value of the plants.

The BHE showed a concentration dependent scavenging activities of the free radicals such as DPPH, super oxide, hydroxyl, nitric oxide, hydrogen peroxide and acted as an efficient chelator of ferric and ferrous ions. It was also found to be effective in reducing the formation of protein carbonyl groups. BHE contained considerable amount of the flavonoid and phenolic compounds which could be accounted for the free radical scavenging activities.

The inhibitory action of the BHE on DNA fragmentation induced by fenton reactants in the hepatic tissue was studied. In the CCl₄ intoxicated animals the extent of DNA damage was detected by the increased mobility of the DNA molecule due to the decrease in the molecular weight. On the other hand the BHE at the concentration of 20µg/ml and 40µg/ml protected the DNA from damage was evidenced by the decreased mobility of the DNA molecule suggested that these extracts have compounds which may combat against free radical-mediated degradation to the deoxyribose sugar moiety of DNA.

The second part of the investigation comprises of the evaluation of non-toxic dosage by acute and chronic toxicity on mice and albino Wistar strain rat models according to the guidelines of OECD. After assessing the dosage by acute and chronic toxicity studies efficacy of the single and the

biherbal drugs have been ascertained by using CCl_4 induced hepatotoxicity model in rats. The hepatoprotective nature of the drugs has been assessed by various biochemical estimations and histological observations. Rats treated with standard drug silymarin have also been utilised in this study to compare the hepatoprotective efficacy of the test drugs.

In the acute toxicity studies death was recorded during the treatment period in the experimental rats which received 500mg/kg body weight of Biherbal extract orally. So from this the ED_{50} dose of 50 mg/kg was selected for the hepatoprotective efficacy studies.

The three month daily oral application of BHE to rats yielded no signs of toxicity. The dosage range and study duration applied in this study are relevant for long-term human use.

Male albino rats were pre-treated with 50 mg/kg of BHE intubation for 14 days and intoxicated with CCl_4 on days 7 to 14. Normal rats were maintained with the BHE to assess the drug toxicity. Standard drug silymarin was used as positive control for comparison.

Biochemical analysis of the blood and serum revealed some important results as follows

In the present investigation a significant reduction in the liver weight, serum urea and bilirubin levels were seen in the BHE pre-treated animals when compared to that of rats intoxicated with CCl_4 . Decreased level of blood

glucose in CCl₄ induced rats were found to be normalized on BHE supplementation.

The total protein and albumin levels in serum were increased in BHE pretreated, CCl₄ insulted animals showing the regeneration of hepatic cells. This increase in the protein level are more pronounced in BHE treated animals, when compared with the group IV and V, which received its individual preparations EAE and PLE.

The serum levels of the liver marker enzymes like AST ALT, ALP, ACP, γ GT 5'-NT, ICD and LDH were significantly increased in CCl₄ intoxicated animals. BHE treatment reverted the increased levels of these enzymes to near normalcy, which was comparable to that of silymarin a standard drug in the present study.

The parallel decrease in the concentration of serum lipids and lipoproteins along with the increase of these parameters in liver tissues of CCl₄ insulted rats were normalized on BHE treatment .The increased serum phospholipids were also brought back to normal levels.

The decrease in liver total protein and glycogen in the rats treated with CCl₄ were normalized on BHE supplementation.

The BHE was effective in reducing the levels of *in vivo* lipid peroxidation products like MDA, CD, iron, nitric oxides and LOOH in the tissues of CCl₄ rats. The *in vitro* nonenzymatic lipid peroxidation induced by

the presence ascorbate, $\text{Fe}^{2+}/\text{Fe}^{3+}$ and H_2O_2 were also significantly reduced by BHE treatment.

Pretreatment with BHE to CCl_4 treated rats increased the activities of SOD, CAT, GPX, GST and GR indicates the enhanced antilipid peroxidative nature of the BHE.

Elevated levels of non-enzymic antioxidants such as vitamin C, vitamin E, Vitamin A, thiols, uric acid, and cereloplasmin in BHE treated animals offer protection against the oxidative injury caused by the free radicals produced by CCl_4 in the present study.

The decreased level of membrane bound enzymes such Na^+/K^+ -ATPase, Mg^{2+} -ATPase and Ca^{2+} -ATPase in the liver tissues of CCl_4 intoxicated rats may be because of the alteration of membrane lipid composition due to the release of free radicals. The rats which received BHE, EAE, and PLE restored the enzyme levels to near normal levels, which could be due to the ability of plant extracts to protect the membranes from oxidative damage through inhibition of lipid peroxidation

The activity of gluconeogenic enzymes and glycolytic enzymes in liver were found to be significantly decreased in CCl_4 intoxicated rats. These decreased enzymes levels were restored to normal levels in BHE pretreated rats indicating that the BHE has got protective action on the mitochondria from damage enabling them to secrete the enzymes and keeping the blood glucose in normal levels.

A significant decrease in the levels of Glycoprotein was observed in CCl₄ treated animals due to the increased load of the toxic metabolites. The increase in glycoprotein content of BHE treated animals suggested the cytoprotective nature of the formulation.

The histopathological observations which showed a faster regeneration of the hepatic cells in rats pretreated with BHE seems to suggest the possibility of BHE being able to condition the hepatic cells towards accelerated regeneration.

CONCLUSION

In conclusion, the BHE afforded protection from CCl_4 induced liver damage, significantly when compared to that of the individual preparations. The beneficial effects of BHE were found comparable to those of silymarin. By trapping oxygen related free radicals, the extract could hinder their interaction with polyunsaturated fatty acids and abolish the enhancement of lipid peroxidative processes. The effect may be due to the presence of flavonoids and phenolic compounds which are strong antioxidants. Antioxidant principles from herbal resources are multifaceted in their effects and provide enormous scope in correcting the imbalance through their consumption. Thus from the foregoing findings, it was observed that BHE exhibited protective effect against CCl_4 induced liver damage, which may be due to its antilipid peroxidative and free radical scavenging activities. It has promising therapeutic application in future to combat the liver related problems associated with free radical formation.

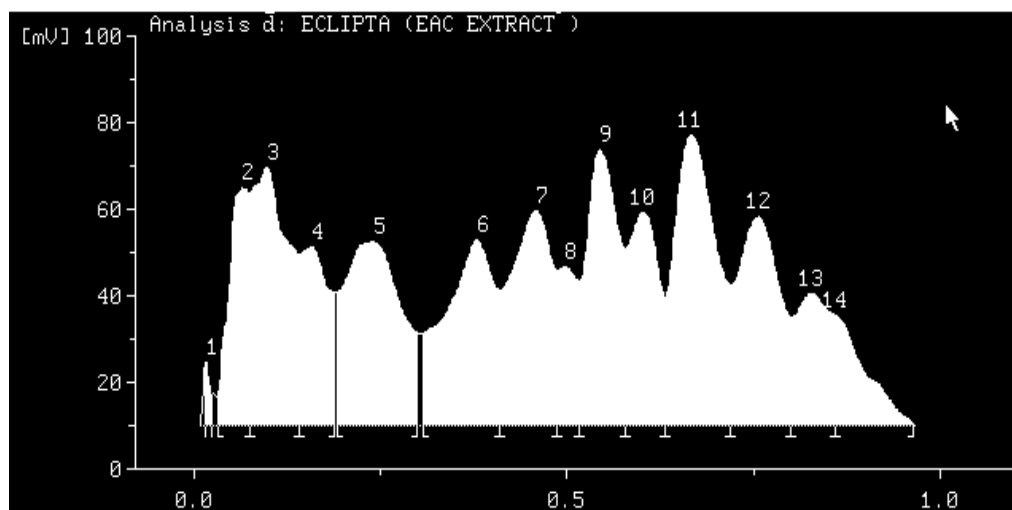
Table 1: Preliminary phytochemical screening of the test drug

S.No	Phytochemicals	EAE Extract	PLE Extract	Bi herbal extract
1.	Alkaloid	+	+	+
2.	Carbohydrate	+	+	+
3.	Protein	-	-	-
4.	Phytosterol	+	+	+
5.	Tannins	+	-	+
6.	Phenolic compounds	+	+	+
7.	Flavonoids	+	+	+
8.	Gums and Mucilage	-	-	-
9.	Glycosides	+	+	+
10.	Saponins	+	+	+
11.	Oils and fats	+	+	+
12.	Terpenes	+	+	+
13	lignin	+	+	+

(+) Indicates the presence of the chemical

(-) Indicates the absence of the chemical

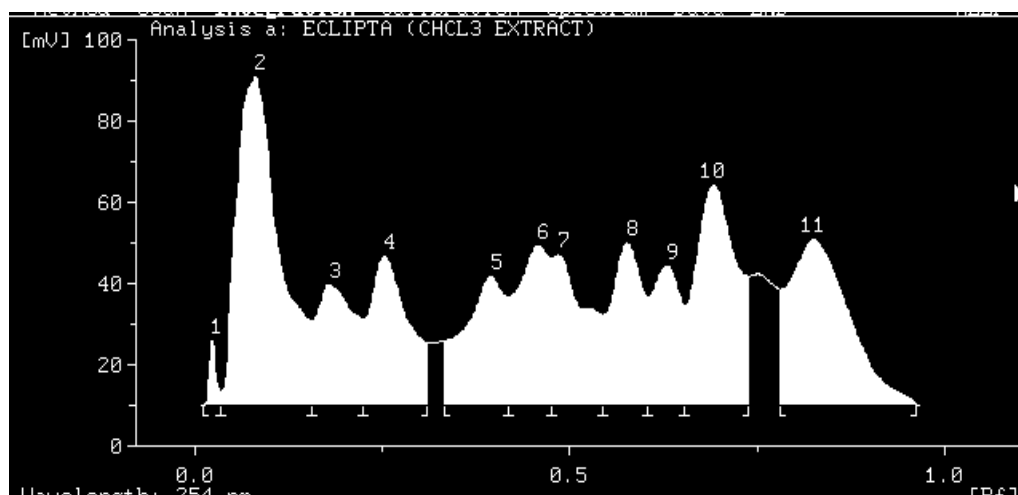
Figure I: The HPTLC finger print analysis of the Ethyl acetate extract of *Eclipta alba*



Track 4, Analysis d: ECLIPTA (EAC EXTRACT)

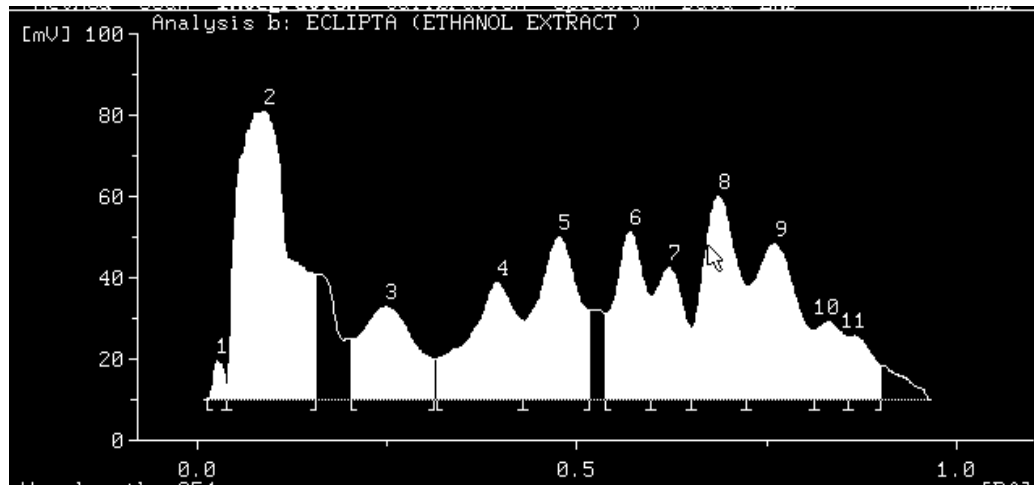
Peak #	start		max			end		area		
	Rf	H	Rf	H	[%]	Rf	H	F	[%]	
1	0.03	0.6	0.06	48.5	9.75	0.07	47.4	931.1	4.79	
2	0.07	47.4	0.09	53.5	10.76	0.14	33.6	2008.4	10.34	
3	0.14	33.6	0.15	35.4	7.11	0.18	25.2	968.0	4.98	
4	0.19	25.2	0.24	36.8	7.40	0.30	16.1	2136.7	11.00	
5	0.30	16.1	0.38	37.9	7.61	0.41	26.4	1881.5	9.68	
6	0.41	26.4	0.46	45.1	9.06	0.49	31.4	1893.5	9.75	
7	0.51	28.9	0.54	59.4	11.93	0.58	36.7	1947.9	10.03	
8	0.58	36.7	0.60	45.3	9.11	0.63	25.9	1398.0	7.20	
9	0.63	25.9	0.67	63.3	12.72	0.72	29.3	2717.3	13.99	
10	0.72	29.1	0.76	44.9	9.01	0.80	21.9	1931.9	9.94	
11	0.80	21.9	0.83	27.6	5.54	0.95	0.2	1614.3	8.31	
Total height =					497.7	total area = 19428.5				

Figure II: The HPTLC finger print analysis of the Chloroform extract of *Eclipta alba*



Peak #	start		max			end		area		
	Rf	H	Rf	H	[%]	Rf	H	F	[%]	
1	0.03	0.6	0.06	48.5	9.75	0.07	47.4	931.1	4.79	
2	0.07	47.4	0.09	53.5	10.76	0.14	33.6	2008.4	10.34	
3	0.14	33.6	0.15	35.4	7.11	0.18	25.2	968.0	4.98	
4	0.19	25.2	0.24	36.8	7.40	0.30	16.1	2136.7	11.00	
5	0.30	16.1	0.38	37.9	7.61	0.41	26.4	1881.5	9.68	
6	0.41	26.4	0.46	45.1	9.06	0.49	31.4	1893.5	9.75	
7	0.51	28.9	0.54	59.4	11.93	0.58	36.7	1947.9	10.03	
8	0.58	36.7	0.60	45.3	9.11	0.63	25.9	1398.0	7.20	
9	0.63	25.9	0.67	63.3	12.72	0.72	29.3	2717.3	13.99	
10	0.72	29.1	0.76	44.9	9.01	0.80	21.9	1931.9	9.94	
11	0.80	21.9	0.83	27.6	5.54	0.95	0.2	1614.3	8.31	
Total height =					497.7	total area = 19428.5				

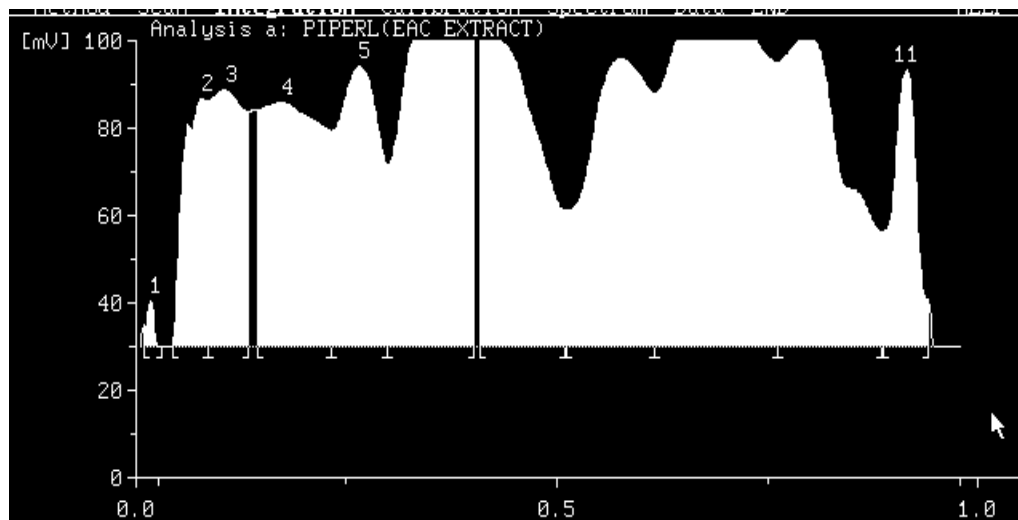
Figure III: The HPTLC finger print analysis of the Ethanolic extract of *Eclipta alba*



TRACK 2, ANALYSIS D: ELLIPTIA (ETHANOL EXTRACT)

Peak #	start		max			end		area	
	Rf	H	Rf	H	[%]	Rf	H	F	[%]
1	0.03	0.0	0.08	67.3	18.98	0.12	30.6	2997.0	22.95
2	0.15	27.4	0.16	27.5	7.77	0.19	11.4	516.4	3.95
3	0.20	11.4	0.24	19.4	5.48	0.31	6.7	1016.0	7.78
4	0.34	9.2	0.39	25.6	7.21	0.43	16.1	1059.4	8.11
5	0.43	16.1	0.47	36.7	10.34	0.51	18.8	1521.0	11.65
6	0.54	18.0	0.57	37.9	10.70	0.60	22.1	1125.0	8.61
7	0.60	22.1	0.62	29.3	8.25	0.65	14.6	856.5	6.56
8	0.65	14.6	0.69	46.9	13.23	0.73	24.8	1656.1	12.68
9	0.73	24.8	0.76	35.1	9.91	0.81	13.9	1573.8	12.05
10	0.81	13.9	0.83	16.1	4.54	0.86	12.5	451.3	3.46
11	0.86	12.5	0.87	12.7	3.58	0.90	5.6	288.3	2.21
Total height =			354.5			total area =			13060.8

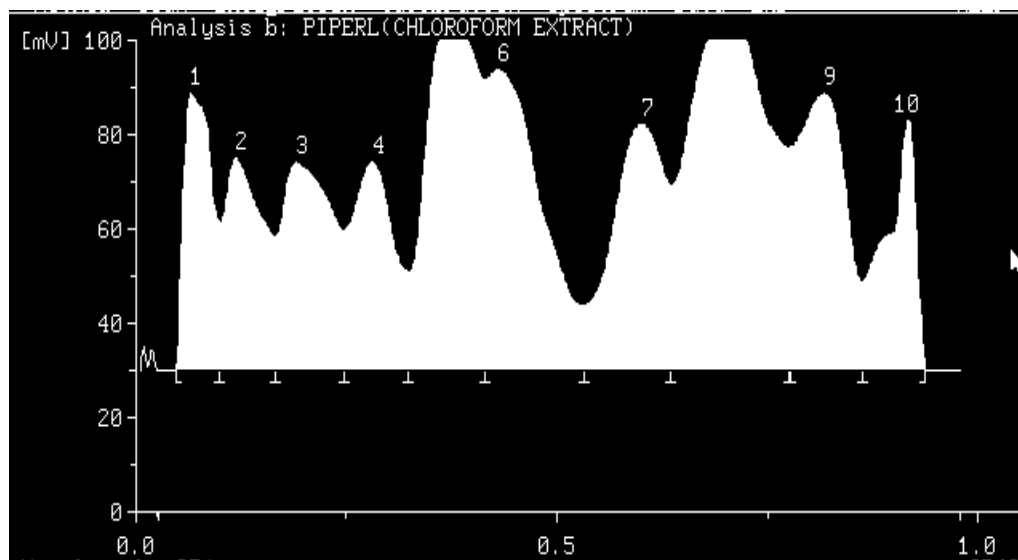
Figure IV: The HPTLC finger print analysis of the Ethyl acetate extract of *P. longum*



Track 1, Analysis a: PIPERL(EAC EXTRACT)

Peak #	start		max			end		area	
	Rf	H	Rf	H	(%)	Rf	H	F	(%)
1	0.01	4.7	0.01	10.5	1.56	0.03	0.0	72.5	0.19
2	0.04	0.1	0.07	56.9	8.48	0.08	56.2	1335.9	3.51
3	0.08	56.2	0.10	58.7	8.75	0.13	53.7	2099.0	5.52
4	0.14	54.1	0.17	55.8	8.32	0.23	49.2	3526.9	9.27
5	0.23	49.2	0.26	64.1	9.55	0.30	41.4	2828.8	7.43
6	0.30	41.4	0.35	76.3	11.37	0.40	71.8	5369.2	14.11
7	0.41	71.9	0.41	71.9	10.71	0.51	31.1	4246.0	11.16
8	0.51	31.1	0.57	65.9	9.82	0.61	57.8	4249.8	11.17
9	0.61	57.8	0.69	76.9	11.45	0.76	64.9	7935.5	20.85
10	0.76	64.9	0.79	70.9	10.57	0.88	26.3	4857.0	12.76
11	0.89	26.4	0.91	63.1	9.41	0.94	10.7	1533.7	4.03
			Total height = 670.9			total area = 38054.2			

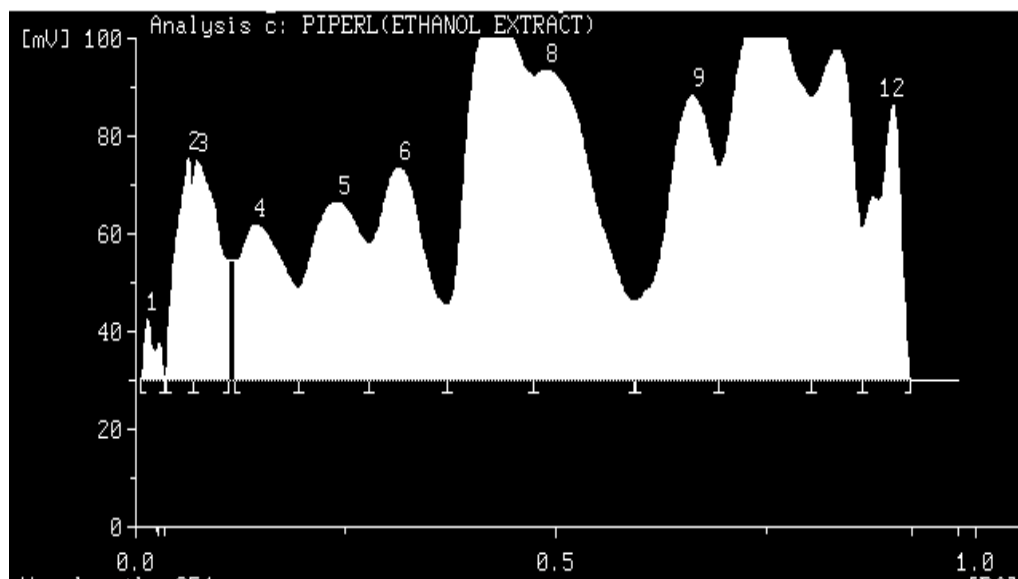
Figure V: The HPTLC finger print analysis of the Chloroform extract of *P. longum*



Track 2, Analysis b: PIPERL(CHLOROFORM EXTRACT)

Peak #	start		max			end		area	
	Rf	H	Rf	H	[%]	Rf	H	F	[%]
1	0.04	0.3	0.06	58.5	10.34	0.09	31.3	1699.4	5.77
2	0.09	31.3	0.11	44.9	7.93	0.16	28.2	1818.7	6.17
3	0.16	28.2	0.19	44.0	7.76	0.24	29.7	2326.3	7.89
4	0.24	29.7	0.28	44.0	7.77	0.32	20.9	1988.3	6.75
5	0.32	20.9	0.37	73.6	13.00	0.41	61.6	4086.2	13.86
6	0.41	61.6	0.43	63.6	11.23	0.53	13.7	3641.9	12.36
7	0.53	13.7	0.60	52.1	9.21	0.63	39.1	2859.1	9.70
8	0.63	39.1	0.70	74.0	13.07	0.77	47.2	6393.1	21.69
9	0.77	47.3	0.81	58.6	10.35	0.86	18.7	3004.7	10.20
10	0.86	18.7	0.91	52.9	9.35	0.94	0.1	1653.4	5.61
Total height =			566.4			total area = 29471.0			

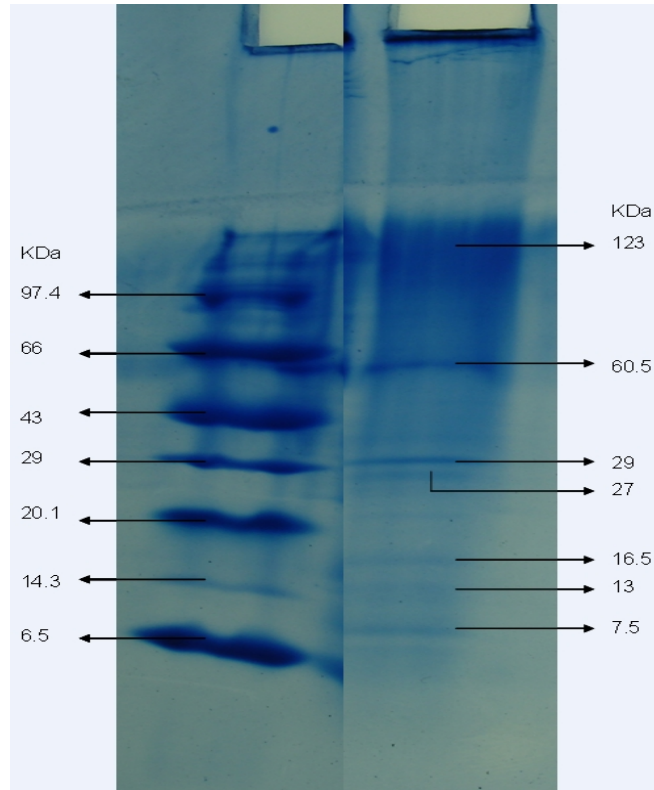
Figure VI: The HPTLC finger print analysis of the ethanolic extract of *P. longum*



Track 3, Analysis C: PIPERL(ETHANOL EXTRACT)

Peak #	start		max			end		area	
	Rf	H	Rf	H	[%]	Rf	H	F	[%]
1	0.00	0.2	0.01	12.5	2.05	0.03	0.0	138.9	0.48
2	0.03	1.1	0.06	45.3	7.44	0.06	39.3	729.0	2.52
3	0.06	39.3	0.07	44.9	7.37	0.11	24.7	1147.2	3.96
4	0.11	24.4	0.14	31.7	5.21	0.19	18.8	1537.5	5.31
5	0.19	18.8	0.24	36.4	5.97	0.27	27.9	1945.0	6.71
6	0.27	27.9	0.31	43.5	7.15	0.37	15.2	2217.1	7.65
7	0.37	15.2	0.42	73.4	12.06	0.47	62.2	4544.2	15.68
8	0.47	62.2	0.48	63.5	10.42	0.59	16.1	4045.0	13.96
9	0.59	16.1	0.66	58.1	9.55	0.69	43.6	2955.3	10.20
10	0.69	43.6	0.75	75.7	12.43	0.80	58.0	5493.2	18.96
11	0.80	58.0	0.83	67.7	11.11	0.86	30.9	2652.8	9.15
12	0.86	30.9	0.90	56.2	9.24	0.92	0.0	1572.7	5.43
			Total height = 609.0			total area = 28977.9			

Figure VII: The SDS-PAGE electrophoresis results of aqueous extract of *E. alba*



Lane 1 - Shows the marker protein bands with their molecular weight ranging 6.50 – 97.4 kDa

Lane 2 - Shows the *Eclipta alba* leaf protein bands with their molecular weight of ranging 7.50 –123 kDa

Protein used as markers with their molecular weight expressed in kDa:

Phosphorylase b-97.4; BSA-66; Ovalbumin-43; Carbonic Anhydrase-29;

Soyabean Trypsin Inhibitor-20.1; Lysozyme-14.3; Aprotinin-6.5

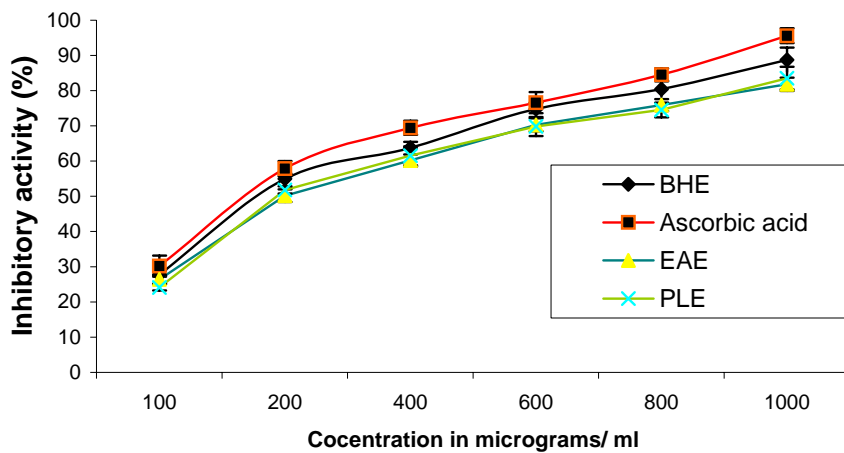
Table II: Concentration of Macronutrients

S.NO	Macronutrients	Leaves of <i>Eclipta alba</i> (Expressed in mgs/100gms)	Seeds of <i>Piper longum</i> (Expressed in mgs/100gms)
1.	Total sugars	27.78	15.56
2.	Total protein	26.23	8.80
3.	Total lipid	24.94	3.89

Table III : Concentration of Minerals present in the plants

S.NO	Macronutrients	Leaves of <i>Eclipta alba</i> (Expressed in ppm)	Seeds of <i>Piper longum</i> (Expressed in ppm)
1.	Aluminum	3.9115	1.399
2.	Barium	0.3915	0.4200
3.	Calcium	560	340
4.	Copper	0.7725	0.574
5.	Chromium	0.1827	0.149
6.	Cobalt	0.0615	0.081
7.	Iron	2.0945	2.633
8.	Lead	0.0412	0.025
9.	Magnesium	21.156	26.115
10.	Molybdenum	0.0890	0.745
11.	Mercury	0.1311	0.259
12.	Manganese	0.710	0.132
13.	Nickel	0.3999	0.181
14.	Potassium	78	117
15.	Sodium	161	138
16.	Silicon	3.9115	1.399
17.	Selenium	0.0115	0.0094
18.	Vanadium	1.6310	2.107
19.	Zinc	3.2710	4.130

Figure VIII: Effect of different plant extracts on DPPH radical scavenging activity



DPPH radical scavenging activity of EAE, BHE, PLE and standard Ascorbic acid Each value represents the mean \pm SD (n = 3).

The EC_{50} value of the BHE was found to be 139 μ g/ml

The EC_{50} value of the standard ascorbic acid was found to be 188 μ g/ml.

Table IV: Super oxide scavenging activity of the different extracts

Concentration in $\mu\text{gms} / \text{ml}$	Inhibitory activity of EAE(%)	Inhibitory activity of PLE(%)	Inhibitory activity of BHE (%)	Inhibitory activity of Ascorbic acid (%)
100	13.97 \pm 2.80	12.67 \pm 1.7	15.48 \pm 1.7a*	17.82 \pm 1.82b*
200	27.84 \pm 1.60	25.84 \pm 2.1	30.47 \pm 1.84a*	34.76 \pm 1.84b*
400	50.50 \pm 2.1	51.89 \pm 1.4	52.75 \pm 1.86a*	58.64 \pm 2.23b*
600	63.80 \pm 2.4	56.81 \pm 1.9	65.82 \pm 2.75a*	72.35 \pm 2.34b*
800	74.78 \pm 1.98	62.54 \pm 1.8	78.75 \pm 1.44a*	84.45 \pm 1.36b*
1000	79.94 \pm 1.50	78.88 \pm 1.6	84.76 \pm 2.35a*	91.34 \pm 1.86b*

Super oxide radical scavenging activity of EAE, BHE, PLE and standard Ascorbic acid. Each value represents the mean \pm SD (n = 3).

Comparison between

- a) BHE vs. EAE, PLE
- b) BHE vs. Ascorbic acid

* $p < 0.05$, ** $p < 0.01$

The EC_{50} value of the BHE was found to be 165 $\mu\text{g}/\text{ml}$

The EC_{50} value of the standard ascorbic acid was found to be 182 $\mu\text{g}/\text{ml}$

Table V: Hydroxyl radical scavenging activity of the different extracts

Concentration in $\mu\text{gms / ml}$	Inhibitory activity of EAE(%)	Inhibitory activity of PLE(%)	Inhibitory activity of BHE (%)	Inhibitory activity of Mannitol (%)
200	23.48 \pm 1.84	21.64 \pm 1.8	25.4 \pm 1.72a*	54.20 \pm 2.30b**
400	46.56 \pm 1.54	44.46 \pm 1.2	50.86 \pm 1.45a*	65.60 \pm 2.41b*
600	61.24 \pm 2.8	62.51 \pm 2.0	64.90 \pm 1.75a*	72.40 \pm 3.82b*
800	72.14 \pm 1.78	65.84 \pm 1.1	78.7 \pm 2.80a*	82.80 \pm 1.72b*
1000	79.86 \pm 1.64	74.64 \pm 1.6	82.82 \pm 1.50a*	91.4 \pm 2.84b*

Hydroxyl radical scavenging activity of EAE, BHE, PLE and standard Mannitol. Each value represents the mean \pm SD (n = 3).

Comparison between

a - BHE vs. EAE, PLE

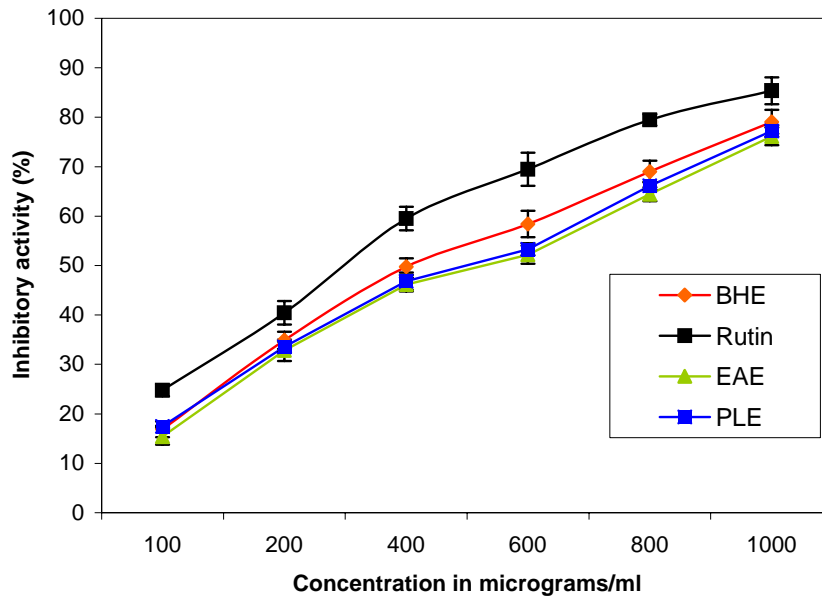
b - BHE vs. Mannitol

* $p < 0.05$, ** $p < 0.01$

The EC₅₀ value of the BHE was found to be 288 $\mu\text{g/ml}$

The EC₅₀ value of the standard Mannitol was found to be 460 $\mu\text{g/ml}$

Figure IX: Nitric oxide radical scavenging activity of the different extracts



Nitric oxide radical scavenging activity of EAE, BHE, PLE and standard Rutin. Each value represents the mean \pm SD (n = 3).

Comparison between

a - BHE vs. EAE, PLE

b - BHE vs. Rutin

* $p < 0.05$, ** $p < 0.01$

The EC_{50} value of the BHE was found to be 431 $\mu\text{g/ml}$

The EC_{50} value of the standard Rutin was found to be 616 $\mu\text{g/ml}$

Table VI: Reducing Power Assay Of The Different Plant Extracts

Concentration in $\mu\text{gms} / \text{ml}$	Absorbance of EAE (O.D)	Absorbance of PLE (O.D)	Absorbance of BHE (O.D)	Absorbance of BHT (O.D)
100	0.17 \pm 0.03	0.13 \pm 0.02	0.25 \pm 0.02a**	0.32 \pm 0.0-3b*
200	0.26 \pm 0.03	0.23 \pm 0.18	0.32 \pm 0.01a*	0.37 \pm 0.03b*
400	0.38 \pm 0.05	0.32 \pm 0.87	0.47 \pm 0.03a*	0.48 \pm 0.02b*
600	0.47 \pm 0.04	0.39 \pm 0.45	0.54 \pm 0.45a*	0.63 \pm 0.20b*
800	0.58 \pm 0.03	0.53 \pm 0.45	0.63 \pm 0.03a*	0.76 \pm 0.05b*
1000	0.72 \pm 0.24	0.69 \pm 0.65	0.81 \pm 0.34a*	0.92 \pm 0.34b*

Reducing capacity of EAE, BHE, PLE and standard BHT.

Each value represents the mean \pm SD (n = 3).

Comparison between

- a) BHE vs. EAE, PLE
- b) BHE vs. BHT

* p <0.05, ** p <0.01

Table VII: Hydrogen peroxide scavenging activity of different plant extracts

Concentration in $\mu\text{g/ml}$	Inhibitory activity of EAE(%)	Inhibitory activity of PLE(%)	Inhibitory activity of BHE(%)	Inhibitory activity of Vitamin E(%)
Control	12.19 \pm 0.40	11.34 \pm 0.69	13.78 \pm 0.34a*	14.90 \pm 0.67b*
100 $\mu\text{g/ml}$	55.56 \pm 0.56	63 .00 \pm 0.87	78 .00 \pm 0.56a*	65.89 \pm 0.89b*
200 $\mu\text{g/ml}$	58.90 \pm 0.56	64.00 \pm 0.67	82.03 \pm 0.67a*	75.96 \pm 0.56b*

Hydrogen peroxide scavenging activity of EAE, BHE, PLE and standard Vitamin E. Each value represents the mean \pm SD (n = 3).

Comparison between

- a) BHE vs. EAE, PLE
- b) BHE vs. Vitamin E

* p <0.05, * * p <0.01

Table VIII: Iron chelating activity of Bi herbal Extract

Concentration in μg/ml	O.D at 525nm	Chelation of Fe²⁺ (%)	O.D at 460nm	Chelation of Fe³⁺ (%)
Control	0.280	0	1.021	0
BHE(20μg/ml)	0.236	18.26±0.194	0.884	13.44±0.093
BHE(40μg/ml)	0.218	30.69± 0.308	0.793	22.33±0.171
BHE(60μg/ml)	0.202	32.02±0.259	0.716	29.94±0.006
BHE(80μg/ml)	0.185	44.25±0.177	0.697	36.01±0.006
BHE(100μg/ml)	0.167	56.08±0.433	0.654	55.19±0.006
EDTA(100μg/ml)	0.077	78.64±0.204	0.149	85.42±0.006

Metal chelating activity of BHE and EDTA .

Each value represents the mean ± SD (n = 3)

Table IX: The average total antioxidant activity of different plant extract

Concentration in μgms / ml	EAE (Absorbance at 500 nm)	PLE (Absorbance at 500 nm)	BHE (Absorbance at 500 nm)	BHT (Absorbance at 500 nm)
100	0.88 \pm 0.05	0.95 \pm 0.03	0.96 \pm 0.04a*	0.82 \pm 0.07b*
200	0.78 \pm 0.06	0.85 \pm 0.45	0.86 \pm 0.02a*	0.74 \pm 0.02b*
400	0.70 \pm 0.05	0.74 \pm 0.05	0.72 \pm 0.04a*	0.79 \pm 0.04b*
600	0.66 \pm 0.03	0.67 \pm 0.56	0.62 \pm 0.02a*	0.66 \pm 0.01b*
800	0.57 \pm 0.04	0.56 \pm 0.45	0.52 \pm 0.02a*	0.48 \pm 0.02b*
1000	0.44 \pm 0.05	0.48 \pm 0.45	0.32 \pm 0.02a*	0.28 \pm 0.04b*

The average total antioxidant activity of EAE, BHE, PLE and standard BHT. Values are represented by mean \pm SEM. (n=3)

Comparison between

a) BHE vs. EAE, PLE

b) BHE vs. BHT

* p <0.05, ** p <0.01

Table X: The Percentage inhibition of protein carbonyl formation by different plant extracts

Concentration in μgms / ml	Inhibitory activity of EAE (%)	Inhibitory activity of PLE (%)	Inhibitory activity of BHE (%)	Inhibitory activity of Vitamin E (%)
100	24.89 \pm 0.04	22.56 \pm 0.7	24.46 \pm 1.8a*	31.15 \pm 1.07b*
200	34.98 \pm 0.03	34.98 \pm 0.02	42.34 \pm 1.23a*	53.68 \pm 1.24b*
400	46.89 \pm 0.03	43.02 \pm 0.45	56.42 \pm 1.35a*	62.22 \pm 1.04b*
600	58.67 \pm 0.02	52.23 \pm 0.4	68.24 \pm 2.17a*	73.18 \pm 1.05b*
800	64.34 \pm 0.03	63.78 \pm 0.23	74.42 \pm 1.08a*	81.26 \pm 1.1b*
1000	74.98 \pm 0.05	73.06 \pm 0.45	81.42 \pm 1.77a*	90.06 \pm 1.05b*

The Percentage inhibition of protein carbonyl formation of EAE, BHE, PLE and Vitamin E. Values are represented by mean \pm SEM. (n=3)

Comparison between

- a) BHE vs. EAE, PLE
- b) BHE vs. Vitamin E

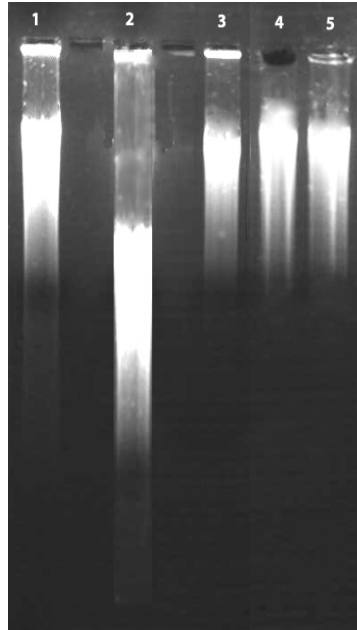
* p <0.05, ** p <0.01

**Table XI : Total Phenolic and flavonoid content of Biherbal
ethanolic extract**

Extracts	Total phenolic content (mg/g)	Total flavonoid content (mg/g)
EAE	53.67±2.67	47.46±2.89
PLE	53.00±2.37	54.98±2.67
BHE	73.40±2.68	55.4±1.57

Each value represents the mean ±SEM. (n=3) Total phenolic content was expressed as mg gallic acid equivalents/g dried extract. Total flavonoid content was expressed as mg catechin equivalent/g dried extract.

Figure-IX: *In vitro* assay of DNA fragmentation study



Lane 1 – untreated Hepatic DNA

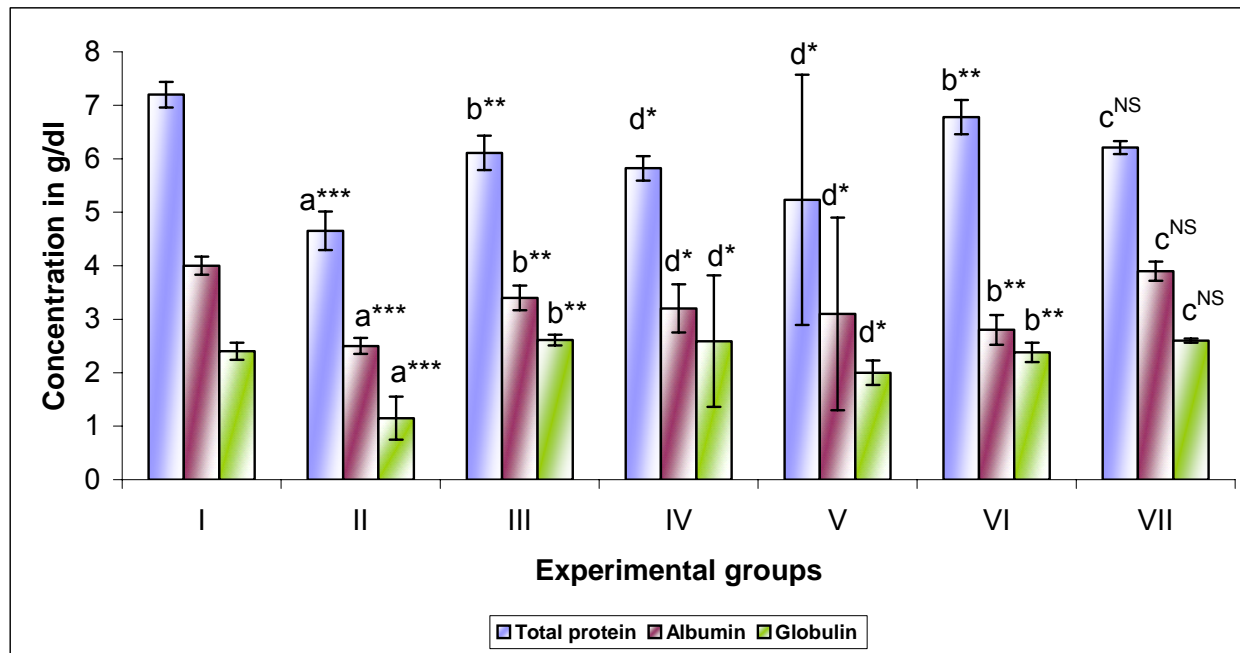
Lane 2 - H₂O₂ induced DNA damage

Lane 3 – DNA damage protection by BHE at concentration of 20µg/ml

Lane 4 - DNA damage protection by BHE at concentration of 40µg/ml

Lane 5 - DNA damage protection by BHT at concentration of 40µg/ml

Figure XI: Serum levels of Protein, Albumin and Globulin indifferent experimental groups of rats

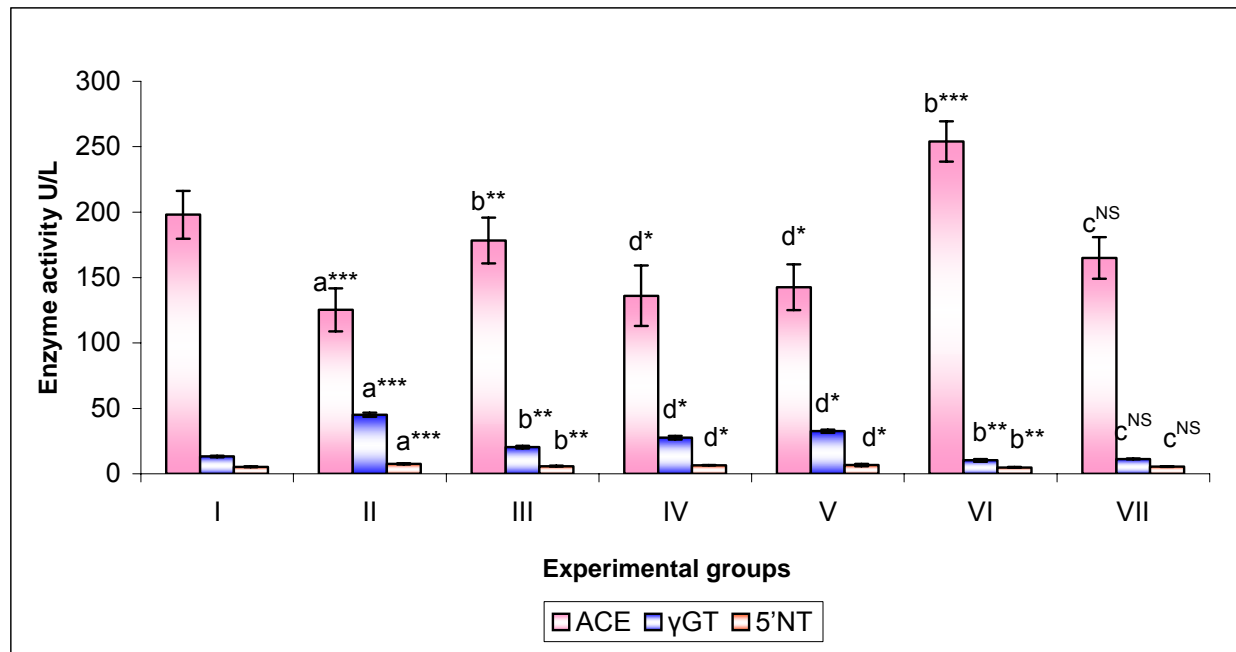


Values are mean \pm SEM of 6 animals in each group Statistical significant test for comparison was done by ANOVA followed by Dunnet's 't' test

- Comparison between
- a - Group I vs Group II
 - b - Group II vs Group III and Group VI
 - c - Group I vs Group VII
 - d - Group III vs Group IV and V

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, NS-Not Significant

Figure XII: Serum activity levels of ACE, γ GT and 5' NT in different experimental rats

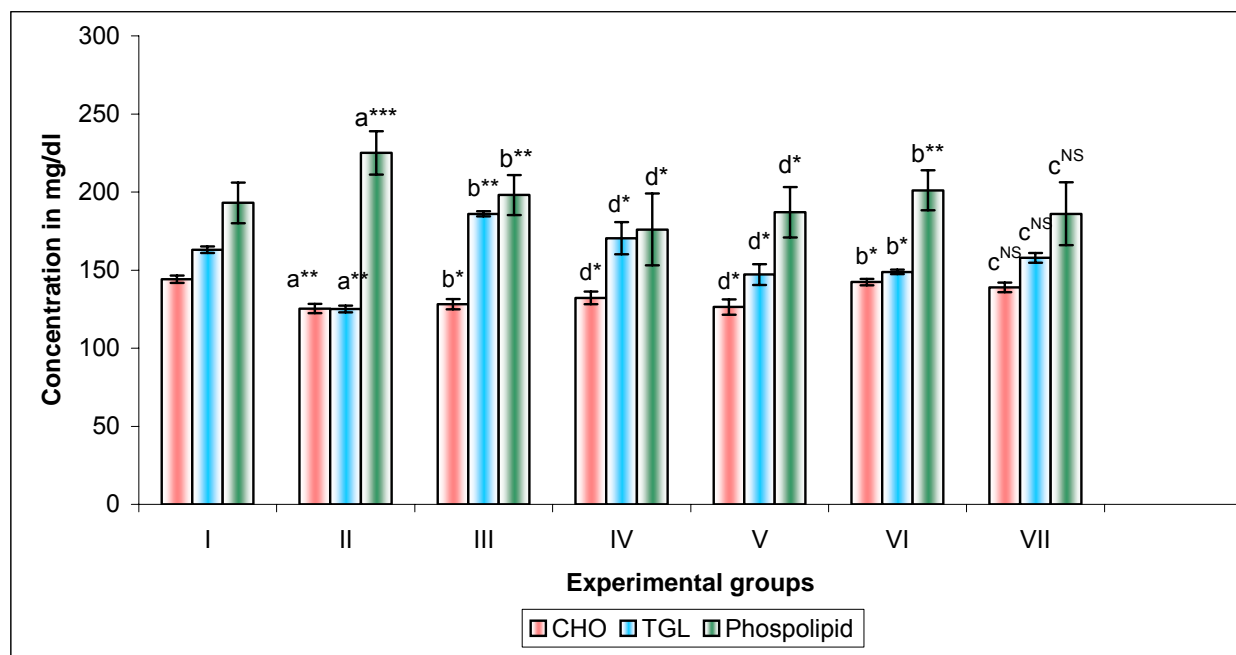


Values are mean \pm SEM of 6 animals in each group Statistical significant test for comparison was done by ANOVA followed by Dunnet's 't' test

- Comparison between
- a - Group I vs Group II
 - b - Group II vs Group III and Group VI
 - c - Group I vs Group VII
 - d - Group III vs Group IV and V

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, NS-Not Significant.

Figure XIII: Serum levels of Total Cholesterol, Triglycerides and Phospholipids in different experimental rats

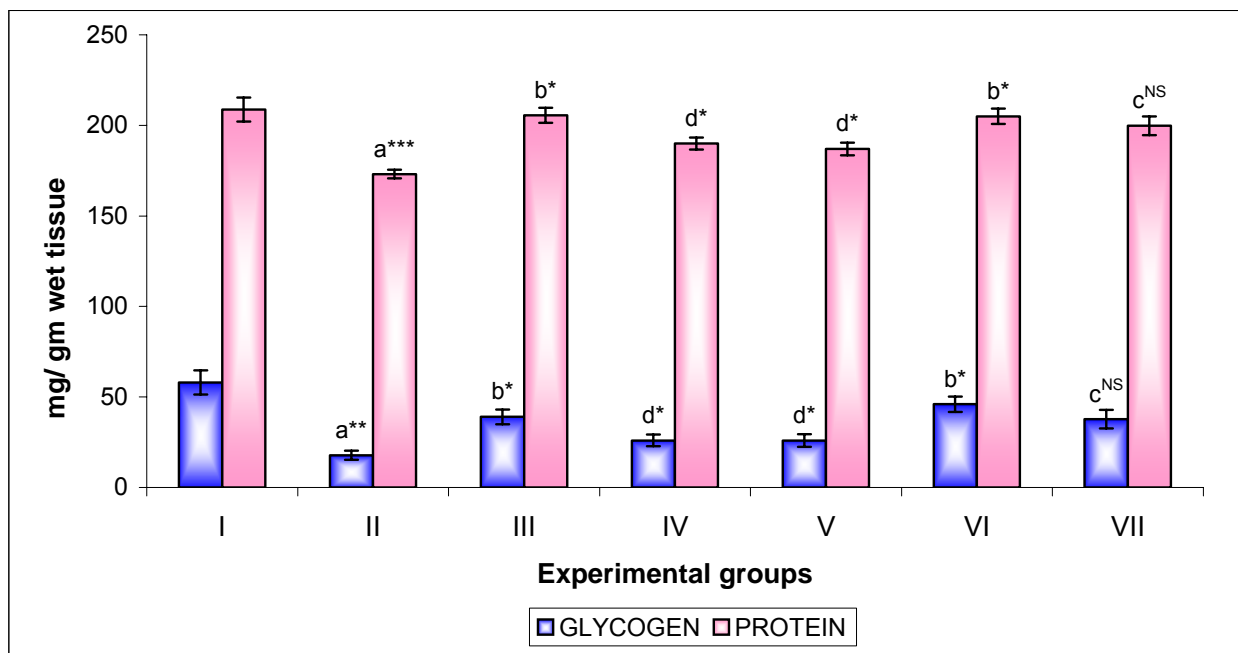


Values are mean \pm SEM of 6 animals in each group Statistical significant test for comparison was done by ANOVA followed by Dunnet's 't' test

- Comparison between
- a - Group I vs Group II
 - b - Group II vs Group III and Group VI
 - c - Group I vs Group VII
 - d - Group III vs Group IV and V

*p<0.05, **p<0.01, ***p<0.001, NS-Not Significant.

Figure XIV: Levels of Liver tissue Glycogen and Protein in different experimental rats

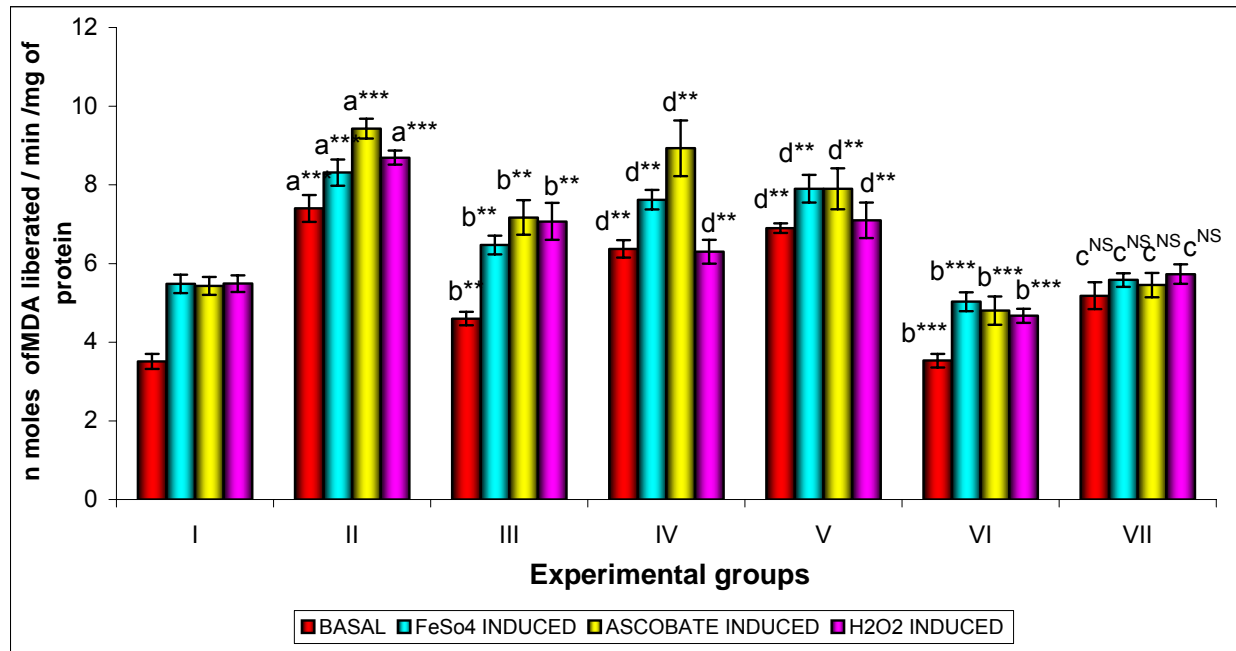


Values are mean \pm SEM of 6 animals in each group Statistical significant test for comparison was done by ANOVA followed by Dunnet's 't' test

- Comparison between
- a - Group I vs Group II
 - b - Group II vs Group III and Group VI
 - c - Group I vs Group VII
 - d - Group III vs Group IV and V

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, NS-Not Significant.

Figure XV: Effect of BHE on Basal, H₂O₂, Ascorbate and FeSO₄induced lipidperoxidation in liver

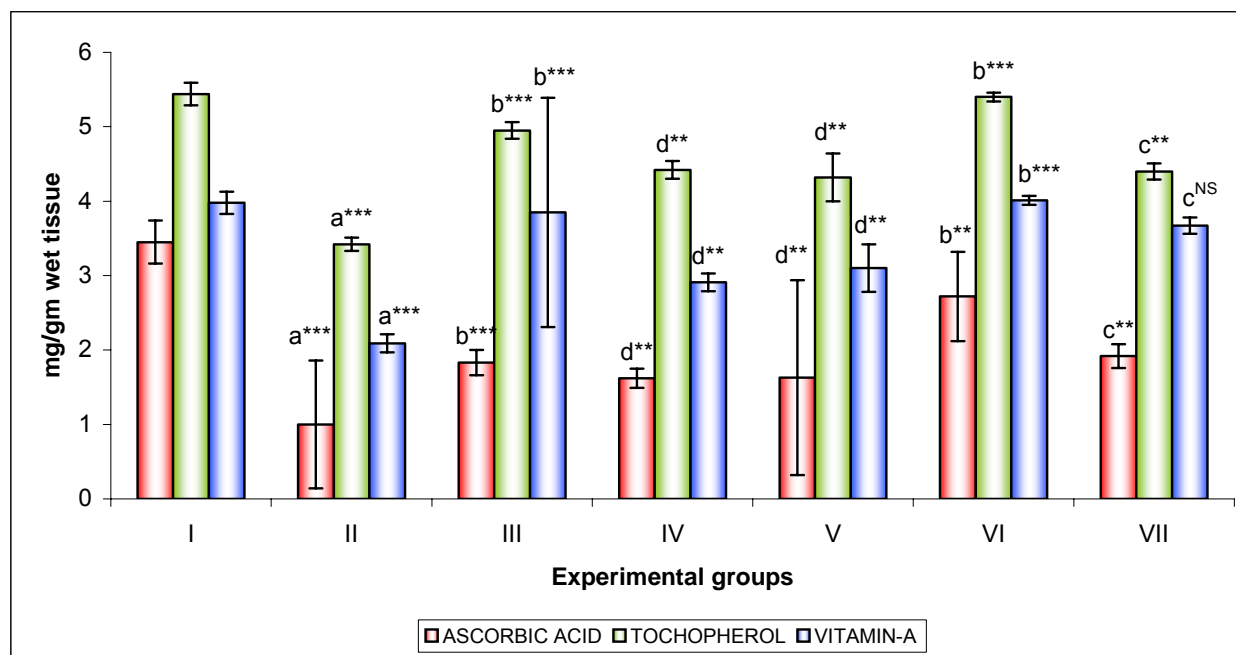


Values are mean \pm SEM of 6 animals in each group Statistical significant test for comparison was done by ANOVA followed by Dunnet's 't' test

- Comparison between
- a - Group I vs Group II
 - b - Group II vs Group III and Group VI
 - c - Group I vs Group VII
 - d - Group III vs Group IV and V

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, NS-Not Significant.

Figure XVI: Levels of Non Enzymic antioxidants in different experimental rats

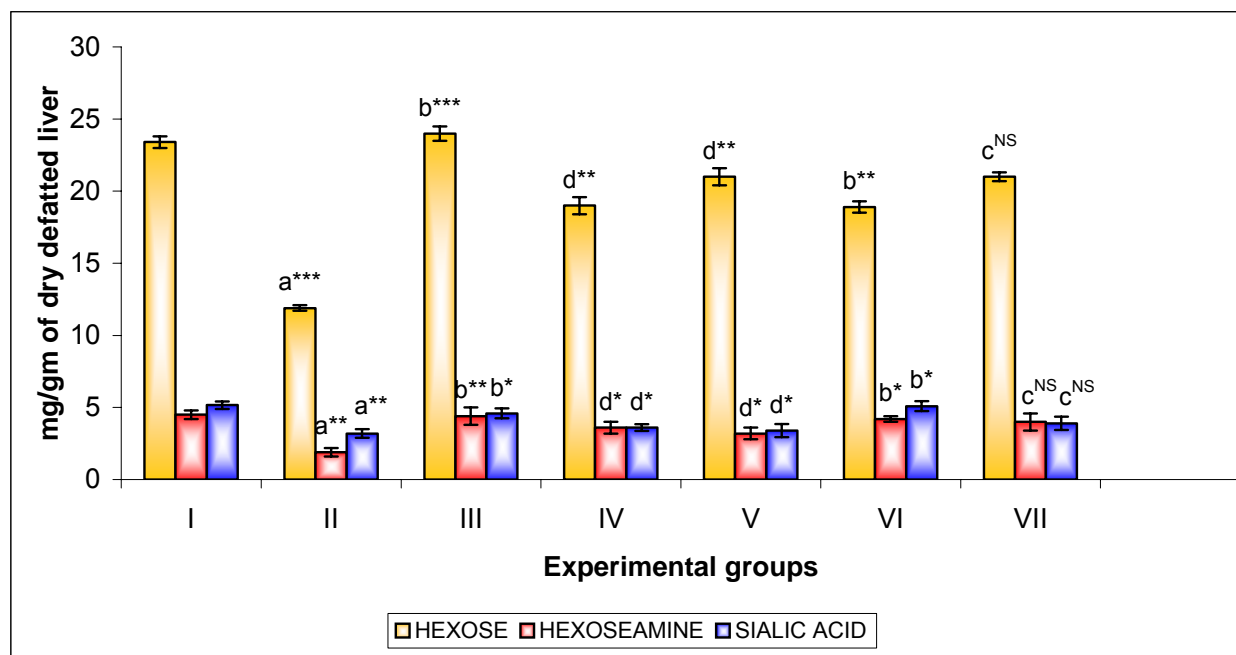


Values are mean \pm SEM of 6 animals in each group Statistical significant test for comparison was done by ANOVA followed by Dunnet's 't' test

- Comparison between
- a - Group I vs Group II
 - b - Group II vs Group III and Group VI
 - c - Group I vs Group VII
 - d - Group III vs Group IV and V

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, NS-Not Significant.

Figure XVII: The levels of liver Glycoproteins in different experimental rats



Values are mean \pm SEM of 6 animals in each group Statistical significant test for comparison was done by ANOVA followed by Dunnet's 't' test

- Comparison between
- a - Group I vs Group II
 - b - Group II vs Group III and Group VI
 - c - Group I vs Group VII
 - d - Group III vs Group IV and V

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, NS-Not Significant.

Table I: Incremental dose finding experiment and its Signs of Toxicity after oral administration of BHE in mice

S. No	Treatment	Dose	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	I	50	+	+	-	+	-	+	-	-	-	-	+	-	-	+	-	-	-	-	+	-
2	II	100	+	+	-	+	-	+	-	-	-	-	+	-	-	+	-	-	-	-	+	-
3	III	250	+	+	+	+	-	+	-	-	-	-	+	-	-	+	-	-	-	-	+	-
4	IV	500	+	+	+	+	-	+	-	+	-	-	+	+	+	+	-	+	-	-	+	3+
5	V	1000	+	+	+	+	-	+	+	+	+	-	+	+	+	+	-	+	+	-	+	3+
6	VI	2000	+	+	+	+	-	+	+	+	+	+	+	+	+	+	-	+	-	-	+	5+
7	VII	4000	+	+	+	+	-	+	+	+	+	+	+	+	+	+	-	+	-	-	+	6+
8	VIII	5000	+	+	+	+	-	+	+	+	+	+	+	+	+	+	-	+	-	-	+	6+

1. Alertness 2. Aggressiveness 3. Pile erection 4. Grooming 5. Gripping 6. Touch Response 7. Increased Motor Activity 8. Tremors 9. Convulsions 10. Muscle Spasm 11. Catatonia 12. Muscle relaxant 13. Hypnosis 14. Analgesia 15. Lacrimation 16. Exophthalmos 17. Diarrhoea 18. Writhing 19. Respiration 20. Number of Deaths (Mortality)

Table II: Organ to body weight ratio of mice, 24 hr after oral administration BHE

Parameters mg/ g wt		Lung	Liver	Spleen	Kidney	Heart
Group	Control	0.75±0.03	3.27±0.22	0.36±0.04	0.68±0.06	0.38±0.03
	BHE	0.72 ± 0.05**	3.33 ± 0.24 *	0.34 ± 0.03	0.74 ± 0.02	0.36±0.02

Values are mean±SEM (n=6); *P<0.05; (**P<0.01) significantly different from control.

Table III: Haematological variables of mice, 24 h after oral administration BHE

Parameters		Hb (g%)	RBC (10⁶/mm³)	PCV (% Volume)	Platelets (10⁵/mm³)	WBC (10³/mm³)	Polymorphs (%)	Lymphocytes (%)	Esonophils (%)
Group	Control	12.7 ± 0.3	4.34 ± 0.12	43.4 ± 1.0	2.84 ± 0.07	9368 ± 388	55.5 ± 1.8	37.1 ± 1.3	0.4±0.2
	BHE	11.7 ± 0.3*	4.59 ± 0.10	42.3 ± 0.8	2.88 ± 0.08	9269± 420**	54.5 ± 2.8	35.1 ± 1.4	0.5±0.2

Values are mean±SEM (n=6); *P<0.05; (**P<0.01) significantly different from control

Table IV: Biochemical variables of mice, 24 hr after oral administration of BHE

Variable	Group	
	Control	BHE
Glucose (mg/dl)	95.54±2.36	85.14±4.21**
Urea (mg/dl)	42.89±2.81	44.66±3.29 ^{ns}
Cholesterol (mg/dl)	64.33±1.48	63.42±3.68 ^{ns}
Protein (g/dl)	6.37±0.16	6.22±0.53 ^{ns}
Phosphorus (mg/dl)	6.42±0.90	5.35±0.61 ^{ns}
Creatinine (mg/dl)	0.77±0.05	0.73±0.1 ^{ns}
Sodium (meq/L)	135±8.09	149.6±10.46**
Potassium (meq/L)	3.43±0.34	3.29±0.26 ^{ns}
AST (IU/L)	35.24±1.8	42.63±2.44**
ALT (IU/L)	32.39±0.47	33.62±0.54 ^{ns}
ALP ((IU/L)	52.62±1.63	63.4±1.35**

Values are mean ± SEM (n = 6); *P<0.01 significantly different from control.

Table V: Organ weights in (g) of rats in the chronic toxicity study of the BHE

Name of the organs	Control		Biherbal extract(25mg/kg)		Biherbal extract (50mg/kg)	
	Female	Male	Female	Male	Female	Male
Lung	0.87 ± 0.04	0.77 ± 0.04	0.74 ± 0.04	0.68±0.10	0.63±0.21	0.59±0.30
Heart	0.38 ± 0.01	0.37 ± 0.01	0.38 ± 0.02	0.45 ± 0.06	0.44 ± 0.04	0.38 ± 0.02
Stomach	1.05± 0.24	1.15± 0.24	1.44± 0.40	1.55± 0.30	1.44± 0.14	1.53± 0.03
Liver	3.64 ± 0.35	3.54 ± 0.35	3.12 ± 0.24	4.54 ± 0.35	4.12 ± 0.24*	4.64 ± 0.35*
Spleen	0.46 ± 0.06	0.56 ± 0.06	0.55 ± 0.04	0.61 ± 0.03	0.63 ± 0.02	0.62 ± 0.06
Pancreas	0.26± 0.04	0.36± 0.04	0.38± 0.02	0.23± 0.03	0.26± 0.24	0.24± 0.14
Brain	0.39± 0.06	0.38± 0.06	0.44± 0.04	0.40± 0.03	0.46± 0.02	0.48± 0.06
Kidney	0.80 ± 0.04	0.81 ± 0.04	0.65 ± 0.08	0.83 ± 0.05	0.85 ± 0.08	0.82 ± 0.03
Ovary	0.25± 0.24	0.26± 0.24	-	0.29± 0.34	-	0.27± 0.14
Testis			0.35± 0.14	-	0.34± 0.24	-

Values are expressed as mean ± S.E.M., n = 6. *p<0.05.

Table VI: Haematological values of male rats in the chronic toxicity study of the BHE

Parameter	Control		Biherbal extract (25mg/kg)		Biherbal extract (50mg/kg)	
	Female	Male	Female	Male	Female	Male
Hb (g%)	12.72± 0.24	13.57± 0.14	12.65± 0.40	14.10± 0.34	12.85± 0.54	14.23± 0.20
RBC (10 ⁶ /mm ³)	4.34± 0.04	4.24± 0.20	4.67± 0.07	4.44± 0.21	4.69± 0.24	4.48± 0.40
PCV (% Volume)	49.55± 0.24	43.44± 0.14	44.88± 0.32	42.34± 0.28	46.32± 0.12	44.23± 0.36
Platelets (10 ⁵ /mm ³)	2.47± 0.34	2.78± 0.31	2.57± 0.12	2.62± 0.06	2.64± 0.24	2.72± 0.34
WBC (10 ³ /mm ³)	5976± 184	6133± 224	6293± 124	6445± 174	6366± 222	6755±178
Polymorphs (%)	46.23± 0.24	48.34± 0.14	49.43± 0.40	48.32± 0.54	47.78± 0.12	46.23± 0.44
Lymphocytes (%)	35.12± 0.41	32.45± 0.23	33.45± 0.27	34.67± 0.34	33.67± 0.26	34.25± 0.24
Eosinophils (%)	0.3± 0.2	0.4± 0.1	0.3± 0.4	0.4± 0.2	0.5± 0.3	0.5± 0.2

Values are expressed as mean ± S.E.M., n = 6. *p<0.05.

Table VII: Clinical blood chemistry values of rats in the chronic toxicity study of the BHE

Parameter	Control		Biherbal extract(25mg/kg)		Biherbal extract (50mg/kg)	
	Female	Male	Female	Male	Female	Male
Glucose (mg/dl)	85.54±3.7	83.34±2.3	77.89±1.7	81.89±4.7	86.12±2.7	82.45±4.3
Urea (mg/dl)	34.67±3.2	34.34±4.7	36.34±2.7	39.23±5.7	38.78±1.7	33.67±2.7
Protein (g/dl)	6.88±7.7	6.55±2.7	6.73±3.7	6.34±1.7	6.38±5.7	6.89±1.7
Cholesterol (mg/dl)	68.79±6.7	70.11±4.7	75.12±5.7	76.35±2.7	76.24±3.7	74.79±5.4
Creatinine (mg/dl)	0.46±0.2	0.35±0.3	0.44±0.2	0.52±0.7	0.49±0.5	0.58±0.9
Sodium (meq/L)	124±8.4	127±9.8	131±6.7	132±9.7	130±3.7	134±4.7
Potassium (meq/L)	3.32±1.7	3.44±0.8	3.27±0.7	3.71±0.5	3.34±0.6	3.42±0.7
AST (IU/L)	33.23±3.7	32.78±4.7	35.67±2.7	36.34±1.7	39.23±3.7	41.23±5.7
ALT (IU/L)	31.13±1.7	34.48±3.7	37.77±2.7	38.54±6.7	43.13±4.7	42.53±6.7
ALP ((IU/L)	51.89±6.7	46.67±7.7	52.24±3.7	49.98±4.7	53.09±2.7	45.98±1.7

Values are expressed as mean ± S.E.M., n = 6. Significantly different from control, p<0.05.

Table VIII: Levels of Glucose, Urea, Bilirubin, Body weight and Liver weight in various experimental rats

Parameters	Group I Normal Control	Group II CCl ₄ (2ml/kg)	Group III BHE(50mg/kg) +CCl ₄ (2ml/kg)	Group IV EAE(50mg/kg)+CCl ₄ (2ml/kg)	Group V PLE(50mg/kg) +CCl ₄ (2ml/kg)	Group VI Silymarin (50mg/kg)+CCl ₄ (2ml/kg)	Group VII BHE (50mg/kg)
Body Weight (gms)	158.13± 4.85	137.33±4.28 a**	145.55± 7.77b**	140.35±4.65 d*	138.68 ± 5.52 d*	153.68± 3.18 b**	155.67± 4.58 c ^{NS}
Liver weight(mg/g body weight)	39.57±0.51	68.45 ±0.63 a**	55.48± 0.45b**	56.35± 0.897 d*	64.13±1.33 d*	34.68±0.82 b**	50.68±0.98c ^{NS}
Glucose (mg/dl)	98.43.33±1.32	84.44±3.40a**	105.43 ±1.08b**	106.98±1.77 d*	105.62±3.32 d*	96.32±3.42 b**	102.21±3.25c ^{NS}
Urea (mg/dl)	18.33±1.40	44.34±2.30a***	31.33±2.27b**	38.66±1.32 d*	37.69±1.79 d*	32.33±2.40 b**	20.20±1.80 c ^{NS}
Bilirubin mg/dl)	0.51±0.03	2.44±0.02a***	1.50±0.03b***	1.45±0.58 d*	1.53±0.24 d*	1.46±0.04 b**	0.77±0.15 c ^{NS}

Values are mean ± SEM of 6 animals in each group Statistical significant test for comparison was done by ANOVA followed by Dunnet's 't' test

Comparison between a - Group I vs Group II
b - Group II vs Group III and Group VI
c - Group I vs Group VII
d - Group III vs Group IV and V

*p<0.05, * *p<0.01 , * * *p<0.001, NS–Not Significant

Table IX: The activity levels of Serum AST, ALT, ALP, ACP, LDH and ICD in different experimental groups of rats

Parameters	Group I Normal Control	Group II CCl ₄ (2ml/kg)	Group III BHE(50mg/kg) +CCl ₄ (2ml/kg)	Group IV EAE(50mg/kg)+CCl ₄ (2ml/kg)	Group V PLE(50mg/kg) +CCl ₄ (2ml/kg)	Group VI Silymarin (50mg/kg)+CCl ₄ (2ml/kg)	Group VII BHE (50mg/kg)
AST(U/L)	45.16 ± 1.21	142.79 ± 4.55a***	86.30 ± 3.45b*	88.29±2.17 d*	86.67±2.75 d*	75.92± 3.46 b*	37.75 ±1.46 c ^{NS}
ALT (U/L)	45.00 ± 1.03	144.50±1.08a***	74.65± 0.98b*	91.05±4.87 d*	92.16±1.5 d*	77.16±0.64 b*	45.50 ± 1.76 c ^{NS}
ALP (IU/L)	75.65 ± 0.52	171.78±0.63a* **	120.75±0.72.b*	142.55±2.05 d*	145.37±3.09 d*	120.37±1.07 b*	75.26±0.48 c ^{NS}
ACP (K.A Units)	4.16±0.33	13.25±1.08a***	6.86±0.25b***	8.97±0.42 d*	9.44±0.61 d*	6.85 ±0 .39 b*	3.24 ± 0.17 c ^{NS}
ICD (IU/L)	2.54±0.53	6.85±0.16 a***	4.74±0.58 b***	5.24±0.45 d *	5.77±0.76 d *	4.84±0.37 b**	2.90±1.43c ^{NS}
LDH (U/L)	142.69±2.87	438.34±2.54a***	255.09 ±2.80.b**	292.76±2.44 d*	314.54±4.75 d*	243.74± 2.98 b*	133.23±2.84 c ^{NS}

Values are mean ± SEM of 6 animals in each group Statistical significant test for comparison was done by ANOVA followed by Dunnet's 't' test

Comparison between

- a - Group I vs Group II
- b - Group II vs Group III and Group VI
- c - Group I vs Group VII
- d - Group III vs Group IV and V

*p<0.05, **p<0.01, ***p<0.001, NS–Not Significant

Table X : Activity levels of Liver tissue, ALT, AST, LDH, ALP and ACP in different experimental rats

Parameters	Group I Normal Control	Group II CCl ₄ (2ml/kg)	Group III BHE(50mg/kg) +CCl ₄ (2ml/kg)	Group IV EAE(50mg/kg) +CCl ₄ (2ml/kg)	Group V PLE(50mg/kg) +CCl ₄ (2ml/kg)	Group VI Silymarin (50mg/kg)+CCl ₄ (2ml/kg)	Group VII BHE (50mg/kg)
GOT(AST)	12.42±0.41	42.53±0.59 a***	24.06±0.82 b***	32.44±0.56 d *	37.78±1.56 d *	18.45 ±0.72 b***	12.84±1.03 c ^{NS}
GPT(ALT)	5.43±0.30	12.47±0.64 a***	6.05±0.22 b***	10.06±0.45 d *	9.67±0.72 d *	8.91±0.16 b***	5.85±0.26 c ^{NS}
LDH	11.82±0.02	21.35±0.02 a***	12.54±0.02 b**	14.45±0.34 d *	15.51±0.67 d *	15.54±0.03 b***	11.60±0.05 c ^{NS}
ALP	13.00±0.57	20.55±1.08 a***	15.34±0.42 b***	18.30±0.72 d *	16.80±0.62 d *	17.74±0.82 b***	13.68±0.72 c ^{NS}
ACP	3.79±0.28	9.97±0.22 a***	4.74±0.21 b***	7.94±0.27 d *	6.97±0.52 d *	5.27±0.26 b***	3.28±0.26 c ^{NS}

Values are mean ± SEM of 6 animals in each group Statistical significant test for comparison was done by ANOVA followed by Dunnet's 't' test

Comparison between a - Group I vs Group II
b - Group II vs Group III and Group VI
c - Group I vs Group VII
d - Group III vs Group IV and V

*p<0.05, **p<0.01, ***p<0.001, NS–Not Significant

Enzyme units

AST, ALT and LDH -μ moles of pyruvate liberated/min/mg protein.

ACP and ALP-μ- moles of phenol liberated /min/ mg protein

Table XI : Activity levels of Liver tissue γ GT, 5'NT and XO in different experimental rats

Parameters	Group I Normal Control	Group II CCl ₄ (2ml/kg)	Group III BHE(50mg/kg) +CCl ₄ (2ml/kg)	Group IV EAE(50mg/k g)+CCl ₄ (2ml/kg)	Group V PLE(50mg/kg) +CCl ₄ (2ml/kg)	Group VI Silymarin (50mg/kg)+CCl ₄ (2ml/kg)	Group VII BHE (50mg/kg)
γ GT	3.56±0.19	7.34±0.17 a***	5.42±0.21 b***	4.97±0.37 d*	5.76±0.74 d*	5.45±0.31 b***	4.32±0.14 c ^{NS}
5'Nucleotidase	1.37±0.14	3.41±0.10 a***	2.49±0.20 b**	3.57±0.2 d*	3.50±0.34 d*	2.25±0.18 b***	1.88±0.14c ^{NS}
Xanthine oxidase	4.17±0.13	5.32±0.23a***	3.99±0.27 b***	4.45±0.12 d*	4.35±0.45 d*	4.01±0.28 b**	3.34±0.22 c ^{NS}

Values are mean \pm SEM of 6 animals in each group Statistical significant test for comparison was done by ANOVA followed by Dunnet's 't' test

Comparison between

- a - Group I vs Group II
- b - Group II vs Group III and Group VI
- c - Group I vs Group VII
- d - Group III vs Group IV and V

*p<0.05, **p<0.01, ***p<0.001, NS–Not Significant

Enzyme units

γ GT μ mole of p- nitroanilide/minute/ mg of protein

5'Nucleotidase- μ moles of phosphorous liberated/minute/mg of protein

Xanthine oxidase - Unit / mg of protein

Table XII : Serum levels of Lipoprotein Cholesterol in different experimental rats

Parameters	Group I Normal Control	Group II CCl ₄ (2ml/kg)	Group III BHE(50mg/kg) +CCl ₄ (2ml/kg)	Group IV EAE(50mg/kg) +CCl ₄ (2ml/kg)	Group V PLE(50mg/kg) +CCl ₄ (2ml/kg)	Group VI Silymarin (50mg/kg)+CCl ₄ (2ml/kg)	GroupVII BHE (50mg/kg)
HDL Cholesterol (mg/dl)	51.37±5.53	30.57±2.43 a**	46.64±3.97 b*	41.64±3.97 d *	40.26±3.6 d*	50.38±2.41 b**	47.27±4.12 c ^{NS}
LDL Cholesterol (mg/dl)	36.67±4.34	74.08±4.13 a***	33.06±5.06 b*	37.79±3.45 d *	39.24±4.5 d*	34.68±2.79 b**	45.77±4.53 c ^{NS}
VLDL Cholesterol (mg/dl)	24.14±2.64	41.00±3.21 a*	22.52±4.12b**	26.94±2.56 d *	24.85±3.56 d *	226.96± 3.45 b*	27.26±2.98 c ^{NS}

Values are mean ± SEM of 6 animals in each group Statistical significant test for comparison was done by ANOVA followed by Dunnet's 't' test

Comparison between

- a - Group I vs Group II
- b - GroupII vs Group III and Group VI
- c - Group I vs Group VII
- d - Group III vs Group IV and V

*p<0.05, **p<0.01 , ***p<0.001, NS–Not Significant

Table XIII: Liver tissue values of Total lipid, Triglycerides, Phospholipids and Cholesterol in different experimental rats

Parameters	Group I Normal Control	Group II CCl ₄ (2ml/kg)	Group III BHE(50mg/kg) +CCl ₄ (2ml/kg)	Group IV EAE(50mg/kg) +CCl ₄ (2ml/kg)	Group V PLE(50mg/kg) +CCl ₄ (2ml/kg)	Group VI Silymarin (50mg/kg)+CCl ₄ (2ml/kg)	Group VII BHE (50mg/kg)
Total Lipid (mg/gm wet tissue)	37.06±5.1	83.69±2.97 a***	47.36±2.29b***	53.59±3.12 d *	55.66±2.65 d *	42.57±2.14 b***	36.55±1.67c ^{NS}
TGL(mg/gm wet tissue)	9.36±0.20	12.87±0.46 a***	10.57±0.25 b**	11.15±0.32 d *	10.85±0.23 d *	10.37±0.71 b**	9.75±0.39 c ^{NS}
Phospholipids (mg/gm wet tissue	14.37±0.36	17.05±0.25 a***	15.54±0.50 b**	16.63±0.52 d *	16.93±0.75 d *	14.15±0.22 b***	14.17±0.26 c ^{NS}
Cholesterol) (mg/gm wet tissue)	6.64±0.30	9.89±0.34 a***	8.66±0.18 b**	9.16±0.26 d *	8.56±0.36 d *	7.56±0.21 b***	6.66±0.25 c ^{NS}

Values are mean ± SEM of 6 animals in each group Statistical significant test for comparison was done by ANOVA followed by Dunnet's 't' test

Comparison between

- a - Group I vs Group II
- b - Group II vs Group III and Group VI
- c - Group I vs Group VII
- d - Group III vs Group IV and V

*p<0.05, **p<0.01, ***p<0.001, NS–Not Significant

Table XIV: Levels of Liver tissue lipid per oxidation products in different experimental rats

Parameters	Group I Normal Control	Group II CCl ₄ (2ml/kg)	Group III BHE(50mg/kg) +CCl ₄ (2ml/kg)	Group IV EAE(50mg/kg)+CCl ₄ (2ml/kg)	Group V PLE(50mg/kg) +CCl ₄ (2ml/kg)	Group VI Silymarin (50mg/kg)+CCl ₄ (2ml/kg)	Group VII BHE (50mg/kg)
Conjugated dienes Δ^{233} / mg of protein	0.25±0.85	0.68±0.07 a***	0.46±0.42 b**	0.58±0.73 d*	0.55±0.47 d*	0.45±0.27 b***	0.24±0.46 c ^{NS}
Hydroperoxides millimoles/ mg of protein	154.56±14.78	365.45±16.60 a***	167.74± 15.84 b**	240.55±23.54d*	254.66±27.56 d*	176.67±19.45 b***	140.55±12.98 c ^{NS}
Nitric oxides nmol/mg of protein)	173.45±16.70	368.45±17.67 a**	185.12±16.87 b**	206.65±20.02 d*	195.76±0.67 d*	175.23±15.45b**	164.65±16.56c ^{NS}
MDA nano moles / mg of protein)	0.95 ± 0.02	0.17 ± 0.02 a***	0.12 ± 0.04 b**	0.13±0.03 d*	0.14±0.01 d*	0.12±0.02 b***	0.11 ± 0.02. c ^{NS}
Iron (mg/gm wet tissue)	141.44±1.17	165.18±1.75a**	150.55±1.35 b**	146.55±1.82 d*	154.33±1.92 d*	145.38±1.25b*	133.55±2.85 c ^{NS}

Values are mean ± SEM of 6 animals in each group Statistical significant test for comparison was done by ANOVA followed by Dunnet's 't' test

Comparison between

- a - Group I vs Group II
- b - Group II vs Group III and Group VI
- c - Group I vs Group VII
- d - Group III vs Group IV and V

*p<0.05, **p<0.01, ***p<0.001, NS–Not Significant

Table XV: Levels of Liver Enzymic antioxidants in different experimental rats

Parameters	Group I Normal Control	Group II CCl ₄ (2ml/kg)	Group III BHE(50mg/kg) +CCl ₄ (2ml/kg)	Group IV EAE(50mg/kg)+CCl ₄ (2ml/kg)	Group V PLE(50mg/kg) +CCl ₄ (2ml/kg)	Group VI Silymarin (50mg/kg)+CCl ₄ (2ml/kg)	Group VII BHE (50mg/kg)
CAT	64.57± 1.23	45.04±1.85a*	54.18±1.35b*	50.63±2.14 d*	47.66±1.63 d*	66.34±1.74b*	63.23±1.22c ^{NS}
SOD	13.33±0.29	10.32±0.48 a**	12.44±0.24b*	11.38±0.18 d*	12.16±0.34 d*	14.45±0.93b*	14.36±0.43c ^{NS}
GST	0.38±0.02	0.26±0.01 a**	0.34±0.01b*	0.27±0.02 d*	0.33±0.23 d*	0.36±0.19b**	0.41±0.010c ^{NS}
GPX	13.60±0.70	10.06±0.39 a**	13.53±0.43b**	12.33±0.42 d*	11.92±0.54 d*	13.45±0.43b**	15.09±0.56c ^{NS}
GR	0.64±0.03	0.13±0.07 a**	0.47±0.10b**	0.45±0.03 d*	0.42± 0.21d*	0.45±0.08 b**	0.53±0.09c ^{NS}

Values are mean ± SEM of 6animals in each group Statistical significant test for comparison was done by ANOVA followed by Dunnet's 't' test

Comparison between

- a - Group I vs Group II
- b - Group II vs Group III and Group VI
- c - Group I vs Group VII
- d - Group III vs Group IV and V

*p<0.05, **p<0.01, ***p<0.001, NS–Not Significant.

Enzyme units

CAT– n moles of H₂O₂ decomposed/min/mg protein,

SOD–units/mg protein. 1 unit of enzyme activity is the amount of enzyme required to inhibit 50% of epinephrine auto-oxidation

GST –n moles of CDNB conjugate formed/min/mg protein,

GP_x - n moles of GSH oxidised /min/mg protein.

GR–n moles of GSH formed/min/mg protein.

Table XVI: Levels of Liver Non-Enzymic antioxidants in different experimental rats

Parameters	Group I Normal Control	Group II CCl ₄ (2ml/kg)	Group III BHE(50mg/kg) +CCl ₄ (2ml/kg)	Group IV EAE(50mg/kg) +CCl ₄ (2ml/kg)	Group V PLE(50mg/kg) +CCl ₄ (2ml/kg)	Group VI Silymarin (50mg/kg)+CCl ₄ (2ml/kg)	Group VII BHE (50mg/kg)
Cereloplasmin (mg/dl)	1.63±0.16	0.75±0.098a**	1.16±0.10b*	0.94±0.15 d*	0.82±0.32 d*	0.94±0.13b*	1.22±0.07c ^{NS}
Total Thiols (µmoles of GSH mgm of Protein)	9.15±0.17	7.50±0.06a*	6.86±0.06b*	5.67±0.23 d*	8.13±0.43 d*	8.99±0.05b*	9.58±0.30c ^{NS}
Uric acid (mg/dl)	3.57±0.16	1.78±0.14 a**	2.35±0.16b*	1.98±0.35 d*	2.05±0.13 d*	2.64±0.17b**	3.54±0.13c ^{NS}
Glutathione (µmoles of GSH/mgm of Protein)	0.54±0.07	0.37±0.08 a*	0.44±0.05b*	0.38±0.04 d*	0.42±0.05 d*	0.46±0.06 b*	0.49±0.12 c ^{NS}

Values are mean ± SEM of 6 animals in each group Statistical significant test for comparison was done by ANOVA followed by Dunnet's 't' test

Comparison between a - Group I vs Group II
b - GroupII vs Group III and Group VI
c - Group I vs Group VII
d - Group III vs Group IV and V

*p<0.05, **p<0.01, ***p<0.001, NS–Not Significant

Table XVII: Levels of Liver Membrane bound ATP ases enzymes in different experimental rats

Parameters μ moles of phosphorous liberated/ min/ mg of protein	Group I Normal Control	Group II CCl ₄ (2ml/kg)	Group III BHE(50mg/kg) +CCl ₄ (2ml/kg)	Group IV EAE(50mg/kg)+ CCl ₄ (2ml/kg)	Group V PLE(50mg/kg) +CCl ₄ (2ml/kg)	Group VI Silymarin (50mg/kg)+CCl ₄ (2ml/kg)	Group VII BHE (50mg/kg)
Total-ATPase	4.84±0.25	2.68±0.09 a***	3.33±0.09 b***	3.28± 0.15 d*	2.95±0.26 d*	3.45±0.07 b***	4.44±0.16c ^{NS}
Na ⁺ /K ⁺ -ATPase	2.53±0.15	1.65±0.16 a**	2.63±0.19 b**	1.81±0.19d*	2.18±0.37 d*	2.09±0.36b*	3.28±0.17c ^{NS}
Mg ²⁺ -ATPase	1.76±0.04	0.83±0.09 a**	1.62±0.11 b**	1.52±0.09 d*	1.41±0.10 d*	1.51±0.14 b*	1.54±0.23c ^{NS}
Ca ²⁺ -ATPase	1.69±0.25	1.78±0.06 a***	1.15±0.64 b**	2.58±0.52 d*	2.17±0.32 d*	1.13±0.08 b*	1.15±0.06c ^{NS}

Values are mean \pm SEM of 6 animals in each group Statistical significant test for comparison was done by ANOVA followed by Dunnet's 't' test

Comparison between a - Group I vs Group II
b - Group II vs Group III and Group VI
c - Group I vs Group VII
d - Group III vs Group IV and V

*p<0.05, **p<0.01, ***p<0.001, NS–Not Significant

Table XVIII: Levels of Liver Glycolytic enzymes in different experimental rats

Parameters	Group I Normal Control	Group II CCl ₄ (2ml/kg)	Group III BHE(50mg/kg) +CCl ₄ (2ml/kg)	Group IV EAE(50mg/kg)+CCl ₄ (2ml/kg)	Group V PLE(50mg/kg) +CCl ₄ (2ml/kg)	Group VI Silymarin (50mg/kg)+CCl ₄ (2ml/kg)	Group VII BHE (50mg/kg)
Hexokinase	405.47±46.65	80.87± 13.14a***	375.13± 15.82b***	206.65±12.43 d*	255.35±11.31 d*	296.05±0.01b*	397.87±10.07c ^{NS}
Phospho gluco isomerase	44.83± 3.79	18.66±1.02a***	33.83±2.70b**	26.22±2.33 d*	26.58± 1.23d*	25.03±0.93 b*	33.33±0.61c ^{NS}
Aldolase	134.11±5.39	89.66±7.57 a***	117.58±6.39 b**	105.78±4.89 d*	106.54±4.90 d*	103.07±7.31 b***	123.34±4.75c ^{NS}

Values are mean ± SEM of 6 animals in each group Statistical significant test for comparison was done by ANOVA followed by Dunnet's 't' test

Comparison between

- a - Group I vs Group II
- b - Group II vs Group III and Group VI
- c - Group I vs Group VII
- d - Group III vs Group IV and V

*p<0.05, **p<0.01, ***p<0.001, NS–Not Significant.

Enzyme units

Hexokinase - n moles of Glucose utilized/min /mg of protein
 Phospho gluco isomerase - n moles of fructose utilized /min /mg of protein
 Aldolase - n moles of glyceraldehyde formed /min/ mg protein

Table XIX: Levels of Liver Gluconeogenic enzymes in different experimental rats

Parameters n moles of phosphorous liberated/ mg of protein / min	Group I Normal Control	Group II CCl₄ (2ml/kg)	Group III BHE(50mg/kg) +CCl₄ (2ml/kg)	Group IV EAE(50mg/kg)+CCl₄ (2ml/kg)	Group V PLE(50mg/kg) +CCl₄(2ml/kg)	Group VI Silymarin (50mg/kg)+CCl₄ (2ml/kg)	Group VII BHE (50mg/kg)
Glucose 6 phosphatase	1.71±0.14	1.14±0.11 a***	1.51±0.10 b**	1.33±0.15 d*	1.35±0.70 d*	1.49±0.11 b**	1.55±0.11c ^{NS}
Fructose 1,6 diphosphatase	1.45±0.13	0.48±0.12 a***	1.22±0.07 b**	1.20 ±0.08 d*	1.13±0.05 d*	1.07±0.15 b*	1.50±0.18c ^{NS}

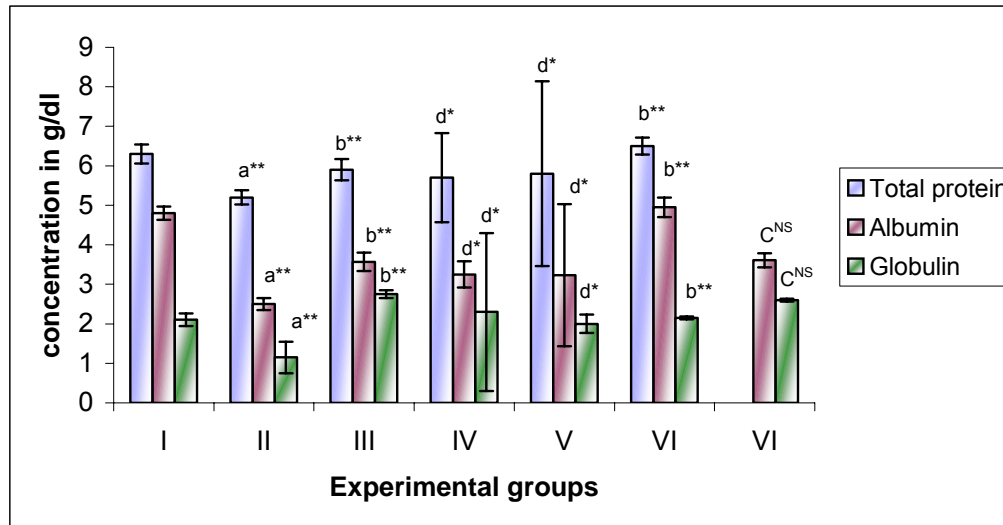
Values are mean ± SEM of 6 animals in each group Statistical significant test for comparison was done by ANOVA followed by Dunnet's 't' test

Comparison between

- a - Group I vs Group II
- b - GroupII vs Group III and Group VI
- c - Group I vs Group VII
- d - Group III vs Group IV and V

*p<0.05, **p<0.01, ***p<0.001, NS–Not Significant

Figure – XI Levels of Serum Protein, Albumin and Globulin in different experimental groups of rats

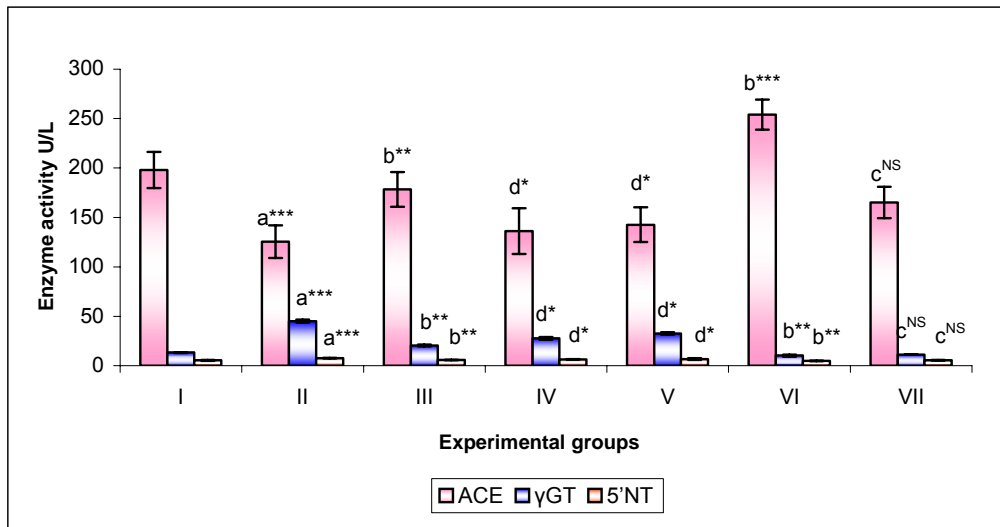


Values are mean \pm SEM of 6 animals in each group Statistical significant test for comparison was done by ANOVA followed by Dunnet's 't' test

Comparison between

- a - Group I vs Group II
- b - Group II vs Group III and Group VI
- c - Group I vs Group VII
- d - Group III vs Group IV and V

Figure XII: Serum activity levels of ACE, γ GT and 5' NT in different experimental rats



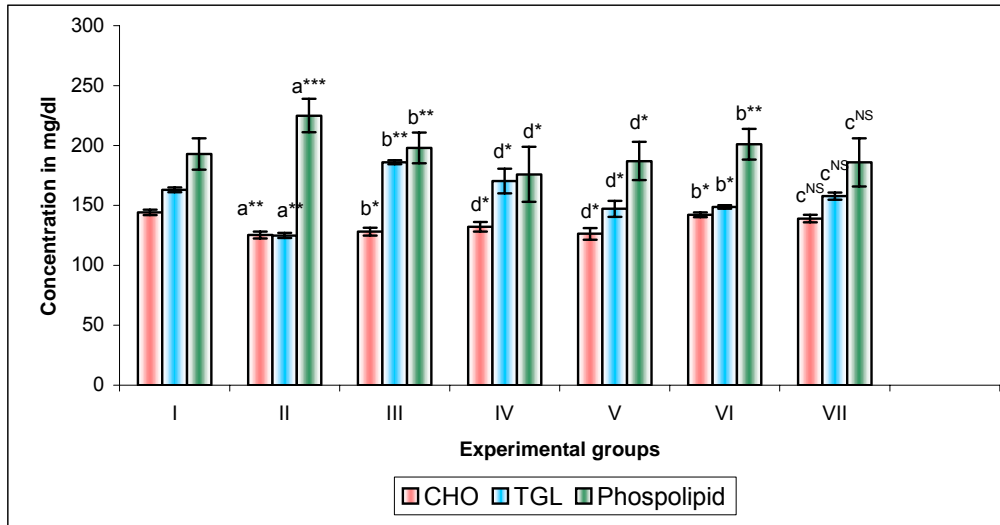
Values are mean \pm SEM of 6 animals in each group. Statistical significant test for comparison was done by ANOVA followed by Dunnet's 't' test.

Comparison between

- a - Group I vs Group II
- b - Group II vs Group III and Group VII
- c - Group I vs Group VI
- d - Group III vs Group IV and V

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, NS-Not Significant

Figure XIII: Levels of Serum Total Cholesterol, TGL and Phospholipids in different experimental rats



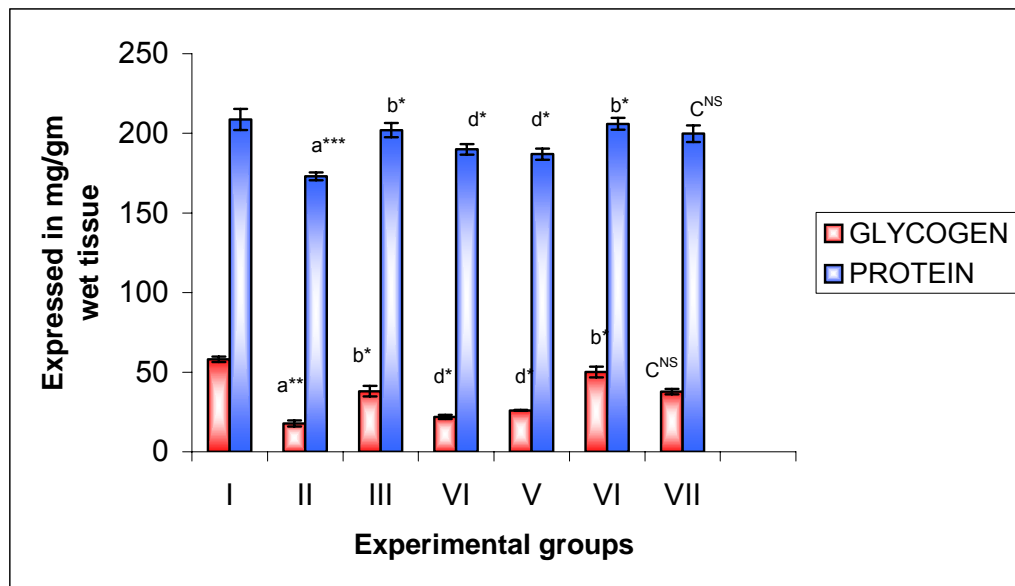
Values are mean \pm SEM of 6 animals in each group Statistical significant test for comparison was done by ANOVA followed by Dunnet's 't' test

Comparison between

- a - Group I vs Group II
- b - Group II vs Group III and Group VI
- c - Group I vs Group VII
- d - Group III vs Group IV and V

* $p < 0.05$, * * $p < 0.01$, * * * $p < 0.001$, NS–Not Significant

Figure XIV: Levels of Liver Glycogen and Protein in different experimental rats



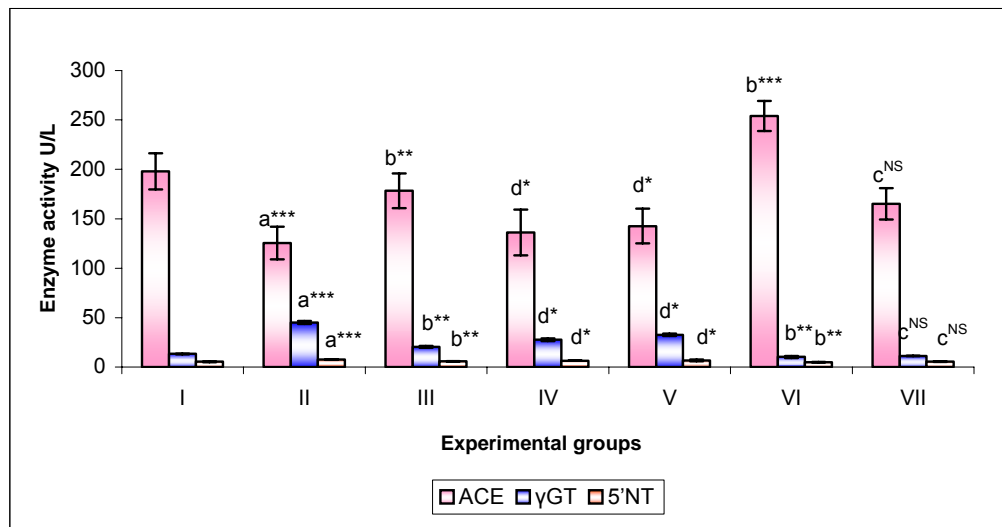
Values are mean \pm SEM of 6 animals in each group. Statistical significant test for comparison was done by ANOVA followed by Dunnet's 't' test.

Comparison between

- a - Group I vs Group II
- b - Group II vs Group III and Group VI
- c - Group I vs Group VII
- d - Group III vs Group IV and V

* $p < 0.05$, * $p < 0.01$, ** $p < 0.001$, NS-Not Significant

Figure XV: Effect of BHE on Basal, H₂O₂, Ascorbate and FeSO₄induced lipidperoxidation in livers of different experimental rats



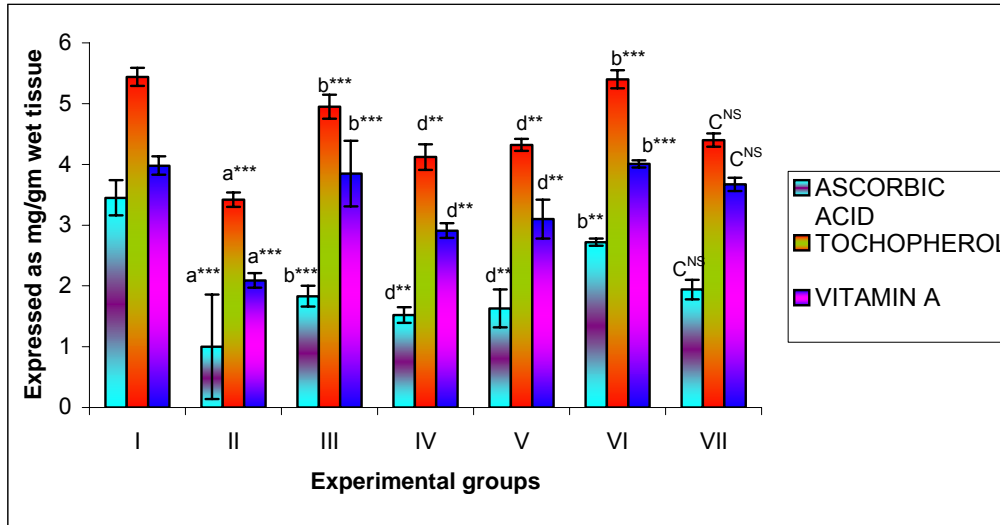
Values are mean \pm SEM of 6 animals in each group Statistical significant test for comparison was done by ANOVA followed by Dunnet's 't' test

Comparison between

- a - Group I vs Group II
- b - Group II vs Group III and Group VI
- c - Group I vs Group VII
- d - Group III vs Group IV and V

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, NS-Not Significant

Figure XVI: Levels of Liver Non Enzymic antioxidants in different experimental rats



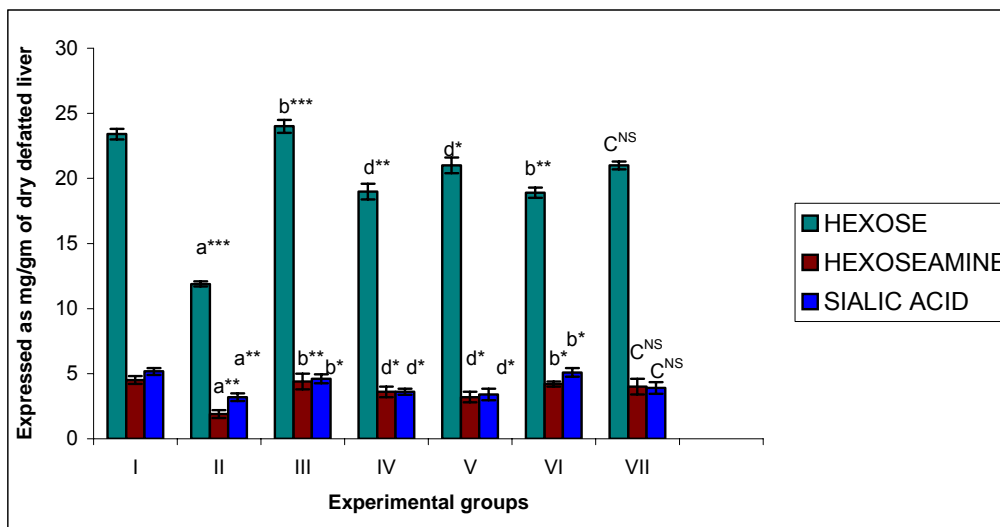
Values are mean \pm SEM of 6 animals in each group. Statistical significant test for comparison was done by ANOVA followed by Dunnet's 't' test.

Comparison between

- a - Group I vs Group II
- b - Group II vs Group III and Group VI
- c - Group I vs Group VII
- d - Group III vs Group IV and V

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, NS—Not Significant

Figure XVII: Levels of liver Glycoproteins in different experimental rats



Values are mean \pm SEM of 6 animals in each group Statistical significant test for comparison was done by ANOVA followed by Dunnet's 't' test

Comparison between

- a - Group I vs Group II
- b - Group II vs Group III and Group VI
- c - Group I vs Group VII
- d - Group III vs Group IV and V

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, NS–Not Significant

HISTOPATHOLOGY OF LIVER TISSUE OF DIFFERENT EXPERIMENTAL ANIMALS

- Figure XVIII a - Section of liver from normal control rats shows normal architecture of hepatocytes.
- Figure XVIII b - Section of liver induced with CCl₄ intoxicated rats shows reactive hyperplastic hepatocytes (binucleate cells).
- Figure XVIII c - Section of liver from BHE pretreated CCl₄ intoxicated rats shows hepatocytes with focal mild fatty change.
- Figure XVIII d - Section of liver from Silymarin pretreated CCl₄ intoxicated rats shows almost normal hepatocytes and occasional binucleate cells.

HISTOPATHOLOGY OF VARIOUS ORGANS OF DIFFERENT EXPERIMENTAL ANIMALS

KIDNEY

- Figure XIX a - Section of kidney from normal control rats shows normal renal tissue with glomeruli and tubules.
- Figure XIX b - Section of kidney induced with CCl₄ intoxicated rats shows renal tissue with tubular damage and tubular cast.
- Figure XIX c - Section of kidney from BHE pretreated CCl₄ intoxicated rats shows renal tissue with tubular epithelial damage. RBC with in the tubules.
- Figure XIXd - Section of kidney from Silymarin pretreated CCl₄ intoxicated shows renal tissue with focal tubular damage, interstitial inflammatory collection. Glomeruli shows epithelial proliferation.

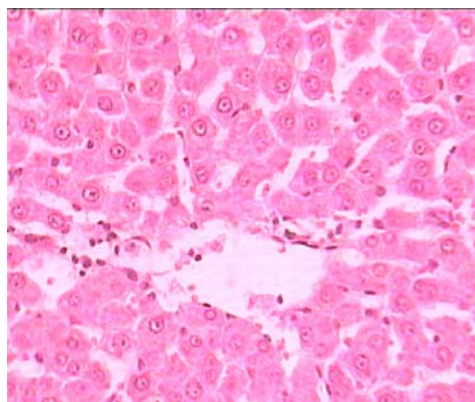
Heart

- Figure XX a - Section of heart from normal control rats shows normal cardiac muscle bundles.
- Figure XX b - Section of heart induced with CCl₄ intoxicated rats shows hyperplasic and hypertrophic cardiac muscles.
- Figure XX c - Section of heart from BHE pretreated CCl₄ intoxicated rats shows congestion and mild inflammatory infiltr.
- Figure XX d - Section of heart from Silymarin pretreated CCl₄ intoxicated shows hypertrophic cardiac muscle bundles.

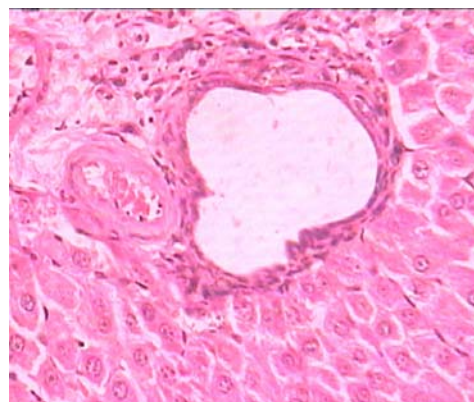
Intestine

- Figure XXI a - Section of intestine from normal control rats shows normal architecture.
- Figure XXI b - Section of intestine induced with CCl₄ intoxicated rats shows mild damaged cells.
- Figure XXI c - Section of intestine from BHE pretreated CCl₄ intoxicated rats shows near normal architecture.
- Figure XXI d - Section of intestine from Silymarin pretreated CCl₄ intoxicated shows near normal architecture.

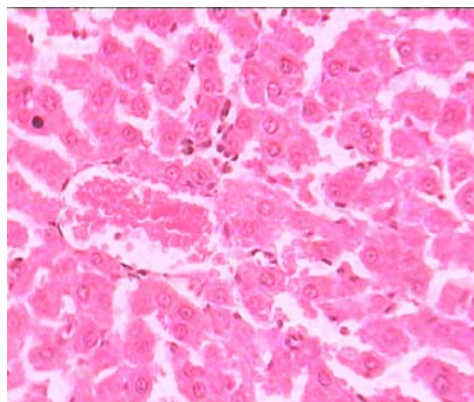
Figure XVIII: Histopathology of Liver tissues of different experimental animals



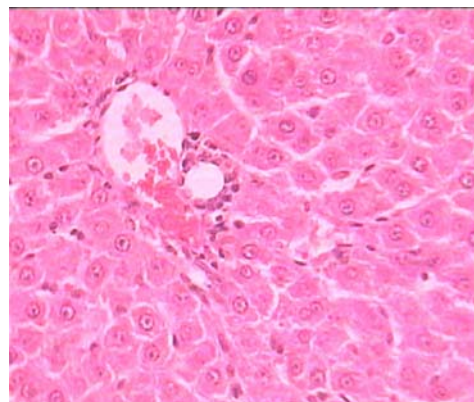
Normal
[a]



CCl₄
[b]

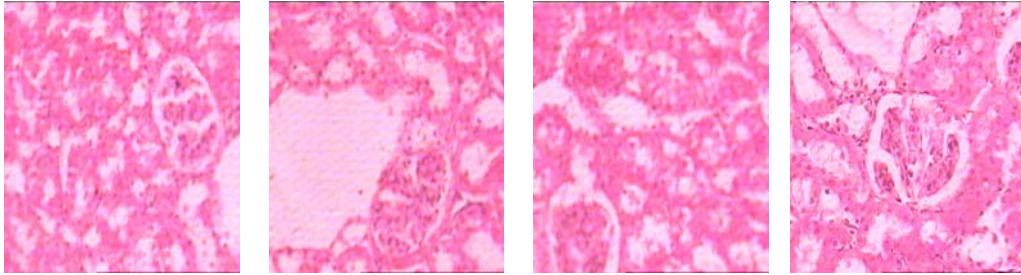


BHE
[c]



Standard
[d]

Figure XIX: KIDNEY



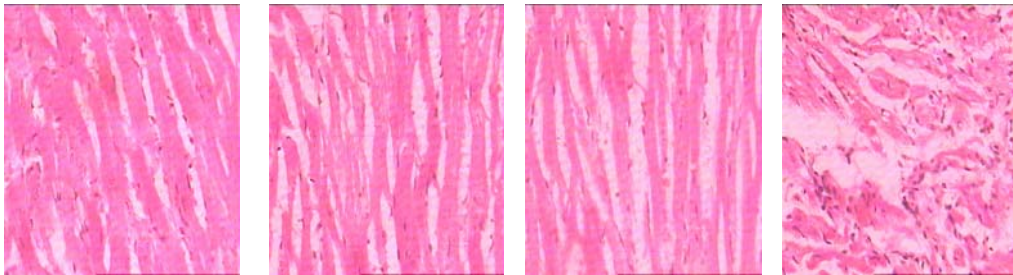
Normal [a]

CCl₄ [b]

BHE [c]

Standard [d]

Figure XX: HEART



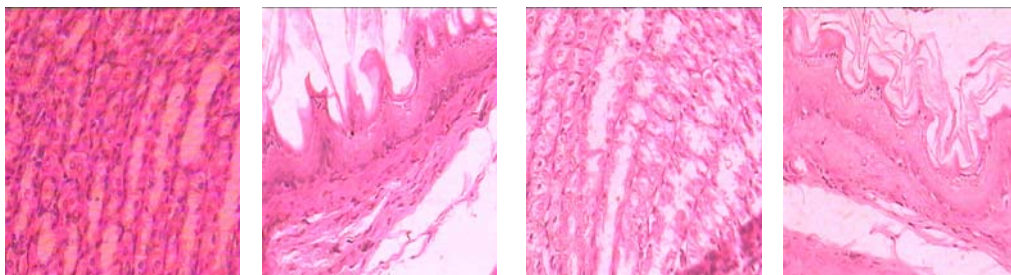
Normal [a]

CCl₄ [b]

BHE [c]

Standard [d]

Figure XXI: INTESTINE



Normal [a]

CCl₄ [b]

BHE [c]

Standard [d]

HISTOPATHOLOGY AND REPORT WITH PHOTOGRAPHS

Figure I: The histology of Lung from the control and treated groups (the 10x and 40x magnifications)

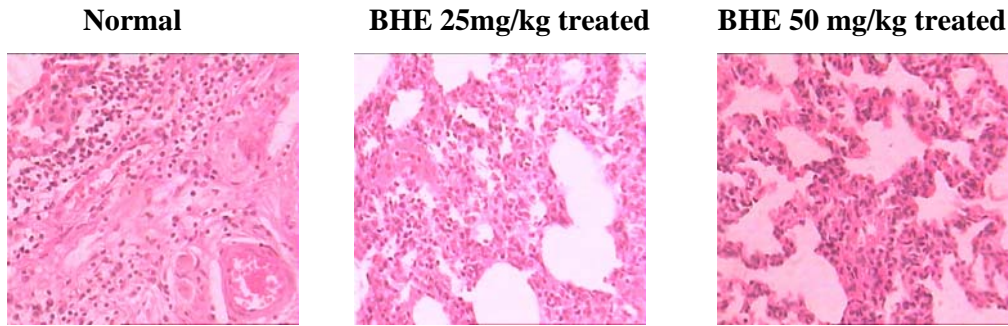
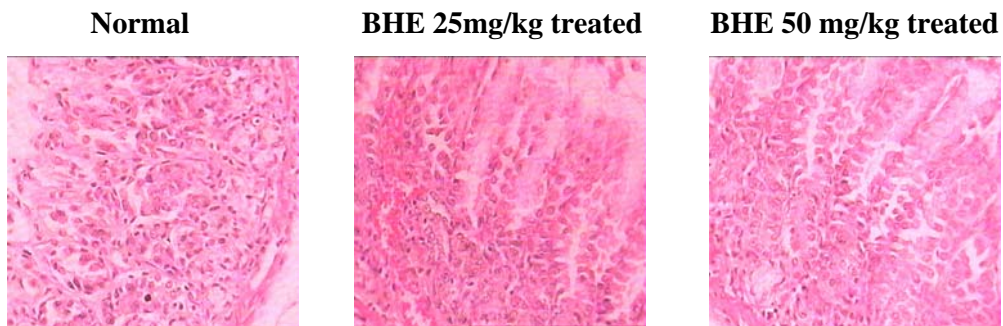


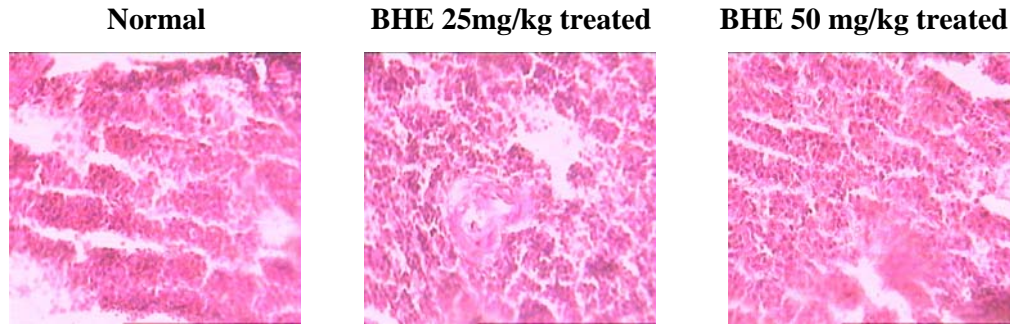
Figure II: The histology of Heart from the control and treated groups (the 10x and 40x magnifications)



Figure III: The histology of Stomach from the control and treated groups (the 10x and 40x magnifications)



**Figure IV: The histology of Spleen from the control and treated groups
(the 10x and 40x magnifications)**



**Figure V: The histology of Liver from the control and treated groups
(the 10x and 40x magnifications)**



**Figure VI: The histology of Pancreas from the control and treated groups
(the 10x and 40x magnifications)**



Figure VII: The histology of Brain from the control and treated groups (the 10x and 40x magnifications)

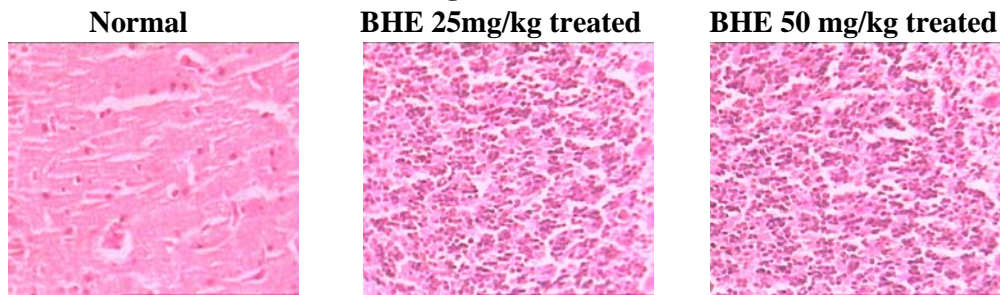


Figure VIII: The histology of Kidney from the control and treated groups (the 10x and 40x magnifications)

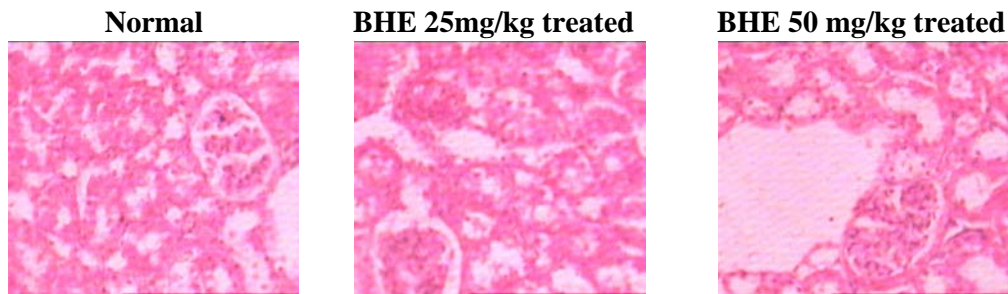


Figure IX. The histology of Ovaries from the control and treated groups (the 10x and 40x magnifications)

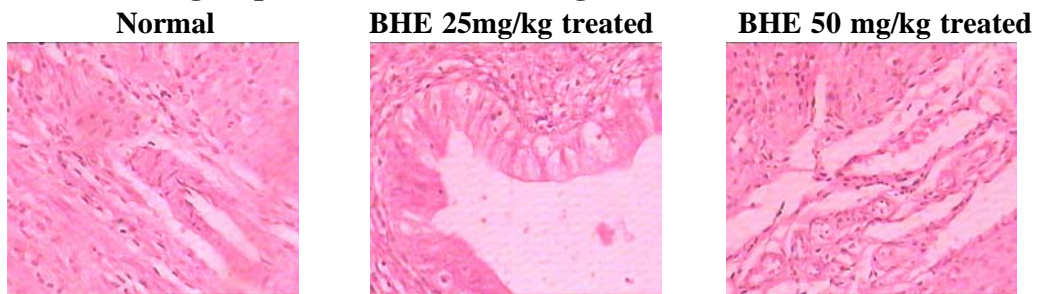
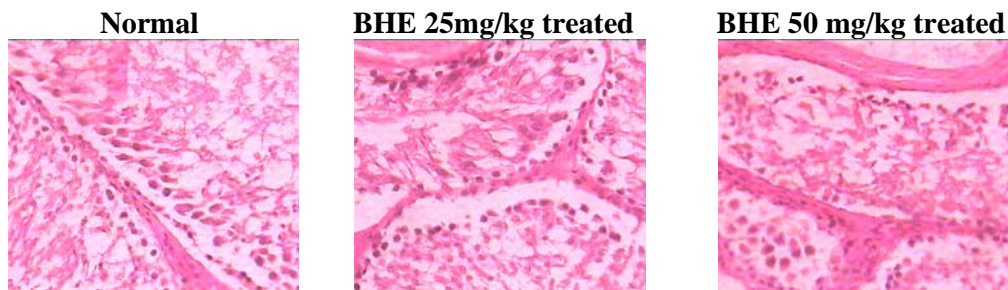


Figure X: The histology of testis from the control and treated groups (the 10x and 40x magnifications)



HISTOPATHOLOGY CHANGES

Normal

- LUNG : shows normal alveoli
HEART : shows normal cardiac muscle bundles.
STOMACH : shows normal mucosal glands.

BHE 25mg/Kg treated

- LUNG : shows congested alveolar wall with mild thickening and mild emphysematous changes (400x)
HEART : shows congestion and mild inflammatory infiltration in between cardiac muscle bundles.
STOMACH : shows near normal mucosal gland with mild exudates

BHE 50mg/Kg treated

- LUNG : shows congestion, narrowed alveolar space and thickened alveolar wall.
HEART : shows hypertrophic cardiac muscle bundles.
STOMACH : shows stomach with superficial erosion and congestion.

Normal

SPLEEN : shows normal spleen with lymphoid aggregation.
LIVER : shows normal hepatocytes.
PANCREAS : shows pancreas with acini and normal islets

BHE 25mg/Kg treated

SPLEEN : shows congestion with lymphoid hyperplasia.
LIVER : shows hepatocytes with focal mild fatty change (400x)
PANCREAS : shows pancreas with acini and normal islets.

BHE 50mg/Kg treated

SPLEEN : shows lymphoid hyperplasia
LIVER : shows almost normal hepatocytes and occasional
binucleate cells.
PANCREAS : shows atrophic islet cells.

Normal

- BRAIN : shows normal brain with nerve fibers and astrocytes
KIDNEY : shows normal renal tissue with glomeruli and tubules.
OVARY : shows ovarian stroma with follicles and corpus leuteum.
TESTIS : shows normal tubules with spermatogenesis.

BHE 25mg/kg treated

- BRAIN : shows brain with micro cystic change and astrocytic proliferation (400x).
KIDNEY : shows renal tissue with focal tubular damage, interstitial inflammatory
OVARY : shows ovarian stroma with follicles and corpus leuteum
TESTIS : shows normal tubules with spermatogenesis.

BHE 50mg/kg treated

- BRAIN : shows brain with edema. Astrocytes show degenerative changes.
KIDNEY : shows renal tissue with tubular epithelial damage. RBC with in the tubules.
OVARY : shows ovarian follicles and corpus leuteum
TESTIS : shows normal tubules with spermatogenesis

RESEARCH PUBLICATIONS

JOURNALS :

International - 4

1. Hepatoprotective activity of methanolic polyherbal extract on CCl_4 – induced hepatic damage in rats in the Journal Biomedicine Vol. 27, No.2, 2007, Page No. 53-56.
2. Hepatoprotective activity Bi- herbal ethanolic extract on CCl_4 induced hepatic damage in rats. African Journal of Biochemistry Research Vol.2 (2), pp. 061-065, February 2008.
3. Antioxidant and antihepatotoxic activity of ethanolic biherbal crude extracts of *Eclipta alba* and *piper longum*, in the Journal Biomedicine Vol. 28, No.1, 2008, Page No. 39-42.
4. Hepatoprotective activity Bi- herbal ethanolic extract on CCl_4 induced hepatic damage in rats. Asian Journal of Biochemistry, 2008, 1-7.