ANTIDIABETIC EFFECT OF Basella rubra IN STREPTOZOTOCIN-INDUCED DIABETIC RATS

BY

A. NIRMALA

A thesis submitted to the Avinashilingam Institute for Home Science and Higher Education for Women - Deemed University Coimbatore - 641 043

In partial fulfilment of the requirements for the degree of

MASTER OF PHILOSOPHY IN BIOCHEMISTRY

AUGUST - 2004

CERTIFICATE

CERTIFICATE

This is to certify that the thesis entitled **"Antidiabetic effect of *Basella rubra* in streptozotocin-induced diabetic rats"** submitted to Avinashilingam Institute For Home Science and Higher Education for Women - Deemed University, Coimbatore, in partial fulfilment of the requirements for the award of the degree of Master of Philosophy in Biochemistry is a record of original research work done by **Ms. A. NIRMALA** during the period of her study in the Department of Biochemistry and Biotechnology, Avinashilingam Institute For Home Science and Higher Education for Women-Deemed University, Coimbatore, 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 to any candidate of any University.

Signature of the Guide

Forwarded

Signature of the

Head of the Department

DECLARATION

I hereby declare that the dissertation entitled **"Antidiabetic effect of *Basella rubra* in streptozotocin-induced diabetic rats"** submitted to the Avinashilingam Institute For Home Science and Higher Education for Women - Deemed University, Coimbatore, in partial fulfilment of the requirements for the award of the degree of **Master of Philosophy in Biochemistry** is a record of original research work done by me under the supervision and guidance of **Dr.S.Saroja** M.Sc., M.Phil., Ph.D., (Madras) Professor and Head, Department of Biochemistry and Biotechnology, Avinashilingam Institute For Home Science and Higher Education for Women-Deemed University, Coimbatore, and it has not formed the basis for the award of any degree / Diploma / Associateship / Fellowship or similar title to any candidate of any University.

A. Nirmala

Signature of the Candidate

Counter signed

Signature of the Guide

ACKNOWLEDGEMENT

Acknowledgement

The author records her whole hearted thanks to GOD Almighty for his grace, guidance, strength and blessings showered on her to putforth this dissertation.

The author expresses her thanks to Dr. K.Kulandaivel, M.A., (Ohio State), Ph.D., (Madras), Chancellor, Dr. M.Chandramani, M.Sc., (Baroda), M.Ed., Ph.D.,(Madras), Vice Chancellor, Dr. Saroja Prabhakaran, M.A., Dip. Ed., (Madras), Ph.D., (Mother Teresa), Registrar and Dr. S.Sivakamasundari, M.Sc., (Annamalai), M.Phil., Ph.D., (Madras), Dean, Faculty of Science, Avinashilingam Institute for Home Science and Higher Education for Women, Deemed University, Coimbatore, for providing all the facilities needed for conducting the study.

The author records her deep sense of gratitude to her co-guide Dr. S. Saroja, M.Sc., M.Phil., Ph.D., (Madras), Professor and Head, Department of Biochemistry and Biotechnology, Avinashilingam Institute for Home Science and Higher Education for Women, Deemed University, Coimbatore, for her meticulous care, constructive criticism, kind advice, constant support and encouragement.

The author is deeply obliged to . co-guide Ms. S.Gayathri devi, M.Sc., M.Phil., lecturer, Department of Biochemistry and Biotechnology, Avinashilingam Institute for Home Science and Higher Education for Women, Deemed University, Coimbatore, for her inspiring and patient guidance and precious assistance during the study.

The investigator records her sincere thanks to all the staff members of the Department of Biochemistry and Biotechnology for their constant encouragement.

The author expresses her sincere gratitude to Ms. P.Radha, M.Sc., and Ms. A. Ramya, M.Sc., Lecturers, Department of Biochemistry and Biotechnology who extended their kind support and help for the completion of the study.

The author also extends her thanks to her friends especially Ms. D. Sophia, who helped her in the course of the study.

She also feels great pleasure in thanking her parents, brother and relatives for their kind co-operation and moral support in making this study a reality.

CONTENTS

CONTENTS

S.NO.	TITLE	PAGE NO.
	LIST OF TABLES	
	LIST OF PLATES	
	LIST OF APPENDICES	
1	INTRODUCTION	1
2	REVIEW OF LITERATURE	3
3	EXPERIMENTAL PROCEDURE	15
4	RESULTS AND DISCUSSION	26
5	SUMMARY AND CONCLUSION	54
	REFERENCES	
	APPENDICES	

LIST OF TABLES

TABLE NO.	TITLE	PAGE NO.
1	NUTRIENTS IN *Basella rubra*	27
2	ENZYMIC ANTIOXIDANTS IN *Basella rubra*	28
3	NON-ENZYMIC ANTIOXIDANTS IN *Basella rubra*	29
4	EXTENT OF INHIBITION OF *IN VITRO* LIPID PEROXIDATION, SUPEROXIDE AND NITRIC OXIDE GENERATION BY *Basella rubra*	30
5	WEIGHT OF THE RATS	31
6	HAEMATOLOGICAL PARAMETERS OF THE RATS	33
7	BLOOD GLUCOSE AND LIVER GLYCOGEN IN THE RATS	34
8	FRUCTOSAMINE LEVELS IN RATS	35
9	GLUCOKINASE, GLUCOSE-6-PHOSPHATASE AND GLUCOSE-6-PHOSPHATE DEHYDROGENASE ACTIVITIES IN THE LIVER OF RATS	36
10	UREA, CREATININE AND BILIRUBIN LEVELS IN THE EXPERIMENTAL RATS	38
11	LEVELS OF AST, ALT AND ALP IN THE RATS	39
12	TOTAL PROTEIN, ALBUMIN AND ALBUMIN/GLOBULIN RATIO IN THE CONTROL AND EXPERIMENTAL RATS	40
13	SERUM LIPID LEVELS IN THE EXPERIMENTAL RATS	42
14	LEVELS OF ENZYMIC AND NON-ENZYMIC ANTIOXIDANTS AND THE EXTENT OF INHIBITION OF *IN VITRO* LIPID PEROXIDATION	44

LIST OF PLATES

PLATE NO.	LIST OF PLATES	PAGE NO.
I	LIVER SECTIONS OF THE RATS	48
II	PANCREAS SECTIONS OF THE RATS	50
III	KIDNEY SECTIONS OF THE RATS	52

LIST OF APPENDICES

APPENDIX NO.	TITLE
I	ESTIMATION OF TOTAL CARBOHYDRATE
II	ESTIMATION OF PROTEIN
III	ESTIMATION OF THIAMINE
IV	ESTIMATION OF RIBOFLAVIN
V	ESTIMATION OF MOISTURE CONTENT
VI	ESTIMATION OF CRUDE FIBRE
VII	ESTIMATION OF CALCIUM
VIII	ESTIMATION OF PHOSPHORUS
IX	ESTIMATION OF TOTAL IRON
X	ESTIMATION OF SODIUM AND POTASSIUM
XI	ESTIMATION OF CHROMIUM
XII	ESTIMATION OF CATALASE
XIII	ESTIMATION OF PEROXIDASE
XIV	ESTIMATION OF SUPEROXIDE DISMUTASE
XV	ESTIMATION OF POLYPHENOL OXIDASE
XVI	ESTIMATION OF GLUTATHIONE PEROXIDASE
XVII	ESTIMATION OF GLUTATHIONE–S-TRANSFERASE
XVIII	ESTIMATION OF ASCORBIC ACID
XIX	ESTIMATION OF TOCOPHEROL
XX	ESTIMATION OF TOTAL CAROTENOIDS
XXI	EXTRACTION AND ESTIMATION OF FLAVONOIDS
XXII	ESTIMATION OF POLYPHENOLS
XXIII	ESTIMATION OF REDUCED GLUTATHIONE
XXIV	ESTIMATION OF TANNINS
XXV	EXTENT OF INHIBITION OF *IN VITRO* LIPID PEROXIDATON
XXVI	DETERMINATION OF INHIBITION OF SUPEROXIDE PRODUCTION
XXVII	DETERMINATION OF INHIBITION OF NITRIC OXIDE GENERATION
XXVIII	ESTIMATION OF HAEMOGLOBIN

XXIX	ENUMERATION OF RED BLOOD CORPUSCLES
XXX	ENUMERATION OF WHITE BLOOD CORPUSCLES
XXXI	ENUMERATION OF DIFFERENTIAL LEUCOCYTE COUNTS
XXXII	PLATELET COUNT
XXXIII	DETERMINATION OF SERUM BILIRUBIN
XXXIV	ESTIMATION OF BLOOD UREA
XXXV	ESTIMATION OF CREATININE
XXXVI	ESTIMATION OF ALKALINE PHOSPHATASE (ALP)
XXXVII	ESTIMATION OF SERUM ASPARTATE TRANSAMINASE AND ALANINE TRANSAMINASE
XXXVIII	ESTIMATION OF BLOOD GLUCOSE
XXXIX	ESTIMATION OF GLYCOGEN
XL	ESTIMATION OF FRUCTOSAMINE
XLI	ESTIMATION OF GLUCOKINASE
XLII	ESTIMATION OF LIVER GLUCOSE-6-PHOSPHATASE
XLIII	ESTIMATION OF LIVER GLUCOSE-6-PHOSPHATE DEHYDROGENASE
XLIV	EXTRACTION OF LIVER LIPIDS
XLV	ESTIMATION OF TOTAL CHOLESTEROL
XLVI	ESTIMATION OF HDL-CHOLESTEROL
XLVII	ESTIMATION OF TRIGLYCERIDES
XLVIII	ESTIMATION OF PHOSPHOLIPIDS
XLIX	DETERMINATION OF ALBUMIN/GLOBULIN RATIO (A/G RATIO)
L	THIOBARBITURIC ACID REACTIVE SUBSTANCES
LI	ASSAY OF SUPEROXIDE DISMUTASE
LII	ESTIMATION OF ASCORBIC ACID
LIII	ESTIMATION OF α-TOCOPHEROL
LIV	HISTOPATHOLOGICAL STUDY

1. INTRODUCTION

Herbs are used by various cultures worldwide as drugs and remedies to cure and mitigate disease, dietary supplements (nutraceuticals, functional foods and beverages) and in overall health care of the world populace (Ghosal, 2002).

Many drugs commonly used today are of herbal origin. Some are made from plant extracts, others are made through transformation of chemicals found within them, while yet others are today synthesized from inorganic materials, but have their historical origins in research into the active compounds found in plants (Hamilton, 2004).

The World Health Organisation (WHO) estimates that 4 billion people i.e. 40 per cent of the world population presently use herbal medicine for some aspect of primary health care (Pei, 2001). The global demand for herbal medicine is not only large but also growing (Srivastava, 2000).

In Indian traditional system of medicine, herbal remedies are prescribed for the treatment of disease including diabetes mellitus (Maiti *et al.*, 2004). The rapidly increasing diabetes mellitus is becoming a serious threat to the health of the mankind in all parts of the world (Li *et al.*, 2004). The prevalence of diabetes mellitus for all age groups world-wide was estimated to be 2.8% in 2000 and 4.4% in 2030. It was estimated by Indian Diabetic Federation (IDF) that in India 31.7 million people had diabetes mellitus in 2000.This would increase to 79.4 million in the year 2030 (Wild *et al.*, 2004).

Diabetes mellitus is a group of metabolic disease characterized by hyperglycemia resulting from defects in insulin secretion, insulin action or both. The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction and failure of various organs especially the eyes, kidneys, nerves, heart and blood vessels (American Diabetes Association, 2004a).

Diabetes mellitus is classified into two types, type 1 and type 2 (Sridhar and Nagamani, 2002; Jeong *et al.*, 2003). Type 1 diabetes accounts for only 5-10% of the diabetics. It results from cellular mediated autoimmune destruction of the β-cells of the pancreas (Sridhar, 2002). At the later stage of the disease, there is little or no insulin secretion as manifested by low or undetectable levels of plasma C-peptide.

Type 1 patients must take exogenous insulin for survival to prevent the development of ketoacidosis (Soliman *et al.*, 2002).

Type 2 diabetes accounts for 90% of those with diabetes (Hirose *et al.*, 2002), previously referred to as non-insulin dependent diabetes (Chiu *et al.*, 2002), encompasses individuals who have insulin resistance (Clore *et al.*, 2002) and usually relative insulin deficiency. Autoimmune destruction of β-cells does not occur (Zimmet *et al.*, 2001).

For the treatment of diabetes mellitus, insulin and various types of hypoglycemic agents such as biguanides and sulfonylureas are found to control the blood sugar level as long as they are regularly administered and they also produce a number of undesirable side effects (Mutalik *et al.*, 2003). Hence in modern medicine, no satisfactory and effective therapy is still available to cure diabetes mellitus (Manonmani *et al.*, 2002).

The treatment of diabetes has been attempted with different indigenous plants and polyherbal formulation. Extracts of drugs from plant sources like *Ferula persica*, *Paronychia argentea*, *Pistacia atlantica* (Hamdan and Afifi, 2004), *Nigella sativa*, *Origanum compactum*, *Vitis vinifera* and *Glycyrrhiza glabra* (Haddad *et al.*, 2001) are used for diabetic treatment.

One such medicinal plant is *Basella rubra,* also known as Malabar spinach. It is a climbing perennial plant and a good source of vitamin C, calcium and iron (Palada and Crossman, 1999). The leaf extract is used in traditional medicine for the treatment of diabetes (Wichtl, 1994). The antidiabetic effect of this plant has not been so far investigated scientifically. In view of this, the present investigation was undertaken to analyze the **"Antidiabetic effect of *Basella rubra* in streptozotocin-induced diabetic rats"** with the following objectives:

1. Quantitative detection of phytochemicals in the leaves of *Basella rubra*
2. Antidiabetic effect of *Basella rubra* in rats
3. Estimation of enzymic and non-enzymic antioxidants
4. Effect of *Basella rubra* on carbohydrate metabolizing enzymes
5. Study of histopathology, if any.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Diabetes mellitus, long considered a minor significance to world health, is now taking its place as one of the main threats to human health in this century (Zimmet, 2000). In America alone, 16 million people have diabetes. Approximately 10% of the diabetic population is composed of type 1 diabetes (IDDM) whereas the remainder has type 2 diabetes (Brown, 1999).

Diabetes mellitus is a common metabolic disorder across the globe, more so in developing countries like India. In fact, India has the dubious distinction (Sridhar *et al.*, 2002) of being home to the largest number of people (31.7 million in 2000, 79.4 million in 2030 in India) with diabetes compared to any other country in the world (United Nation Population Division, 1999).

Classification of diabetes mellitus

Diabetes mellitus has been classified (American Diabetes Association, 2004a) as follows:

1. Type 1 or Insulin Dependent Diabetes Mellitus
2. Type 2 or Non-Insulin Dependent Diabetes Mellitus
3. Gestational diabetes

Other specific types

i. Genetic defects of β-cell function
ii. Genetic defects in insulin action
iii. Disease of the exocrine pancreas
iv. Endocrinopathis
v. Drug or chemical induced
vi. Infections
vii. Uncommon form of immune mediated diabetes

Type 1 or Insulin Dependent Diabetes Mellitus

Type 1 diabetes mellitus is characterized by severe insulinopenia and dependence on exogenous insulin to preserve life (Peveir *et al.*, 1992). Many patients with type 1 diabetes mellitus are obese and children and adolescents with type 1 diabetes mellitus are more prone to high body mass index (BMI) than their non-diabetic counterparts and unaffected siblings (Holl *et al.*, 1994).

It is an immune mediated, multifactorial disease involving severe destruction of the insulin producing pancreatic (>90%) β-cells (Lernmark, 1999). Obesity by itself leads to insulin resistance and increase the requirements of insulin to attain good glycemic control (Smith, 1996). Many factors might affect the BMI in patients with type 1 diabetes mellitus. These include glycemic control, insulin dose, quality and quantity of food consumed and different daily activity and exercise (Halleux *et al.*, 1998).

Type 2 or Non-Insulin Dependent Diabetes Mellitus

Type 2 diabetes is a heterogenous disorder characterized by insulin resistance and or defective insulin secretion (Gerich, 1998). It is the commonest form of diabetes accounting for 90-95% of cases. The etiology of this condition is broadly understood as an interaction between genes and environmental factors (O'Ruhilly, 1997). However, no single gene has yet been incriminating for the common form of type 2 diabetes. It is strongly believed that the condition is heterogenous and that multiple genes are involved (Chowdhury and Bhattacharaya, 2003).

Type 2 diabetes mellitus is characterized by elevated plasma concentration of triglycerides (TG) (Laakso *et al.*, 1993), low concentration of HDL and predominance of LDL particle (Pontrelli *et al.*, 2002).

In many, but not all, individuals with type 2 diabetes mellitus, the plasma glucagon concentration is increased in absolute terms and, when viewed relative to the prevailing hyperglycemia and hyperinsulinemia, (Cherrington, 1999) the majority of type 2 diabetes mellitus patients have hyperglucagonemia. Individuals with type2 diabetes are and also at a significantly higher risk for coronary heart disease, peripheral vascular disease, and stroke, and they have a greater likelihood of having hypertension, dyslipidemia and obesity (American Diabetes Association, National Institute of Diabetes and Digestive and Kidney disease, 2004) and hypercoagulability and microalbuminuria (Zimmet *et al.*, 2001).

Gestational diabetes mellitus

Significant hormonal changes during pregnancy can lead to blood sugar elevation in genetically predisposed pregnancy is called gestational diabetes. Gestational diabetes mellitus occurs transiently during pregnancy. Forty to fifty percent of women with gestational diabetes will eventually develop diabetes mellitus later in life, especially those

who require insulin during pregnancy and those who are over weight. Offsprings of women with gestational diabetes mellitus are at increased risk of obesity, glucose intolerance and diabetes in late adolescence and young adulthood (American Diabetes Association, 2002a; American Diabetes Association, 2004b).

Risk factors

Risk factors that could be operating at cellular level initiate and promote progression of diabetes vascular disease include insulin resistance *is a primary risk factor* and hyperinsulinemia, altered fatty acid metabolism, dyslipidemia, hypertension, ketoacidosis, osmotic effects, vasoactive hormones and dysfunction in sympathetic regulation of glucose and fat metabolism (Jakus, 2000).

Symptoms of diabetes mellitus

The early symptoms of untreated diabetes mellitus are elevated blood sugar level and loss of glucose in the urine. The major symptoms of diabetes include excessive thirst, frequent urination (polyuria), increased appetite (polyphagia), weakness and fatigue and weight loss. Other symptoms may include muscle cramps, impaired vision, poor wound healing and in women itching due to vaginal yeast infection. Extremely elevated glucose levels can lead to lethargy and diabetic coma (The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 2001).

Complications of diabetes mellitus

Long-term complications of diabetes include retinopathy with potential loss of vision; nephropathy leading to renal failure; peripheral neuropathy with risk of foot ulcers, amputation (Nathan, 1993) and charcot joints and autonomic neuropathy causing gastrointestinal, gastrourinary and cardiovascular symptoms and sexual dysfunction (Geiss, 1997). Glycation of tissue proteins and other macromolecules and excess production of polyol compounds from glucose are among the mechanisms thought to produce tissue damages from chronic hyperglycemia. Patients with diabetes have an increased incidence of atherosclerotic, cardiovascular, peripheral vascular and cerebrovascular diseases. Hypertension, abnormalities of lipoprotein metabolism and periodontal diseases are often found in people with diabetes (The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 2001).

Diabetic retinopathy

Diabetic retinopathy is the most frequent cause of new cases of blindness among adults aged 20-74 years. During the first two decades of disease, nearly all patients with type 1 diabetes and >60% of patients with type 2 diabetes have retinopathy (Fong *et al.*, 2004). Vision loss due to diabetic retinopathy results from several mechanisms.

First, central vision may be impaired by macular edema or capillary nonperfusion. Second, the new blood vessels of proliferating diabetic retinopathy and contraction of the accompanying fibrous tissue can distort the retina and lead to fractional retinal detachment producing severe and often irreversible vision loss. Third, the new blood vessels may bleed, adding further complication of preretinal or vitreous hemorrhage (American Diabetes Association, 2002 b).

Diabetic neuropathy

Diabetic neuropathies are among the most frequent complications of long-term diabetes. The earliest and the best-measured signs of diabetic neuropathies are decreased sensory and nerve conduction velocities. There is substantial evidence that diabetic neuropathy is also due to sorbitol accumulation (Cogan *et al.* 1984). In rats, increasing the sorbitol concentration in the sciatic nerve is directly related to decrease nerve conduction velocity, possibly as a result of decreased myoinositol concentration (Yue *et al.*, 1984). Although some studies have shown inositol supplementation to improve nerve conduction velocity, addressing the underlying accumulation of sorbitol is of greater importance (Wyngaarden *et al.*, 1992).

Diabetic nephropathy

About 20-30% of patients with type 1 or type 2 diabetes develop evidence of nephropathy, but in type 2 diabetes, a considerably smaller fraction of these progress to end-stage renal disease (American Diabetes Association, 2004c).

Diabetic retinopathy is characterized by proteinuria (>300 mg/24 hr), increased blood pressure and progressive decline in renal function. The pathological hallmark of diabetic nephropathy in the renal glomerulus is the expansion of the mesangial matrix and thickening of the capillary basement membrane (American Diabetes

Association, 2002c). A causal relationship between oxidative stress and diabetic nephropathy has been established by the following observations:

1. Lipid peroxides and γ-hydroxy deoxyguanosine, are increased in the kidneys of diabetic rats with albuminuria
2. High glucose directly increases oxidative stress in glomerular mesangial cells, a target cell of diabetic nephropathy
3. Oxidative stress induces mRNA expression of TGE-β_1 and fibronectin which are the genes implicated in diabetic glomerular injury and
4. Inhibition of oxidative stress ameliorates all the manifestations associated with diabetic nephropathy (Ha and Kim, 1999).

Macrovascular disease

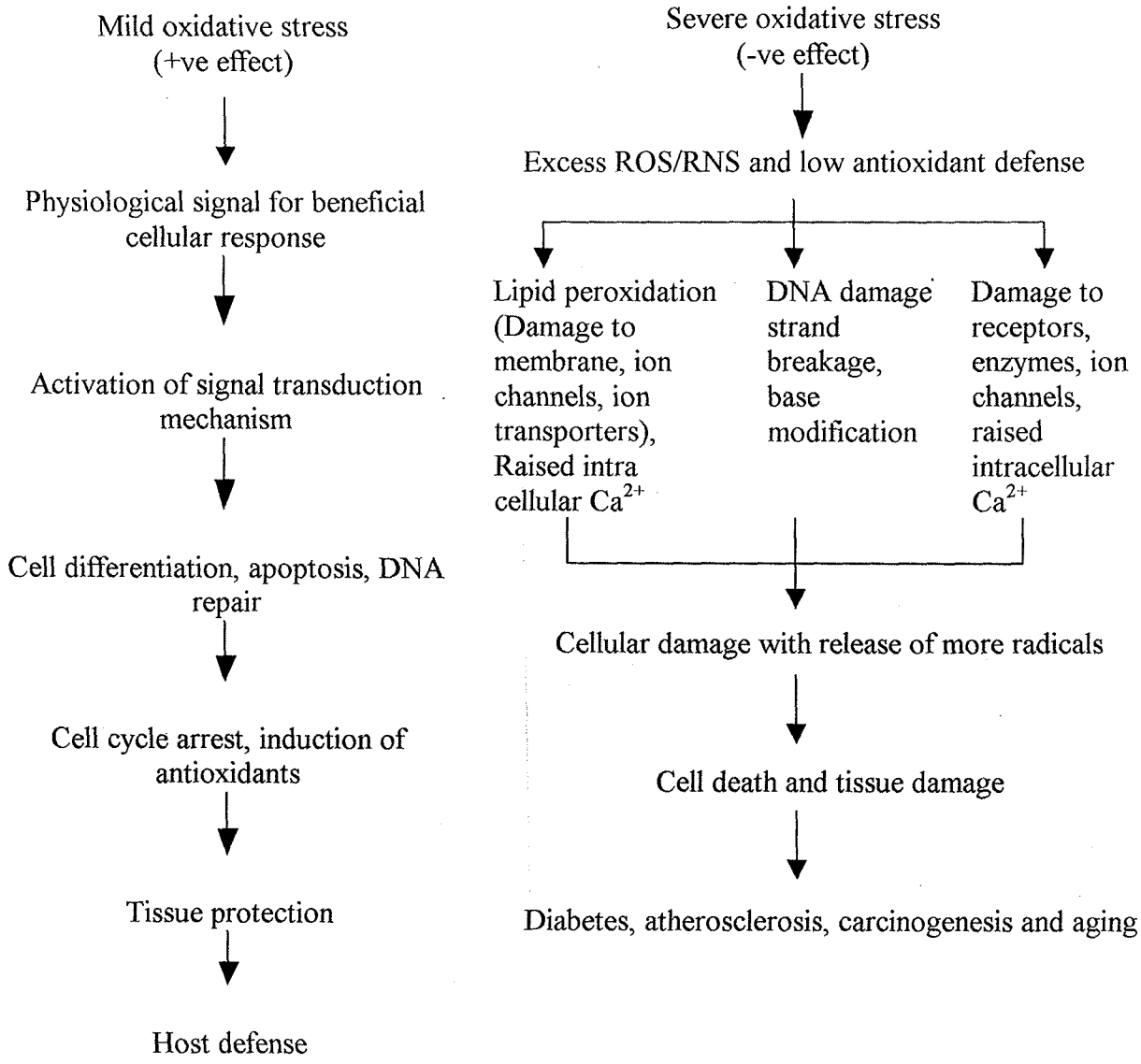
Cardiovascular disease is the leading cause of morbidity and mortality in type 2 diabetic individuals. Many of the established risk factors for atherosclerosis are more prevalent in the diabetic population, including hypertension, visceral obesity, low HDL and increased plasma LDL (Niki and Noguchi, 2002). However, both the Multiple Risk Factor Intervention Trial (MRFIT) and the Goteborg study have shown that at every level of known risk factors, the cardiovascular risk is 4-6 times higher for diabetic state contributing to accelerated atherosclerosis (Stamler *et al.*, 1993).

Free radicals and oxidative stress

Free radicals are defined as atoms or molecules that contain one or more unpaired electrons, making them unstable and highly reactive. The most important ROS are the superoxide anion radicals, hydrogen peroxide (H_2O_2), alkoxyl ($RO^{\bullet}$), peroxyl ($ROO^{\bullet}$) and hydroxyl radicals ($^{\bullet}OH$) (Frei, 1994) and hypochlorous acid (HOCl), other non-oxygen species existing as reactive nitrogen species (RNS) such as nitric oxide (NO) and peroxy nitrite (ONOO) have also important bioactivity (Evans and Halliwell, 1999).

In health, balance between pro-oxidants and antioxidant is critical for the survival and functioning of aerobic organism (Sies, 1985). During diabetes mellitus resistant hyperglycemia, causes increased production of free radicals via autooxidation of glucose (Wolf and Dean, 1987) and non-enzymic protein glycation (Wolf *et al.*, 1991), which may lead to disruption of cellular function (Kinalski *et al.*, 2000) and oxidative damage to membranes (Oberley, 1988).

Responses and signals during oxidative stress (Irshad and Chaudhuri, 2002)



Oxidative stress in diabetes mellitus

Oxidative stress is produced during normal metabolic process in the body and also induced by a variety of environmental factors and chemicals. Oxidative stress has been shown to have a significant effect in the causation of diabetes as well as diabetes related complications in human beings (Wilson, 1998). Oxidative stress in diabetes has been shown to co-exist with a reduction in the antioxidant status (Coppy *et al.*, 2000). Oxidative stress has been shown to produce glycation of proteins, inactivation of enzymes and alteration in structural function of collagen in basement membrane (Boynes, 1991).

Oxidative stress may have significant effect on the glucose transport protein (GLUT) or insulin receptor. Scavengers of oxidative stress may have an effect in reducing the increased serum glucose level in diabetes and may alleviate the diabetes as well as reduce its secondary complications (Sabu and Kuttan, 2000).

Antioxidant therapy and diabetic complications

Antioxidants therapy with vitamin E might normalize diabetic retinal hemodynamics (Clermont, 1998) and also decrease the severity of retinopathy (Kowluru *et al.*, 2001). Aminoguanidin has antioxidative property and inhibits the development of retinopathy, nephropathy and neuropathy (Guillgusseau, 1994). Aminoguanidin inhibits lipid peroxidation.

Treatment using a variety of antioxidant compounds such as vitamin E, α-lipoic acid, adduct of lipoic acid and gamma-linolenic acid, glutathione, probucol, N-acetyl cysteine and transition metal chelators results in a significant reduction in neuropathic symptoms in human and experimental diabetic animals (Tomlinson, 1998).

The protective effect of antioxidants on renal injury in diabetic animals has been recently reported. Taurine and vitamin C effectively reduced glomerular hypertrophy, albuminuria, glomerular collagen and TGF-β_1 accumulation in rats with streptozotocin-induced diabetes. Administration of vitamin E prevented glomerular hyperfiltration, albuminuria and renal PKC activity (Dominguez, 2000).

Antioxidants

Antioxidant has been defined as "any substance that delays or inhibits oxidative damage to a target molecule" (Gutteridge and Halliwell, 1990). All antioxidants generally influence the redox status, thereby protecting cells against reactive oxygen species (ROS) under certain circumstances, while promoting ROS generation in others (Herbert, 1996). Antioxidants are of two types namely enzymic antioxidant such as superoxide dismutase (SOD) (Offer *et al.*, 2000), catalase (CAT), glutathione peroxidase (GPx) (Ramanathan *et al.*, 1999) and non-enzymic antioxidants such as reduced glutathione (GSH) (Kinalski *et al.*, 2000), ascorbic acid, α-tocopherol (Halliwell and Aruoma, 1991), vitamins, estrogen (Kose *et al.*, 1993), β-carotene (Palozza *et al.*, 2001) and flavonoids (Chen *et al.*, 2002).

Enzymic antioxidants

Superoxide dismutase (SOD)

SODs are a family of metalloenzymes that convert superoxide ion into H_2O_2. They are found virtually in all aerobic organisms. There are four families of SOD: Cu-SOD, Cu-Zn-SOD, Mn-SOD and Fe-SOD. Human SOD is the Cu-Zn-SOD enzyme. SOD is considered to be a stress protein, which is synthesized in response to oxidative stress. SOD is reported to inhibit $^{\bullet}OH$ production (Ray and Husain, 2002).

Glutathione peroxidase (GSH-Px)

Glutathione peroxidases are selenoenzymes. GPx activity is being reduced in selenium deficiency. Low levels of selenium have been associated with a high risk of cardiovascular disease (Mannisto *et al.*, 2000).

GSH-Px catalyse the reduction of hydroperoxides at the expense of GSH (Ursini *et al.*, 1995).

$$ROOH + 2\,GSH \longrightarrow ROH + H_2O + GSSG$$

GSH peroxidase resides in the cytosol and mitochondrial matrix. It acts as an enzyme protecting hemoglobin from oxidative destruction by H_2O_2; as a contraction

factor of mitochondria; catalase reduction of H_2O_2 and organic peroxides including those derived from unsaturated lipids to alcohol; protects biomembranes from oxidative attack and prevents lipid peroxidation by scavenging H_2O_2 and slowing down dependent free radical attack on the lipids (Rana *et al.*, 2002).

Catalase (CAT)

Catalase is an enzyme, which is present in most cells, and catalyses the decomposition of hydrogen peroxide to water and oxygen. It is found to be important in the inactivation of many environmental mutagens (Nagao *et al.*, 1986). Plasmid DNA strand scission caused by xanthine/XO has been reported to be prevented by both catalase and SOD. CAT also prevented chromosomal aberration caused by hypoxanthine/XO in Chinese hamster cells (Lwata *et al.*, 1984). It has also been reported that while CAT is inactivated by $^{\bullet}OH$, GPx and SOD are considerably less affected by the ROMs (Piegeolet and Corbisier, 1990).

Non-enzymic antioxidants

Vitamin C

Vitamin C is actively taken up in high concentration by secretory cells of the islets of Langerhans where it is believed to play a role in antioxidant defense (Bailey and Flatt, 1986). In diabetes mellitus, vitamin C metabolism is abnormal and subjects have been shown to have low vitamin C and high dehydro-2-ascorbic acid concentration in plasma (Sinclair *et al.*, 1994). Vitamin C is a potent inhibitor of protein glycation, which has the particular advantage of low inherent toxicity in humans even in mega doses (Davie *et al.*, 1992).

Vitamin E (α-tocopherol)

Vitamin E is the most important antioxidant in an earlier line of defence in lipid peroxidation by scavenging peroxyl radicals (Metin *et al.*, 2002). It acts as a chain breaking antioxidant and has been shown to be beneficial in arresting neurodegenerative disease (Packer, 1994).

Treatment for diabetes mellitus

Allopathy

The modern drugs (sulphonylureas and biguanides) including insulin and oral hypoglycemic agents, control the blood sugar level as long as they are regularly administered and they also produce a number of side effects or undesirable effects (Reynolds, 1997). Despite their effectiveness, sulfonylureas have unwanted side effects and toxicity, including nausea, vomiting, haematological and dermatological reactions, obstructive jaundice, hyponatremia, and intolerance of alcohol and weight gain. Common side effects of biguanides include nausea, vomiting and epigastric distress. There is also the risk of developing lactic acidosis and hepatic diseases (Shorr *et al.*, 1996).

Herbal medicine

In ancient times, plants have been an exemplary source of medicine. Ayurveda and other Indian systems of traditional medicine mention the use of plants in the treatment of various human ailments, which includes neotropics, antihypertensives, antidiabetic and anti-inflammatory agents (Grover *et al.*, 2002). Many drugs commonly used today are of herbal origin. Indeed about 25 per cent of the prescription drugs contain at least one active ingredient derived from plant material. Some are made from plant extracts; others are synthesized to mimic a natural plant compound (Shukla *et al.*, 2000).

World health organization has suggested the evaluation of the potential of plants as effective therapeutic agents, especially in areas, where we lack safe modern drugs. WHO notes that of the 119 plant derived pharmaceutical medicines, about 74 per cent are used in modern medicine in ways that are correlated directly with their traditional uses as plant medicines by native cultures (WHO, 1994).

In the ongoing search for more effective and safer drugs, attention is being paid to new and safe drugs (Malhotra, 1991). Some recent work in drug development relates to species of *Commiphora* (used as hypolipidaemic agent), *Bacopa* and *Curcuma.*

Some examples of hypoglycemic medicinal plants used for the treatment of diabetes mellitus are *Taraxacum officinale* (dendelion), *Glycyrrhiza glabra* (licorice), *Syzygium cumini* (Jambal), *Opuntia streptacantha* (prickly pear), *Panax ginseng*, *P. quinquefolium* (ginseng) and *Globularia alypum* (globularia) (Ivorra *et al.*, 1989).

A number of herbal preparation and plant extracts have been used in the treatment of diabetes mellitus.

Tinospora cordifolia is a plant widely used in Ayurveda and is known as Amrita/ Gulancha/ Guduchi. *T.cordifolia* is claimed to be useful in Ayurvedic literature, for treatment of diabetes (Devasagayam and Sainis, 2002).

Abroma augusta is effective in the treatment of diabetes and in amenorrhoea. The leaves contain octacosonol, tarasexerol, β-sitosterol acetate and mixture of long chain fatty diols. The aqueous extract of *A.augusta* has antidiabetic activity (Hussain *et al.*, 2001).

In Ayurveda, extracts of more than one plant are also used in combination for treatment. A combination of *Coccinia indica* (leaves) and *Abroma augusta* (root extract) is used in the treatment of diabetes (Hussain, 2002a; Hussain, 2002b).

Aegle marmelos correa (Bael) is indigenous to India. Indigenous people use both leaves and fruits of this plant to treat (250 mg/kg body weight) diabetes mellitus (Ponnachan *et al.*, 1993).

Allium sativum is proclaimed to have Rasayana effect in Ayurveda. It is among the oldest of all cultivated plants. It has been used as a medicinal agent since thousands of years. It contains S-allyl cysteine sulphoxide, which has demonstrated antidiabetic effect in experimentally induced rats (Ali *et al.*, 2000). *Allium cepa* (onion) contains sulfur compounds, which are believed to be responsible for health benefits, including antidiabetic property (Brown, 1999).

Aloe vera (L) burn fil leaf extract has been reported to have blood glucose lowering effect in streptozotocin-induced type 2 diabetes mellitus (Okyar *et al.*, 2001).

Hypoglycemic plants such as *Aralia elata* (root, bark, young shoots), *Aesculus hippocastanum* (seeds), *Kochiascoparia* (fruit), *Polygala senega var. latifolia* (roots), *Beta vulgaris* (leaves and roots) have been reported by Yoshikava *et al.* (2001).

Gymnema sylvestre is a herb that has a long history of use in India for controlling diabetes. The common name is gurmar ("sugar destroying"). It suppresses the taste of sugar. Extract of (400mg/day) *Gymnema sylvestre* has been shown to effectively lower blood glucose levels in both type 1 and type 2 diabetic patients (Baskaran *et al.*, 1990; Shanmugasundaram *et al.*, 1990; Upadhyay *et al.*, 1996).

Fenugreek (*Trigonella foenum graecum*) has been described in the Greek and Latin pharmacopoeias (5g of powdered fenugreek seeds) for the treatment of both type 1 and type 2 diabetes (Bordia, 1997). Research also suggests that it lowers plasma cholesterol and triglyceride levels.

Bitter gourd (*Momordica charantia*) has been extensively used in folk medicine as a remedy for diabetes mellitus (Grover and Yadav, 2004).

Many plants have been cited in the literatures that are effective in the treatment of diabetes mellitus. *Basella rubra* has also been used to treat diabetes mellitus. But so far no indepth scientific investigation has been done to elucidate its efficacy as an antidiabetic plant. *Basella rubra* was therefore chosen for this study.

EXPERIMENTAL PROCEDURE

3. EXPERIMENTAL PROCEDURE

Type 2 diabetes is a common metabolic condition whose incidence, associated mortality and morbidity continue to rise (Marks and Raskin, 1998). Insulin and various types of hypoglycemic agents such as biguanides and sulfonylureas including some of the recently developed ones are available for the treatment of diabetes. But none is ideal in the treatment due to the toxic side effects and some times diminution in responses after prolonged use (David, 1996). The disadvantages of the presently available drugs are that they have to be given throughout the life and produce side effects (Eshrat, 2003).

Basic principle in the use of crude plant products or polyherbal preparations in traditional medicine is that the adverse effects of one component will be nullified by the protective effect of the other components without interfering with their therapeutic properties (Mutalik *et al.*, 2003). Herbs can be very effective in helping to manage elevated blood glucose level (Kaczmar, 1998; Matsuda *et al.*, 2002).

Indian plants, which are most effective, and the most commonly studied in relation to diabetes and their complications are *Cajanus cajan*, *Ficus bengalensis*, *Allium cepa*, *Aloe vera*, *Pterocarpus marsupium* and *Syzigium cuminii*. All these plants have shown varying degree of hypoglycemic and antihyperglycemic activity (Grover *et al.*, 2002).

Basella rubra belonging to *Basellaceae* family is found to contain medicinal properties. No scientific investigation has so far been done to document the hypoglycemic effect of this plant. The present study was designed to investigate the "Antidiabetic effect of *Basella rubra* in streptozotocin-induced diabetic rats".

The following experimental procedure was adopted.

PHASE I

3.1.Quantitative detection of phytochemicals in the leaves of *Basella rubra*

3.1.1. Assessment of nutrients

3.1.2. Determination of the enzymic and non-enzymic antioxidants

3.1.3. Determination of the extent of inhibition of *in vitro* lipid peroxidation, superoxide and nitric oxide generation

PHASE II

3.2.Hypoglycemic activity of *Basella rubra*

i) Experimental design

ii) Induction of diabetes in rats

iii) Preparation of the pulp of *Basella rubra*

iv) Administration of the pulp to rats

3.2.1. Assessment of haematological parameters in rats

3.2.2. Estimation of biochemical parameters

3.2.3. Enzymes of carbohydrate metabolism

3.2.4. Assessment of lipid profile

3.2.5. Effect of *Basella rubra* on protein in rats

3.2.6. Estimation of TBARS

3.2.7. Assay of antioxidants

3.2.8. Histopathological study

3.2.9. Statistical analysis

PHASE I

3.1.1.Assessment of nutrients

Assessment of nutrients in *Basella rubra* such as carbohydrate, protein, vitamins (thiamine and riboflavin), moisture content, crude fibre and minerals, calcium, phosphorus, iron, sodium, potassium and chromium was done.

3.1.1.1.Carbohydrate

The amount of carbohydrate present in the leaf sample was determined by the method of Hedge and Hofreiter (1962). The procedure has been elaborated in Appendix I.

3.1.1.2.Protein

The protein content in the sample was determined by the method of Lowry *et al.*, (1951) as detailed in Appendix II.

3.1.1.3. Vitamins

The vitamins, thiamine and riboflavin were analyzed in the leaf sample.

3.1.1.3.1. Thiamine

Thiamine content in the leaf sample was assessed by the method of Raghuramulu *et al.* (1983) as per the procedure given in Appendix III.

3.1.1.3.2. Riboflavin

The amount of riboflavin was analyzed fluorimetrically by the method of Raghuramulu *et al.* (1983). The procedure has been dealt in detail in Appendix IV.

3.1.1.4. Moisture content

The moisture content of the leaf sample was quantified by the method of Raghuramulu *et al.* (1983) as detailed in Appendix V.

3.1.1.5. Crude fibre

The crude fibre content was estimated by the method of Raghuramulu *et al.* (1983). The procedure has been elaborated in Appendix VI.

3.1.1.6. Estimation of the mineral content

The ash solution was prepared and used for the quantification of minerals such as calcium, phosphorus, iron, sodium, potassium and chromium.

Five gram of the leaves was weighed accurately into silica crucible (which had been previously heated to about 600°C, cooled and weighed). The crucible was then placed on a clay pipe triangle and heated over the low flame till all the materials was completely charred. This was followed by heating in a muffle furnace for about 6 hours at 600°C. The crucible was then cooled in a desiccator and weighed. To ensure completeness of ashing, the crucible was again heated in a muffle furnace for half-an-hour, cooled and weighed. This was repeated till two consecutive weights were the same and the ash was almost white or greyish white in colour.

The ash was moistened with a small amount of glass-distilled water (0.5 ml). Added 5 ml of 2N HCl and evaporated to dryness on a boiling water bath. Added another 5 ml of 2N HCl and evaporated to dryness. 4 ml of 2N HCl and 1 ml of water were added and the solution warmed over boiling water bath. Filtered using Whatman No.40 filter paper into 100 ml standard flask, cooled and made up the volume to 100 ml with distilled water and used for the estimation of minerals (Raghuramulu *et al.* 1983).

3.1.1.6.1. Calcium

The amount of calcium was estimated by the method of Clark and Collip (1925) as detailed in Appendix VII.

3.1.1.6.2. Phosphorus

The phosphorus was colorimetrically quantified by Fiske and Subbarow method (Oser, 1971). The procedure is explained in Appendix VIII.

3.1.1.6.3. Iron

The iron content was analyzed by the method of Oser (1971). The procedure is given in Appendix IX.

3.1.1.6.4. Sodium and Potassium

The amount of sodium and potassium was analyzed using a flame photometer as per the method of Raghuramulu *et al.* (1983) as given in Appendix X.

3.1.1.6.5. Chromium

The chromium content in the leaf sample was assessed by atomic absorption spectroscopy method (Krishna and Ranjan, 1991). The procedure is explained in Appendix XI.

3.1.2. Determination of enzymic and non-enzymic antioxidants

3.1.2.1. Enzymic antioxidants

The levels of enzymic antioxidants, catalase, peroxidase, superoxide dismutase, polyphenol oxidase, glutathione peroxidase and glutathione-S-transferase were determined.

3.1.2.1.1. Catalase

The catalase activity was measured by the method of Luck (1974), which is described in Appendix XII.

3.1.2.1.2. Peroxidase

The amount of peroxidase was estimated by the method of Reddy *et al.* (1995) as per the procedure given in Appendix XIII.

3.1.2.1.3.Superoxide dismutase

The superoxide dismutase activity was determined as per the method of Misra and Fridovich (1972). The detailed procedure is given in Appendix XIV.

3.1.2.1.4. Polyphenol oxidase

Polyphenol oxidase was estimated by the method of Esterbauer *et al.* (1977), the procedure for which is elaborated in Appendix XV.

3.1.2.1.5.Glutathione peroxidase

The glutathione peroxidase was quantified by the method of Rotruck *et al.* (1984). The procedure is explained in Appendix XVI.

3.1.2.1.6. Glutathione-S-transferase

The glutathione-S-transferase content was assessed by the method of Beutler (1984) as per the Appendix XVII.

3.1.2.2. Levels of non-enzymic antioxidants

Non-enzymic antioxidants namely ascorbic acid, α-tocopherol, polyphenol, flavonoids, tannins, total carotenoids and reduced glutathione were analyzed in *Basella rubra* leaf sample.

3.1.2.2.1. Ascorbic acid

Ascorbic acid was estimated by the method of Roe and Kuether (1953). The procedure is explained in Appendix XVIII.

3.1.2.2.2. α-tocopherol

It was estimated by the method given by Rosenberg (1992), which is explained in Appendix XIX.

3.1.2.2.3. Total carotenoids

The total carotenoids level was determined by the method of Zakaria *et al.* (1979), which is given in the Appendix XX.

3.1.2.2.4. Flavonoids

The flavonoids content was estimated by the method of Cameron *et al.* (1943). The procedure is detailed in Appendix XXI.

3.1.2.2.5. Polyphenols

The amount of polyphenols was estimated by the method of Malick and Singh (1980), which is elaborated in Appendix XXII.

3.1.2.2.6. Reduced glutathione

The method of Moron *et al.* (1979) was followed for the estimation of reduced glutathione, which is described in Appendix XXIII.

3.1.2.2.7. Tannins

The tannins were estimated by the method of Schanderl (1970) as explained in Appendix XXIV.

3.1.3. Determination of the extent of inhibition of *in vitro* lipid peroxidation, superoxide production and nitric oxide generation

3.1.3.1. Inhibition of *in vitro* Lipid peroxidation

The extent of inhibition of lipid peroxidation in cells by the leaf extract of *Basella rubra* was analyzed by the method of Okhawa *et al.* (1979). The detailed procedure is given in Appendix XXV.

3.1.3.2. Inhibition of superoxide production

The extent of inhibition of superoxide production was estimated by the method of Varley (1969). The procedure is described in Appendix XXVI.

3.1.3.3. Inhibition of nitric oxide generation

The extent of inhibition of nitric oxide generation was estimated by the method of Green and Hill (1984). The procedure is given in Appendix XXVII.

PHASE II

3.2.Hypoglycemic activity of *Basella rubra*

Male albino rats were selected for the study. They were of the same age and weight (150-200g). The animals were fed with stock diet and water *ad libitum*. After keeping in the laboratory condition for a few weeks for acclimatization, the experiment was initiated. Rats were categorized into 4 groups, each group consisting of 6 rats.

i) The experimental design was as follows.

Groups	Treatment
I	Control normal, healthy rats (non diabetic rats)
II	Diabetic control
III	Non diabetic rats administered with the leaf pulp of *Basella rubra*
IV	Diabetic rats administered with the leaf pulp of *Basella rubra*

ii) Induction of diabetes

Diabetes was induced in rats after 18 hours fasting by intraperitoneal administration of streptozotocin (60 mg/kg body weight dissolved in 10mM citrate buffer pH 4.5) (Archana *et al.*, 2001).

iii) Preparation of the leaf extract

Basella rubra was raised in the university campus under normal condition. Fresh leaves were collected for the study whenever required. Fresh leaves of *Basella rubra* (400mg) were made into a pulp with 10 ml of water by grinding it in a mortar and pestle. This pulp was utilized for the experiment.

iv) Administration of *Basella rubra*

Rats of groups III and IV were administered with the pulp of *Basella rubra* (400mg) orally by intubation. In the case of group IV, the pulp was administered simultaneously with the induction of diabetes. This was continued for 30 days.

3.2.1.Assessment of haematological parameters in rats

The experimental and control rats were sacrificed at the end of the 30th day of the experiment. Blood was collected by cardiac puncture under chloroform anesthesia. Serum was separated and stored at -20°C for analysis. Then the organs, liver, kidney and pancreas were dissected out, removed the blood by blotting and preserved for biochemical and histopathological studies.

3.2.1.1. Estimation of haemoglobin

Haemoglobin content of the blood was estimated by cyanmethaemoglobin method of Drabkin and Austin (1932). The procedure is described in Appendix XXVIII.

3.2.1.2 Enumeration of red blood corpuscles

The erythrocyte count was determined accurately by diluting a measured quantity of blood with an isotonic solution (Sanderson and Phillips, 1981) as per the procedure given in Appendix XXIX.

3.2.1.3 Enumeration of white blood corpuscles

The total and differential counts of white blood cells were done using Truk's fluid (glacial acetic acid 5 ml + Gentian violet 1% + water 95 ml), which can destroy the RBCs by following the method of Sanderson and Phillips (1981). The procedure is detailed in Appendices XXX and XXXI.

3.2.1.4. Platelet count

The blood platelets were counted in Neubauer counting chamber (Sanderson and Phillips, 1981) as described in Appendix XXXII.

3.2.2. Biochemical parameters

3.2.2.1. Determination of serum bilirubin

Serum bilirubin was estimated by the method of Malloy and Evelyn (1937) as per the procedure given in Appendix XXXIII.

3.2.2.2. Estimation of blood urea

Blood urea was quantified by DAM-TSC method (Netlson, 1957). The procedure is explained in Appendix XXXIV.

3.2.2.3. Estimation of creatinine

Creatinine was estimated by alkaline picrate method (Owen e*t al.*, 1954), which is described in Appendix XXXV.

3.2.2.4. Estimation of alkaline phosphatase (ALP)

Alkaline phosphatase was determined by the method of King and Armstrong (Raghuramulu *et al.*, 1983), which is detailed in Appendix XXXVI.

3.2.2.5. Estimation of serum aspartate transaminase (AST) and alanine transaminase (ALT)

AST and ALT were estimated by the method of Reitman and Frankel (1957). The detailed procedure is given in Appendix XXXVII.

3.2.2.6. Estimation of blood glucose

The total blood glucose was quantified by orthotoluidine method (Raghuramulu *et al.*, 1983) as per the procedure given in Appendix XXXVIII.

3.2.2.7 Estimation of glycogen

The liver glycogen was estimated by the method of Good *et al.* (1933). The procedure is given in Appendix XXXIX.

3.2.2.8. Estimation of fructosamine

Fructosamine was estimated in serum by the method of Armbruster (1987). The procedure is explained in Appendix XL.

3.2.3. Enzymes of carbohydrate metabolism

3.2.3. 1 Estimation of glucokinase

Glucokinase activity was assayed in the liver by the method of Brandstrup *et al.* (1957). The procedure is given in Appendix XLI.

3.2.3.2 Estimation of liver glucose-6-phosphatase

The liver glucose-6-phosphatase was assayed according to the method of Koida and Oda (1959). The procedure is given in Appendix XLII.

3.2.3.3 Estimation of liver glucose-6-phosphate dehydrogenase

Liver glucose-6-phosphate dehydrogenase was determined by the method of Kornberg and Horecker (1955). The detailed procedure is given in the Appendix XLIII.

3.2.4. Lipid profile

3.2.4.1 Extraction of liver lipids

Lipids were extracted from the liver by the method of Radin (1981). The procedure is explained in Appendix XLIV.

3.2.4.2 Estimation of total cholesterol

The total cholesterol content of the serum was determined by CHOD-PAP method (Flegg, 1972) as detailed in Appendix XLV.

3.2.4.3 Estimation of HDL cholesterol

The amount of HDL cholesterol in serum was determined by CHOD-PAP method (Flegg, 1972). The procedure is given in Appendix-XLVI.

3.2.4. 4 Estimation of VLDL and LDL cholesterol

The VLDL and LDL cholesterol values were determined by using the formula

$$\text{VLDL (mg/dl)} = \frac{\text{Triglycerides}}{5}$$

$$\text{LDL (mg/dl)} = \text{total cholesterol} - \frac{\text{Triglycerides}}{5} - \text{HDL}$$

3.2.4.5 Estimation of triglycerides

The triglycerides were estimated by the method of GPO-PAP (Bucolobe and David, 1973; Fossati and Principe, 1982). The procedure is described in Appendix-XLVII.

3.2.4.6 Estimation of phospholipids

Phospholipid level was estimated (from lipid extract) by the method of Zilversmit and Davis (1950). The procedure is given in Appendix XLVIII.

3.2.5. Effect of *Basella rubra* on protein in rats

3.2.5.1 Estimation of protein

The serum protein content was estimated by the method of Lowry *et al.* (1951). The procedure is given in Appendix - II

3.2.5.2 Determination of albumin/globulin ratio (A/G ratio)

The A/G ratio in serum was determined by the procedure of King and Wootton (1959) as explained in Appendix- XLIX.

3.2.6. Estimation of Thiobarbituric acid reactive substance (TBARS)

Production of thiobarbituric acid reactive substances (TBARS) is a commonly used technique to assess lipid peroxidation (Bagchi *et al.*, 1998). Lipid peroxidation was induced by Fe^{2+} ascorbate system in human red blood cells (RBC) (Acharya *et al.*, 2003).

TBARS was estimated by the method of Nichans and Samuelson (1968). The detailed procedure is given in Appendix L.

3.2.7. Assay of antioxidants

3.2.7.1. Enzymic antioxidants

During diabetes mellitus persistent hyperglycemia causes an increased production of free radicals via autooxidation of glucose and non-enzymic protein glycation, which may lead to disruption of cellular functions and oxidative damage to membranes (Oberley, 1988). The levels of reactive oxygen species are controlled by antioxidant enzymes like SOD, CAT and GPx and non-enzymic scavengers such as reduced glutathione (GSH) (Kotlar *et al.*, 1998; Barlow-Walden *et al.*, 1995). The antioxidant enzymes were therefore assayed.

3.2.7.1.1 Superoxide dismutase (SOD)

Superoxide dismutase activity was determined by the method of Kakkar *et al.* (1984). The procedure is explained in Appendix-LI.

3.2.7.1.2 Catalase (CAT)

The activity of catalase was determined by the method of Luck (1974). The procedure for the same is given in Appendix-XII

3.2.7.1.3 Glutathione peroxidase (GPx)

Glutathione peroxidase was estimated by the method of Rotruck *et al.* (1984). Detailed procedure is given in Appendix-XVI

3.2.7.2. Non-enzymic antioxidants

3.2.7.2.1. Estimation of reduced glutathione

The glutathione content was determined by the method of Moron *et al.* (1979) as described in Appendix-XXIII

3.2.7.2.2. Ascorbic acid

Ascorbic acid was estimated by the method of Omaye *et al.* (1979), which is explained in Appendix- LII.

3.2.1.7.2.3. α-tocopherol

α-tocopherol was estimated by the method of Baker *et al.* (1951) as detailed in Appendix -LIII.

3.2.8.Histopathological studies

The control and experimental rats were sacrificed at the end of the experimental period. Sections of the organs like liver,kidneyand pancreas were done by the method of Culling (1979). The procedure is given in Appendix LIV.

3.2.9.Statistical analysis

The data obtained are expressed as mean ± SD. The significance of the various treatments was found by ANOVA.

RESULTS AND DISCUSSION

4. RESULTS AND DISCUSSION

Basella rubra is a medicinal plant, which is used in traditional medicine for the treatment of diabetes. This study is an attempt to scientifically investigate the "**Antidiabetic effect of *Basella rubra* in streptozotocin-induced diabetic rats**". The observations made and the results obtained are presented and discussed under the following headings:

PHASE I

4.1 Quantitative detection of phytochemicals in the leaves of *Basella rubra*

4.1.1 Assessment of nutrients in *Basella rubra*

4.1.2 Determination of the levels of enzymic and non-enzymic antioxidants

4.1.3 Determination of the extent of inhibition of *in vitro* lipid peroxidation, superoxide production and nitric oxide generation by *Basella rubra*

PHASE II

4.2 Therapeutic effect of *Basella rubra* in STZ induced diabetic rats

4.2.1 Weight of the rats

4.2.2 Hematological parameters

4.2.3 Blood glucose and liver glycogen in rats

4.2.4 Level of fructosamine

4.2.5 Activities of carbohydrate metabolizing enzymes

4.2.6 Levels of urea, creatinine and bilirubin

4.2.7 Activities of the enzymes, aspartate transaminase, alanine transaminase and alkaline phosphatase

4.2.8 Total protein, albumin and globulin

4.2.9 Lipid profile

4.2.10 Antioxidants and the extent of inhibition of *in vitro* lipid peroxidation

4.2.11 Histopathological study

PHASE I

4.1 Quantitative detection of phytochemicals in the leaves of *Basella rubra*

4.1.1 Assessment of nutrients in *Basella rubra*

The amount of nutrients present in *Basella rubra* was analysed and it is given in Table I.

Table I

NUTRIENTS IN *Basella rubra*

S.No.	Nutrient	Quantity/100g Fresh weight
1	Total carbohydrate (g/100g)	3.1 ± 0.12
2	Protein (g/100g)	2.9 ± 0.25
3	Moisture content (g/100g)	81.0 ± 1.63
4	Fibre content (g/100g)	12.7 ± 2.10
5	Thiamine (mg/100g)	0.05 ± 0.01
6	Riboflavin (mg/100g)	0.31 ± 0.02
7	Calcium (mg/100g)	71.4 ± 1.07
8	Phosphorus (mg/100g)	17.8 ± 1.03
9	Iron (mg/100g)	1.57 ± 0.01
10	Sodium (mg/100g)	52.75 ± 1.30
11	Potassium (mg/100g)	204.8 ± 1.77
12	Chromium (mg/100g)	0.004 ± 0.001

Values are mean ± SD of six replicates

Basella rubra contains appreciable quantity of carbohydrate, protein, moisture, fibre content, vitamins and minerals such as calcium, phosphorus, iron, sodium, potassium and chromium. The fibre content of *B. rubra* is 25.3 g/100g. Fibre is found to be good for diabetic patients. The water soluble fibre can help improve blood sugar control and lower the level of cholesterol.

Supplementation of potassium improves insulin sensitivity, responsiveness and secretion; insulin administration induces a loss of potassium; and a high potassium intake reduces the risk of heart diseases and atherosclerosis (Norbiato *et al.*, 1984).

B. rubra is also found to contain chromium (0.004mg/100g). Chromium is vital to proper blood sugar control as it functions as a key constituent of the "glucose tolerance factors". In diabetes, supplementing the diet with chromium has been shown to decrease fasting glucose levels, improve glucose tolerance, lower insulin levels and decrease total cholesterol and triglyceride level while increasing HDL cholesterol levels (Mooradian, 1994; Anderson, 1992). Chowdhury and Bhatacharya (2003) have also reported that the micronutrient chromium is known to play a role in carbohydrate metabolism, more specifically in insulin action. Supplementation with trivalent chromium often causes a modest improvement of glycaemic control in diabetic patients.

4.1.2 Enzymic antioxidants

The activities of various enzymic antioxidants in *Basella rubra* are presented in Table II.

Table II

ENZYMIC ANTIOXIDANTS IN *Basella rubra*

S.No	Enzymic antioxidants	Activity (U/mg protein)
1	Catalase *	6.30 ± 0.200
2	Peroxidase **	0.40 ± 0.025
3	Superoxide dismutase ***	6.90 ± 0.258
4	Glutathione-S-transferase ♦	0.27 ± 0.012
5	Glutathione peroxidase ♦♦	105.6 ± 0.186
6	Polyphenol oxidase ♦♦♦	0.24 ± 0.025

Values are mean ± SD of six replicates

* Amount of enzyme that brings about a decrease in absorbance of 0.05 at 240 nm

** Change in absorbance/min/mg protein

*** Amount of SOD that causes 50% reduction in the extent of NBT oxidation

♦ µMoles of CDNB-GSH conjugate /min/mg protein

♦♦ µGram of GSH consumed/min/mg protein

♦♦♦ Amount of enzyme that transform 1µmole of dihydrophenol to 1µmole of quinone/min

From the above table it is clear that the enzymic antioxidants catalase, peroxidase, superoxide dismutase, glutathione-S-transferase, glutathione peroxidase and polyphenol oxidase are high in *Basella rubra.*

Arivazhagan *et al.* (2000) have reported that SOD protects tissues against oxygen free radicals by catalyzing the removal of superoxide radical ($O_2^{\bullet-}$), which damages the membrane and biological structure.

Catalase has been shown to be responsible for the detoxification of significant amounts of H_2O_2, SOD and catalase are two major scavenging enzymes that remove the toxic free radicals *in vivo*. Reduced activity of SOD and catalase in liver and kidney have been observed during the diabetes and this may result in a number of deleterious effects due to the accumulation of superoxide radicals ($O_2^{\bullet-}$) and hydrogen peroxide (Shanthakumari *et al.*, 2003).

Basella rubra being a good source of these enzymes may be effective in the treatment of hyperglycemia.

Non-enzymic antioxidants

The non-enzymic antioxidants in *Basella rubra* were assessed and illustrated in Table III.

Table III

NON-ENZYMIC ANTIOXIDANTS IN *Basella rubra*

S.No	Non-enzymic antioxidants	Quantity (mg/g)
1	Ascorbic acid	31.0 ± 1.290
2	Vitamin E	4.51 ± 0.260
3	Carotenoids	5.6 ± 0.157
4	Polyphenols	317.9 ± 1.995
5	Flavonoids	26.0 ± 0.460
6	Tannins	121.3 ± 1.10
7	Glutathione	10.03 ± 0.022

Vaues are mean ± SD of six replicates

From the above table, it is evident that *Basella rubra* is a good source of non-enzymic antioxidants such as ascorbic acid, polyphenols, flavonoids, tannins and glutathione, whereas vitamin E and carotenoids are present in low amounts.

It has been confirmed that vitamin C protects the body from damage due to inflammation, and high doses of vitamin C can improve the immune function (Cartmell and Kingsworth, 2000).

Vitamin E is an important antioxidant in the cell membrane (Garg *et al.*, 1996). Mukai *et al.* (1993) have reported that vitamin E (α-tocopherol) is one of the most important and least toxic of all lipid soluble antioxidant vitamins. It scavenges peroxyl radical intermediates in lipid peroxidation and is responsible for protecting polyunsaturated fatty acids (PUFA) present in cell membrane and low-density lipoprotein against lipid peroxidation.

Since *Basella rubra* is found to contain high levels of these non-enzymic antioxidants, it would be effective in treating the diseases caused by free radicals and also in the management of diabetes.

4.1.3 Assessment of inhibition of *in vitro* lipid peroxidation, superoxide and nitric oxide generation by *Basella rubra*

Membrane lipids are particularly susceptible to lipid peroxidation. The damage caused by lipid peroxidation is highly detrimental to the functioning and survival of the cell and its organelles. Presence of PUFA in the phospholipids of the bilayer of biological membranes is the basis of membrane fluidity. Lipid peroxidation therefore, affects the biophysical properties of membranes (Devasagayam *et al.*, 2003).

The extent of inhibition of *in vitro* lipid peroxidation, superoxide and nitric oxide generation is depicted in Table IV.

Table IV

EXTENT OF INHIBITION OF *in vitro* LIPID PEROXIDATION, SUPEROXIDE AND NITRIC OXIDE GENERATION BY *Basella rubra*

S.No	Sample	Percent inhibition of		
		***In vitro* lipid peroxidation**	**Superoxide generation**	**Nitric oxide generation**
1	*Basella rubra*	73.4	67	10.6

It can be inferred from the table that the extent of inhibition of *in vitro* lipid peroxidation, superoxide scavenging activity was found to be higher (73.4% and 67% respectively) than the nitric oxide generation (10.6%) by *Basella rubra.*

Our results are in accordance with the findings of Govindarajan *et al.* (2003) who have reported that alcoholic extract of *Picorrhiza kurrooa* Royle ex Benth inhibited the lipid peroxidation, superoxide scavenging and nitric oxide generation activity.

Thus, it is evident that the presence of antioxidants in *Basella rubra* may contribute to the effective inhibition of *in vitro* lipid peroxidation, superoxide scavenging activity and nitric oxide generation.

PHASE II

4.2 Therapeutic effect of *Basella rubra* in streptozotocin-induced diabetic rats

The initial weight of the rats (before induction of diabetes with streptozotocin) and their final weight at the end of the experimental period are given in Table V.

Table V

WEIGHT OF THE RATS

GROUP	Treatment	Weight (g)	
		Initial	Final
I	Non-diabetic control	194.3 ± 2.50	230.4 ± 2.98[a]
II	Diabetic control	196.3 ± 1.96	154.4 ± 2.76[c]
III	Non-diabetic + *Basella rubra*	198.0 ± 3.36	220.0 ± 2.58[b]
IV	Diabetic + *Basella rubra*	196.0 ± 3.36	228.2 ± 2.77[a]
CD (0.05)		3.5	3.6

Values are mean ± SD

Values with different superscripts differ significantly ($P<0.05$)

The body weight of the STZ-induced diabetic rats (Diabetic control) has been significantly reduced ($P<0.05$) when compared to that of the non-diabetic control rats. The rats treated with *Basella rubra* alone are similar to that of the non-diabetic controls. Diabetic rats (Group IV) treated with *Basella rubra* showed a significant increase ($P<0.05$) in their body weight compared to that of the initial body weight and close to that of non-diabetic control rats fed with *Basella rubra* (Group III). This prevention in the loss of body weight of diabetic rats fed with *Basella rubra* is indicative of its therapeutic effect in STZ-induced diabetes mellitus.

Several reports have shown that the body weight decreased in diabetic rats (Palanivel *et al.*, 2001; Laviola *et al.*, 2001 and Raju *et al.*, 2001).

Our results are similar to that of Babu *et al.* (2003) who have reported that body weight has been slightly increased in the control rats compared to initial body weight, whereas in the diabetic control rats, there was a significant decrease in the body weight. In diabetic rats treated with ethanolic extracts of *Cassia kleinii*, no reduction in body weight was observed.

4.2.2 Haematological parameters

Haematological parameters of the rats are presented in Table VI.

Table VI

HAEMATOLOGICAL PARAMETERS OF THE RATS

Group	Treatment	Haemoglobin (g/dl)	WBC /mm^3	RBC 10^6/mm^3	Polymorphs (%)	Lymphocytes (%)	Eosinophils (%)	Platelets 10^9/L
I	Non-diabetic control	15.7 ± 2.06^a	8529±56.2^a	5.8 ± 0.39^a	50.7 ± 3.38^a	41.41 ± 2.37^a	1.71 ± 0.37^a	863 ± 26.7^a
II	Diabetic control	10.0 ± 1.87^b	8006±44.7^b	4.3 ± 0.24^c	57.4 ± 3.76^b	36.31± 3.06^b	2.86 ± 0.32^b	832 ± 30.2^b
III	Non-diabetic + *Basella rubra*	16.0 ± 2.08^a	8504±37.5^a	5.4 ± 0.25^a	52.5 ± 3.176^a	44.48 ± 3.99^a	1.80 ± 0.36^a	850 ± 31.31^a
IV	Diabetic + *Basella rubra*	14.0 ± 1.57^a	8569±42.5^a	4.8 ± 0.36^b	51.9 ± 3.51^a	43.65 ± 3.47^a	1.78 ± 0.24^a	858 ± 32.8^a
CD $_{(0.05)}$		3.8	110.2	0.43	4.34	4.3	0.43	14.1

Values are mean ± SD

Values with different superscript differ significantly ($P<0.05$)

The haemoglobin, red blood cells, leucocytes and platelet content of the diabetic rats were found to be lower than that of the control rats. But the diabetic rats on treatment with *Basella rubra* showed a significant increase in the levels of all the haematological parameters.

Our results are similar to that of Mutalik *et al.* (2003). They have demonstrated that the feeding the diabetic rats with dianex, a polyherbal formulation for 30 days resulted in a significant increase in RBC count and blood haemoglobin.

4.2.3 Blood Glucose and Liver Glycogen in rats

The levels of blood glucose and liver glycogen content in the rats are given in Table VII.

Table VII

BLOOD GLUCOSE AND LIVER GLYCOGEN IN THE RATS

Group	Treatment	Glucose (mg/dl)	Glycogen (mg/g liver)
I	Non-diabetic control	80.0 ± 3.65^{a}	53.3 ± 2.01^{a}
II	Diabetic control	252.0 ± 4.16^{c}	38.6 ± 2.60^{b}
III	Non-diabetic + *Basella rubra*	82.0 ± 4.76^{a}	54.7 ± 2.15^{a}
IV	Diabetic + *Basella rubra*	110.1 ± 3.93^{b}	52.0 ± 2.58^{a}
CD (0.05)		5.46	3.19

Values are mean ± SD

Values with different superscripts differ significantly ($P<0.05$)

The blood glucose level was found to be increased drastically (252 mg/dl) in the diabetic rats compared to that of the control rats (80mg/dl), and also the non-diabetic rats treated with *Basella rubra* (82 mg/dl). Treatment of the diabetic rats with *Basella rubra* reduced the blood glucose level significantly. However, it did not reach the control level of blood glucose.

The liver glycogen content in diabetic rats was lower than that of the control groups. The diabetic rats treated with *Basella rubra* showed a significant increase ($P< 0.05$) in their liver glycogen content.

Zhang et al. (2003) and Kiho *et al.* (1992) also have reported that treatment of alloxan induced diabetic rats with *Rehmannia glutinosa* oligosaccharide for 15 days resulted in a significant decrease in blood glucose level and increase in hepatic glycogen content.

Our results are corroborative with the report of Lemhadri et al. (2004), who showed that administration of the aqueous extract of *Origanum vulgare* to STZ-induced diabetic rats caused a significant reduction in the blood glucose level.

Aqueous extracts of other plants such as *Carum carvi* and *Capparis spinosa* (Eddouks *et al.*, 2004), *Mithania coagulans* (Hemalatha *et al.*, 2004), *Artemisia herba alba* (Twaij and Badr., 1988), *Eclipta alba* (Shukla *et al.*, 2000), *Fraxinus excelsior* (Eddouks and Maghrani., 2004), *Hibiscus rosasinensis* (Sachdewa *et al.*, 2001) have also been found to be beneficial in the treatment of diabetes mellitus.

4.2.4 Fructosamine

Fructosamine levels in the serum of rats are depicted in Table VIII.

Table VIII

SERUM FRUCTOSAMINE LEVELS IN RATS

Group	Treatment	Fructosamine (mg/100 ml)
I	Non-diabetic control	250.5 ± 3.94^{a}
II	Diabetic control	324.4 ± 4.02^{c}
III	Non-diabetic + *Basella rubra*	254.8 ± 8.93^{a}
IV	Diabetic + *Basella rubra*	299.7 ± 5.84^{b}
$CD_{(0.05)}$		7.9

Values are mean ± SD

Values with different superscripts differ significantly ($P<0.05$)

It is evident from the above table that the fructosamine level is significantly higher in the diabetic rats when compared to that of the other groups of rats. In diabetic rats treated with *Basella rubra,* the fructosamine level got reduced significantly ($P< 0.05$). However, it did not reach the control level.

This indicated that although *Basella rubra* is effective in lowering the fructosamine level, either the dose (400mg) administered or the duration of treatment is not sufficient.

Our results are consistent with that of Ryan *et al.* (2002) who have also reported that fructosamine level is lowered in Type I diabetic rats that were given CMT-8 daily for 3 weeks.

4.2.5 Carbohydrate metabolizing enzymes

Carbohydrate metabolizing enzymes such as glucokinase, glucose-6-phosphatase and glucose-6-phosphate dehydrogenase activities in liver of the rats are given in Table IX.

Table IX

GLUCOKINASE, GLUCOSE-6-PHOSPHATASE AND GLUCOSE-6-PHOSPHATE DEHYDROGENASE ACTIVITIES IN LIVER OF THE RATS

Group	Treatment	Glucokinase (U/mg protein)	Glucose-6-phosphatase (U/mg protein)	Glucose-6-phosphate dehydrogenase (U/mg protein)
I	Non-diabetic control	46.4 ± 2.8 [a]	124.6 ± 3.55 [a]	101.6 ± 2.57 [a]
II	Diabetic control	13.2 ± 2.1 [b]	201.8 ± 3.16 [b]	70.9 ± 2.29 [b]
III	Non-diabetic + *Basella rubra*	45.5 ± 3.9 [a]	125.6 ± 3.24 [a]	102.5 ± 3.50 [a]
IV	Diabetic + *Basella rubra*	43.6 ± 4.3 [a]	124.7 ± 2.67 [a]	98.1 ± 2.67 [a]
$CD_{0.05}$		4.5	4.2	4.2

Values are mean ± SD
Values with different superscripts differ significantly ($P<0.05$)

1 unit of:

Glucokinase = µmoles of glucose phosphorylated per hour
Glucose –6-phosphatase = µmoles of phosphate liberated per hour
Glucose-6-phosphate dehydrogenase = 50% reduction in NADP

A significant ($P<0.05$) decrease in the activities of glucokinase and glucose-6-phosphate dehydrogenase was recorded in the diabetic rats compared to the rest of the groups.

This is in line with the findings of Ugochukwu and Babady (2003) who have reported that diabetic rats showed a significant decrease in the activities of both the hepatic glucose-6-phosphate dehydrogenase and glucokinase.

Aqueous extract of *Enicostemma littorale* blume treatment in diabetic rats significantly decreased liver glucose-6-phosphatase activity (Maroo *et al.*, 2003).

Results of this study are in agreement with Prakasam *et al.* (2002) who have reported that oral administration of aqueous extract of *Casearia esculenta* root for 45 days in diabetic rats resulted in a significant reduction in blood glucose, glucose-6-phosphatase and fructose-1,6-bisphosphatase and an increase in the activity of liver glucokinase. Also, working with *Boerhaavia diffusa* (BLE) (Pari and Satheesh, 2004), have shown that the activity of the hepatic enzymes such as glucokinase was significantly increased and glucose-6-phosphatase, fructose 1,6-bisphosphatase were significantly decreased in diabetic rats.

On the contrary, glucose-6-phosphatase activity has been enormously elevated in diabetic rats. But in the other groups, it was found to be within normal limits.

Treating the diabetic rats with *Basella rubra* has been found to be beneficial as all the three carbohydrate-metabolizing enzymes assessed were found to be on par with the non-diabetic controls. This has proved that *Basella rubra* is an antidiabetic medicinal plant. Glucose-6-phosphatase activity in pancreatic islets could therefore be an important factor in the control of glucose metabolism and consequently of glucose dependent insulin secretion (Petrolonis *et al.*, 2004).

4.2.6 Urea, creatinine and bilirubin in the experimental rats

The levels of urea, creatinine and bilirubin in serum of the experimental rats treated with *Basella rubra* is depicted in Table X.

Table X

UREA, CREATININE AND BILIRUBIN LEVELS IN SERUM OF THE EXPERIMENTAL RATS

Group	Treatment	Urea (mg/dl)	Creatinine (mg/dl)	Bilirubin (mg/dl)	
				Total	Direct
I	Non-diabetic control	33.55 ± 3.14[a]	0.87 ± 0.03[a]	0.93 ± 0.055[a]	0.33 ± 0.043[a]
II	Diabetic control	74.30 ± 3.92[b]	1.03 ± 0.28[b]	1.62 ± 0.064[c]	0.47 ± 0.067[b]
III	Non-diabetic + *Basella rubra*	30.45 ± 3.04[a]	0.81 ± 0.06[a]	0.90 ± 0.056[b]	0.32 ± 0.040[a]
IV	Diabetic + *Basella rubra*	35.08 ± 4.72[a]	0.80 ± 0.06[a]	0.94 ± 0.047[a]	0.31 ± 0.057[a]
$CD_{0.05}$		4.98	0.19	0.01	0.3

Values are mean ± SD

Values with different superscripts differ significantly ($P<0.05$)

Urea, creatinine and bilirubin have been increased in diabetic rats. This condition was reversed on treatment with *Basella rubra.*

Bolkent *et al.* (2004) have also found that *Aloe vera* leaf gel and pulp extracts decreased the urea and creatinine level in diabetic rat to near normal values. Administration of Dianex to diabetic rats by Mutalik *et al.* (2003) caused reduction in the elevated levels of creatinine and urea in diabetic rats.

Bilirubin in the serum is used as an indicator of hepatic and biliary function. Data published on bilirubin levels in diabetes are contradictory; there are reports of lower, higher (Mansour *et al.*, 2002) and unchanged (Watkins and Sherman, 1991) serum level in both animal and human diabetes.

In the present study, serum bilirubin level had been significantly elevated in diabetic rats. This was brought to the normal level on administration of *Basella rubra* revealing that this medicinal herb is also hepatoprotective.

4.2.7 Activities of the enzymes, aspartate transaminase, alanine transaminase and alkaline phosphatase

The levels of enzymes such as AST, ALT and ALP in serum of the control and experimental rats treated with *Basella rubra* is depicted in Table XI.

Table XI

SERUM LEVELS OF AST, ALT AND ALP IN THE RATS

Group	Treatment	AST (IU/L)	ALT (IU/L)	ALP (IU/L)
I	Non-diabetic control	54.1 ± 4.09^{a}	29.0 ± 2.58^{a}	195.5 ± 4.92^{a}
II	Diabetic control	78.01 ± 3.86^{c}	48.3 ± 5.34^{b}	254.5 ± 4.36^{b}
III	Non-diabetic + *Basella rubra*	58.3 ± 4.22^{a}	30.0 ± 4.65^{a}	194.7 ± 4.37^{a}
IV	Diabetic + *Basella rubra*	$60.3 \pm 3.85^{a,b}$	33.3 ± 3.77^{a}	192.5 ± 6.27^{a}
$CD_{0.05}$		5.3	5.6	6.7

Values are mean ± SD

Values with different superscripts differ significantly ($P<0.05$)

1 unit AST= µm oxaloaceticacid liberated/min/L serum
ALT= µm pyruvate liberated/min/L serum
ALP= mm p-nitrophenol liberated/ min/L serum

The levels of AST, ALT and ALP were found to be significantly ($P<0.05$) higher in the diabetic rats than that of the control rats. But in the diabetic rats treated with *Basella*, the levels of these enzymes were found to be decreased.

Mori *et al.* (2003) have reported that raised levels of enzymic activity (AST, ALT and ALP) were observed in the alloxan-induced diabetic rats as well as in diabetic patients.

Such alteration of transaminase activity in the tissues is explicable in terms of energy metabolism as these enzymes play a role in gluconeogenesis. In diabetes, the stores of glycogen in the liver and muscles are diminished and in compensation, levels of

AST and ALT are raised to produce alternative glucose precursors. Administration of insulin stimulates alanine production in muscle and liver tissue, leading to the restoration of near normal levels of these transaminases (Salmuddin *et al.*, 1996).

Hence the decrease in the activity of these enzymes in the diabetic rats administered with *Basella* shows that it is effective in reverting the activity of these enzyme to near normal values.

4.2.8 Total protein albumin and albumin / globulin ratio

Table XII depicts the levels of protein, albumin, globulin and A/G ratio in the control and experimental rats.

Table XII

TOTAL PROTEIN, ALBUMIN AND ALBUMIN/GLOBULIN RATIO IN THE CONTROL AND EXPERIMENTAL RATS

Group	Treatment	Total protein (mg/dl)	Albumin (mg/dl)	Globulin (mg/dl)	A/G ratio
I	Non-diabetic control	7.2 ± 0.3^{a}	4.4 ± 0.39^{a}	2.8 ± 0.15^{a}	1.57 ± 0.018^{a}
II	Diabetic control	6.0 ± 0.2^{c}	2.8 ± 0.43^{b}	3.2 ± 0.23^{c}	0.87 ± 0.041^{c}
III	Non-diabetic + *Basella rubra*	7.5 ± 0.2^{b}	4.4 ± 0.44^{a}	3.1 ± 0.23^{b}	1.41 ± 0.046^{b}
IV	Diabetic + *Basella rubra*	7.0 ± 0.1^{a}	4.3 ± 0.32^{a}	2.7 ± 0.33^{a}	1.59 ± 0.048^{a}
$CD_{0.05}$		0.24	0.52	1.98	0.04

Values are mean ± SD

Values with different superscripts differ significantly ($P<0.05$)

The level of protein, albumin, globulin and A/G ratio in all the samples tested was found to be reduced in diabetic rats when compared to control rats. The lowered levels of albumin, globulin and A/G ratio were found to be increased to normal levels in diabetic rats treated with *Basella* (Group III and IV).

Rasch and Mogensen, (1980) have reported that the plasma A/G ratio level was less in diabetes than in the normal controls. Our results are also similar to that of

Palanivel *et al.* (2001) who have reported that the level of protein and albumin/globulin ratio was lower in the STZ induced diabetic rats than in the normal control rats. Increased protein catabolism in diabetes might have induced a direct adverse effect on the synthesis and secretion of albumin.

4.2.9 Lipid profile

The levels of lipids assessed in the different groups of rats are presented in Table XIII.

Table XIII

SERUM LIPID LEVELS IN THE EXPERIMENTAL RATS

Group	Treatment	Total cholesterol (mg/dl)	HDL cholesterol (mg/dl)	LDL cholesterol (mg/dl)	VLDL cholesterol (mg/dl)	Triglycerides (mg/dl)	Phospholipids (mg/g liver)
I	Non-diabetic control	103.3 ± 3.8 [a]	26.55 ± 4.08 [a]	59.69 ± 3.84 [a]	17.13 ± 2.34 [a]	85.65 ± 3.30 [a]	192.8 ± 3.97 [a]
II	Diabetic control	137.0 ± 4.1 [b]	12.86 ± 3.70 [b]	91.61 ± 4.33 [b]	32.53 ± 2.66 [c]	162.65 ± 2.65 [c]	230.35 ± 3.56 [c]
III	Non-diabetic + *Basella rubra*	100.0 ± 3.3 [a]	25.98 ± 3.68 [a]	57.12 ± 4.40 [a]	16.74 ± 3.38 [a]	83.71 ± 4.77 [a]	190.8 ± 3.65 [a]
IV	Diabetic + *Basella rubra*	106.0 ± 3.7 [a]	26.56 ± 3.58 [a]	59.40 ± 3.60 [a]	20.01 ± 2.77 [b]	120.06 ± 3.76 [b]	200.9 ± 3.68 [b]
CD (0.05)		14.73	4.99	5.44	1.51	4.8	6.7

Values are mean ± SD

Values with different superscripts differ significantly ($P<0.05$)

A significant increase in the levels of serum cholesterol, VLDL cholesterol, LDL cholesterol, triglycerides and phospholipids was found in diabetic rats compared to the non-diabetic controls treated and untreated with *Basella rubra*. However, serum HDL cholesterol concentrations were significantly ($P<0.05$) lower in diabetic rats. Concentration of all these parameters was reversed in the diabetic rats on administration with *Basella rubra*.

Our results are in accordance with Mary *et al.* (2002) who have reported that herbal formulation; liposem treatment resulted in a significant dose dependent reduction in total lipids, LDL and VLDL cholosterol and phospholipids with a concomitant rise in HDL-cholesterol in diabetic rats.

Halim (2003) showed that the treatment of diabetic rats with water extract of *A. augusta* and *A. indica* for 8 weeks improved serum lipid profile, while HDL cholesterol was unaffected.

Kameswararao *et al.* (2003) reported that serum cholesterol, triglyceride, LDL and VLDL cholesterol levels were significantly higher in diabetic rats compared to those in normal rats, while HDL cholesterol level was decreased in the diabetic rats. On treatment with *Momordica cymbalaria* to the diabetic rats, a significant reduction in serum total cholesterol, LDL cholesterol and VLDL cholesterol and triglyceride and a significant increase in HDL cholesterol were observed. The root of *Withania somnifera* has been shown to be a potential source of hypocholesterolemic and hypoglycemic agents (Andallu and Radhika, 2000).

Basella rubra has also been found to have antihyperlipidemic effect in this study.

4.2.10. Assessment of antioxidants and extent of inhibition of *in vitro* lipid peroxidation

The levels of enzymic and non-enzymic antioxidants and the extent of inhibition of *in vitro* lipid peroxidation in liver of the rats are given in Table XIV.

Table XIV

LEVELS OF ENZYMIC AND NON-ENZYMIC ANTIOXIDANTS AND THE EXTENT OF INHIBITION OF *IN VITRO* LIPID PEROXIDATION IN LIVER OF THE RATS.

Group	Treatment	SOD (U/mg protein)	CAT (U/mg protein)	GPx (U/mg protein/min)	Vitamin E (mg/g)	Vitamin C (µg/g)	Reduced glutathione (nanomoles/g tissue)	TBARS (nanomoles/ 100g tissue)
I	Non-diabetic control	6.48 ± 1.26^{a}	75.0 ± 2.94^{a}	8.6 ± 0.39^{a}	0.37 ± 0.053^{a}	0.08 ± 0.028^{a}	8.67 ± 2.58^{a}	0.70 ± 0.20^{a}
II	Diabetic control	2.92 ± 0.11^{b}	34.98 ± 4.46^{c}	4.7 ± 0.48^{b}	0.18 ± 0.028^{c}	0.05 ± 0.029^{b}	5.03 ± 1.28^{b}	1.94 ± 0.17^{b}
III	Non-diabetic + *Basella rubra*	6.96 ± 0.99^{a}	73.90 ± 3.19^{a}	9.1 ± 1.95^{a}	0.53 ± 0.039^{b}	0.08 ± 0.031^{a}	8.77 ± 2.28^{a}	0.68 ± 0.04^{a}
IV	Diabetic + *Basella rubra*	6.08 ± 0.85^{a}	62.98 ± 2.96^{b}	8.7 ± 0.25^{a}	0.51 ± 0.030^{b}	0.07 ± 0.029^{a}	8.25 ± 2.30^{a}	0.77 ± 0.06^{a}
$CD_{(0.05)}$		1.20	4.55	1.36	0.11	0.04	2.85	0.18

Values are mean ± SD
Values with different superscripts are significantly different ($P<0.05$)
SOD (U/mg protein): amount of SOD that causes 50% reduction in the extent of NBT oxidation
CAT (U/mg protein): amount of enzyme that brings about a decrease in absorbance of 0.05 at 240 nm
GPx (U/mg protein): µmoles of NADH oxidized/mg protein/min

The enzymic antioxidants SOD, CAT, GPx levels and non-enzymic antioxidants (Vitamin E, Vitamin C, reduced glutathione (GSH)) were lower in diabetic rats compared to that of the control rats (Groups I and Group III). These enzymic and non-enzymic antioxidants levels in diabetic rats treated with *Basella rubra* significantly ($P< 0.05$) increased to a level closer to the normal values.

Scavenging enzymes like SOD, CAT and GPx metabolize the free radicals formed during normal conditions to harmless intermediates. A change in this normal process in diabetes leads to overproduction of superoxide ion and hydrogen peroxide, which in turn form the harmful hydroxyl radicals. Similar to the finding in this study, a decrease has been observed in the activity of SOD, CAT and GPx in some of the tissues in diabetic rats by Sekar and Govindasamy (1990).

Kumari and Augusti (2002) assessed the activities of SOD and CAT in the liver and heart of normal, diabetic and treated diabetic rats. They too found these enzymes to be significantly low in liver and heart of the diabetic control rats ($P<0.001$).

Illing *et al.* (1991) opines that reduced GPx activity in diabetic condition, may be due to inactivation of the enzyme involved in disposal of O_2 species and also insufficient availability of GSH.

CAT, GPx, SOD level in erythrocytes was found to be low in alloxan-treated diabetic rats when compared with normal rats. Diabetic rats treated with 75% methanolic extracts of *A. marmelos* significantly ($P< 0.001$) increased the levels of SOD, CAT and GPx (Sabu and Kuttan, 2004). Erikksson and Borg (1991) have also shown that the activities of SOD, CAT and GPx got lowered in STZ-diabetic rats, on administration of *Aegle marmelos* for 30 days.

Decreased GSH concentration contributes to the pathogenesis of complications associated with the chronic diabetic state (Meister, 1983).

Reduced glutathione synthesized mainly in the liver is an important non-enzymic antioxidant in the antioxidative defence system. Kaplowitz *et al* Sokhtens (1985) have remarked that the marked depletion of GSH observed in the tissues of alloxan-diabetic rats, may be due to the utilization of this compound by the two antioxidant enzymes GPx and GST as their substrate.

Vitamin C at high doses has been shown to reduce the accumulation of sorbitol in the erythrocytes of diabetics and to inhibit the glycosylation of proteins (Davie *et al.*, 1992).

Transport of Vitamin C into cell is facilitated by insulin. Many diabetics do not have enough intracellular Vitamin C. Therefore, a relative Vitamin C deficiency exists in many diabetics despite adequate dietary consumption (Cunningham, 1991).

Lipid peroxidation is a free radical induced process leading to oxidative deterioration of polyunsaturated fatty acid. Increased lipid peroxidation under diabetic conditions may be due to increased oxidative stress in the cells as a result of the depletion of antioxidants scavenger systems (Krishnakumar *et al.*, 1999).

From the table, it is inferred that lipid peroxidation in diabetic rats was higher than in the control rats. The diabetic rats treated with *Basella rubra* significantly ($P< 0.05$) reduced lipid peroxidation.

Our results are similar to those obtained by Shanthakumari *et al.* (2003) who have reported significant elevation in plasma and tissue TBARS in diabetic rats when compared with control rats. Oral administration of *P. betle* (150 mg/kg weight) for 30 days showed significant reduction in TBARS in diabetic rats. Earlier, there have been many reports documenting elevated serum lipid peroxide level and diminished antioxidant status in diabetic subjects (Bukan *et al.*, 2003).

The inhibition of antioxidant enzymes in diabetes results in over production of reactive oxygen species that might lead to the accumulation of lipid peroxide products. The increased level of TBARS is an index of lipid peroxidation. The increased levels of LPO in diabetic rats indicate the degenerative status in diabetes.

Basella rubra has facilitated the scavenging of free radicals in diabetic rats by the elevation of SOD, CAT, GPx. *Basella rubra* being rich in Vitamin C can effectively reduce the formation and accumulation of sorbitol and thereby the secondary complications in diabetes. Also, the dehydroascorbate, which converts the oxidized glutathione to reduced glutathione helps in maintaining the GSH level in the treated diabetics.

The harmful effects of lipid peroxides in diabetes can be drastically reduced by the administration of *Basella rubra*, which is an excellent source of antioxidants.

4.2.11 Histopathological study

A. Liver

Group

I Non-diabetic control

Sections studied show structure of liver. The hepatocytes are seen as cords radiating from the central veins. The portal tracts are composed of portal vein radicle, hepatic artery radicle and bile duct radicle. The central veins appear normal. No pathological changes are observed – **Normal histology.**

II Diabetic control

Sections studied show structure of liver. The hepatocytes show global microvesicular steatosis. The portal tracts appear normal and the central veins appear congested – **Global microvesicular steatosis.**

III Non-diabetic + *Basella rubra*

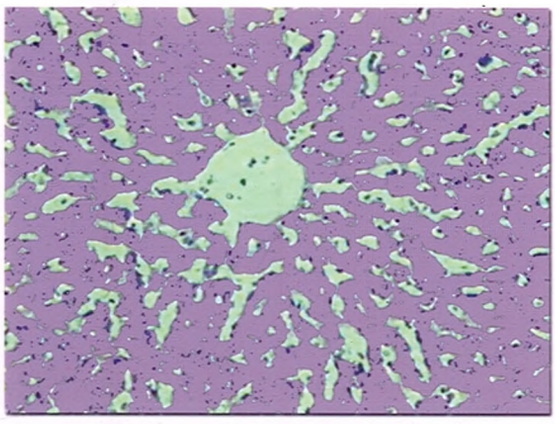
Sections studies show structure of liver. The hepatocytes, portal tracts and central veins appear normal. No pathological changes are observed – **Normal histology.**

IV Diabetic + *Basella rubra*

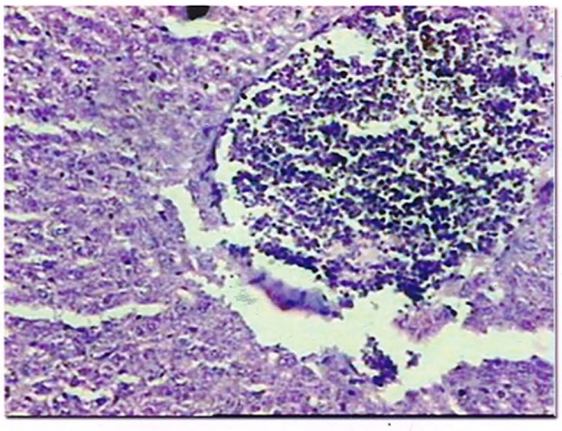
Sections studied show structure of liver. The hepatocytes, portal tracts and central veins appear normal. No steatosis is observed – **Normal histology.**

PLATE - I

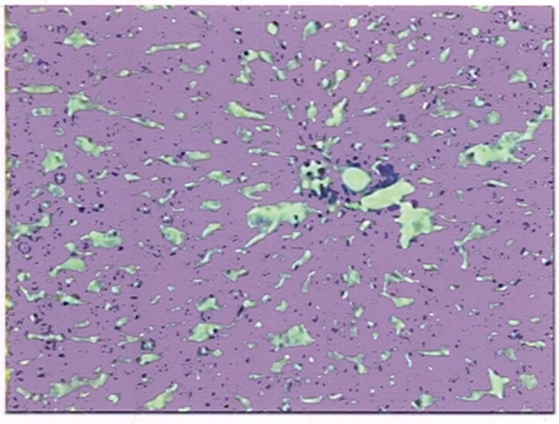
LIVER SECTIONS OF THE RATS



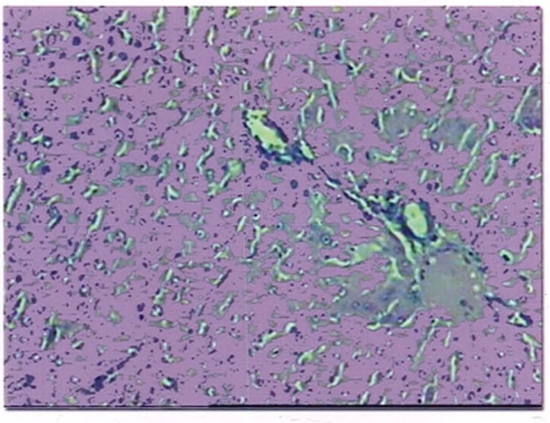
Non diabetic control



Diabetic control



Non diabetic + *Basella rubra*



Diabetic + *Basella rubra*

B. Pancreas

Group

I Non-diabetic control

Sections studied show predominant exocrine pancreatic tissue composed of acini with draining ductules. The endocrine component is found as scattered nodules within the substance of the exocrine pancreas. No pathological changes are observed – **Normal histology.**

II Diabetic control

Sections studied show predominant exocrine pancreatic tissue composed of acini with draining ductules. The endocrine component is found as scattered nodules within the substance of the exocrine pancreas within and around which there is focal mild infiltration by mononuclear cells, of which lymphocytes predominate – **Insulitis.**

III Non-diabetic + *Basella rubra*

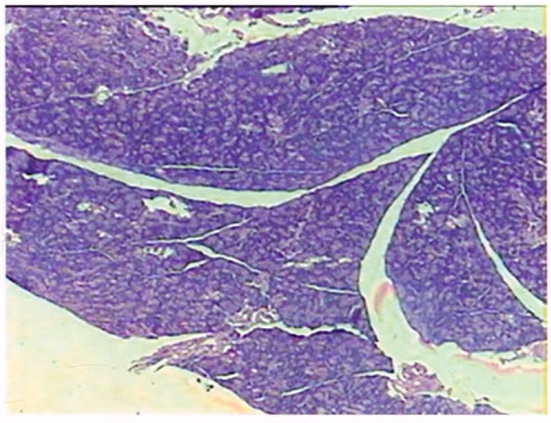
Sections studied show predominant exocrine pancreatic tissue composed of acini with draining ductules. The endocrine component is found as scattered nodules within the substance of the exocrine pancreas. No pathological changes are observed – **Normal histology.**

IV Diabetic + *Basella rubra*

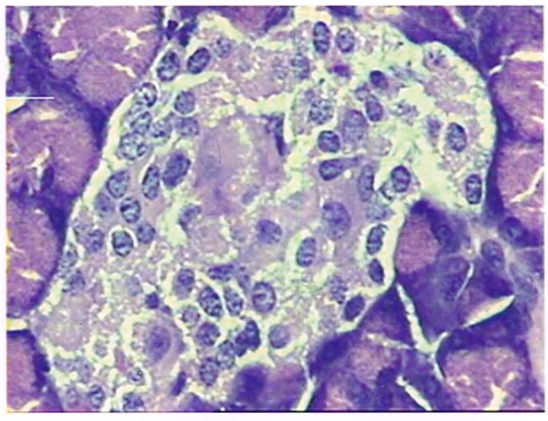
Sections studied show predominant exocrine pancreatic tissue composed of acini with draining ductules. The endocrine component is found as scattered nodules within the substance of the exocrine pancreas. No insulitis is observed – **Normal histology.**

PLATE - II

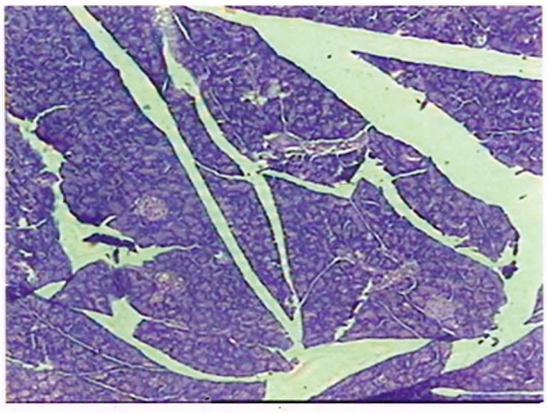
PANCREAS SECTIONS OF THE RATS



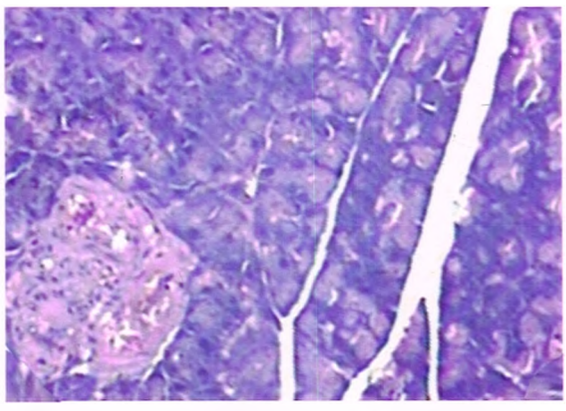
Non diabetic control



Diabetic control



Non diabetic + *Basella rubra*



Diabetic + *Basella rubra*

C. Kidney

Group

I Non-diabetic control

Sections show structure of kidney. The glomeruli, tubules, blood vessels and intestitium appear normal. No pathological changes are observed – **Normal histology.**

II Diabetic control

Sections show structure of kidney. The glomeruli, tubules, blood vessels and intestitium appear normal. No pathological changes are observed – **Normal histology.**

III Non-diabetic + *Basella rubra*

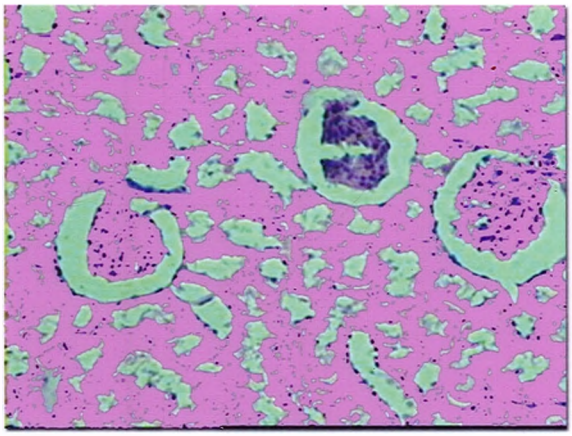
Sections show structure of kidney. The glomeruli, tubules, blood vessels and intestitium appear normal. No pathological changes are observed – **Normal histology.**

IV Diabetic + *Basella rubra*

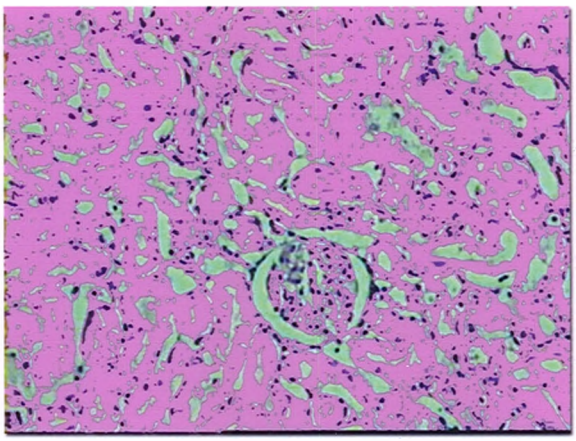
Sections show structure of kidney. The glomeruli, tubules, blood vessels and intestitium appear normal. No pathological changes are observed – **Normal histology.**

PLATE - III

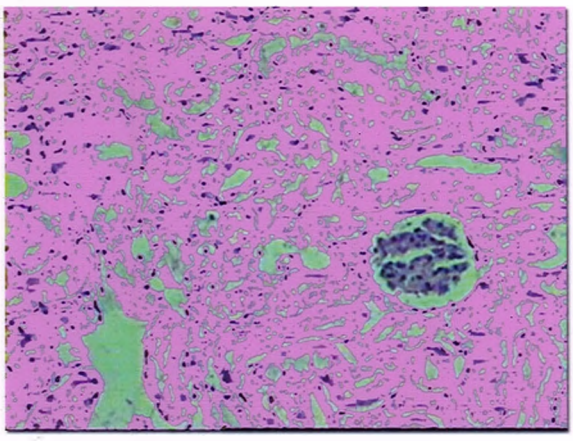
KIDNEY SECTIONS OF THE RATS



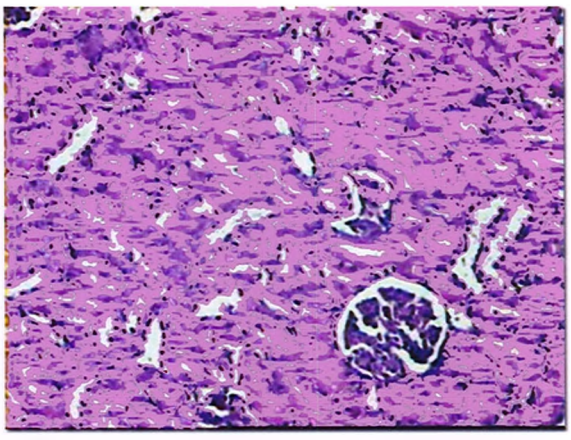
Non diabetic control



Diabetic control



Non diabetic + *Basella rubra*



Diabetic + *Basella rubra*

The histological study of the liver, pancreas and kidney of the different groups of rats revealed that no abnormality in the structure of these organs in the non-diabetic control rats treated/not treated with *Basella rubra*. It is evident that the leaves of *Basella rubra* are safe for medicinal use.

Insulitis was observed in the pancreas and global microvascular steatosis in the liver of the diabetic rats. But structure of kidney was of normal histology.

Treatment of the diabetic rats with *Basella rubra* ameliorated the STZ-induced diabetes in the pancreas and liver. This again reinforces the finding that *Basella rubra* is effective in the treatment of diabetes mellitus.

SUMMARY AND CONCLUSION

5. SUMMARY AND CONCLUSION

Basella rubra is an important medicinal plant. According to the ethno botanical data, *Basella rubra* possesses hypoglycemic effect, which has never been experimentally demonstrated and hence its hypoglycemic potential remains untapped. Therefore, it was considered worthwhile to undertake a study to evaluate the hypoglycemic effect of *Basella rubra* in streptozotocin induced hypoglycemic rats. Summary and conclusion of the findings of this study are given in this chapter.

Analysis of *Basella rubra* for nutrients showed that appreciable amounts of protein, fibre content, minerals such as calcium, phosphorus, iron, potassium, sodium and chromium and vitamins, thiamine and riboflavin are present in it.

Basella rubra was also found to be a good source of both enzymic and non-enzymic antioxidants. The activity of the enzymic antioxidants such as catalase and peroxidase were higher than superoxide dismutase. The non-enzymic antioxidants like ascorbic acid, α-tocopherol, flavonoids, polyphenols and tannins were also present in higher amounts. The high levels of both the enzymic and non-enzymic antioxidants indicate that *Basella rubra* has excellent free radical scavenging activity.

Protein, fibre, chromium, potassium, thiamine, riboflavin and antioxidants (enzymic, non-enzymic) in *Basella rubra* are found in rich amounts. Besides this, the extent of inhibition of *in vitro* lipid peroxidation and superoxide and nitric oxide scavenging activity makes *Basella rubra* suitable for treating diabetes mellitus.

In diabetic rats administered with *Basella rubra* loss in body weight was prevented. The present study clearly proves that the treatment of diabetic rats with *Basella rubra* showed significantly decreased levels of blood glucose, total cholesterol, triglycerides, VLDL, LDL cholesterol and lipid peroxidation when compared to diabetic untreated rats and increase in liver glycogen, fructosamine and HDL cholesterol levels were also observed. The levels of carbohydrate metabolizing enzymes such as glucokinase and glucose-6-phosphate dehydrogenase were found to be lower in diabetic rats when compared to rats supplemented with *Basella rubra.*

In diabetic rats, increased levels of urea, creatinine and bilirubin (total, direct) were observed. The activities of AST, ALT and ALP were also found to be increased in rats induced with streptozotocin. But their levels were decreased significantly to near normal in *Basella rubra* treated rats.

Decreased levels of total protein, albumin, globulin, and A/G ratio were recorded in diabetic rats. On treatment with *Basella rubra,* their levels were significantly increased.

Enzymic (SOD, CAT, GPx) and non-enzymic (α-tocopherol, vitamin C, reduced glutathione) antioxidants were found to be significantly reduced in the streptozotocin induced diabetic rats and a higher percentage for lipid peroxidation was noted. Concentrations of all these parameters were found to be reversed to near normal in the diabetic rats administered with *Basella rubra.*

Histopathological examination also revealed the effect of *Basella rubra* in reversing the degeneration of the tissue.

Hence, to conclude *Basella rubra*, which is used as a green leafy vegetable, can also be used to manage hyperglycemia and hyperlipidemia.

REFERENCES

REFERENCES

Acharya, K., Samui, K., Rai, M., Dutta, B. and Acharya, R. (2003), Antioxidants and nitric oxide synthase activation properties of *Auricularia auricula*, INDIAN J EXP BIOL, 42: 538.

Ali, M., Thomson, M. and Afazal, M. (2000), Garlic and onions: their effect on eicosanoid metabolism and its clinical relevance, Prostaglandins, Leukot Essent. Fatty acid ,62:55.

American Diabetes Association (2002a), Gestational Diabetes Mellitus, Diabetes Care, 25: S94-S96.

American Diabetes Association (2002b), Diabetic Retinopathy, Diabetes Care, 25: S90-S93.

American Diabetes Association (2002c), Diabetic Nephropathy, Diabetes Care, 25: S85-S89.

American Diabetes Association (2004a), Diagnosis and Classification of Diabetes mellitus, Diabetes Care, 27 (Supp 1): S5-S10.

American Diabetes Association (2004b), Gestational Diabetes Mellitus, Diabetes Care, 27: S88-S90.

American Diabetes Association (2004c), Nephropathy in Diabetes, Diabetes Care, 27: S79-S83.

American Diabetes Association, National Institute of Diabetes and Digestive and Kidney disease., (2004), Prevention or delay of type 2 diabetes, Diabetes Care, 27: S47.

Andallu, B. and Radhika, B. (2000), Hypoglycemic, diuretic and hypocholesterolemic effect of Winter Cherry (*Withania somnifera*, Dunal) root, INDIAN J EXP BIOL, 38: 607-609.

Anderson, R.A. (1992), Chromium, glucose tolerance and diabetes, Biological Trace Element Research, 32: 19-24.

Archana, S., Rashmi, N. and Khemani, L.D. (2001), Hypoglycemic effect of *Hibiscus rosasinensis L.* leaf extract in glucose and streptozotocin-induced hyperglycemic rats. INDIAN J EXP BIOL, 39: 284-286.

Arivazhagan, P., Thilagavathy, T. and Panneerselvam, C. (2000), Antioxidant lipoate and tissue antioxidants in aged rats, J. Nutr. Biochem., 11: 122-7.

Armbruster, D.A. (1987), Fructosamine: Structure, analysis and clinical usefulness, Clin. Chem., 33: 2153-2163.

Babu, Y., Gangadevi, T. and Subramaniam, A. (2003), Antidiabetic activity of ethanol extract of *Cassia kleinii* in streptozotocin-induced diabetic rats and isolation of an active fraction and toxicity evaluation of the extract, Indian J Pharmacol, 35: 290-296.

Bagchi, D., Garg, A., Krohn, R.L., Bagchi, M., Bagchi, D.J., Balmoori, J. and Stohs, S.J. (1998), Protective effect of grape seed proanthocyanidins and selected antioxidants against TPA-induced hepatic and brain lipid peroxidation and DNA fragmentation and peritoneal macrophage activation in mice, Gen Pharmacol., 30: 771.

Bailey, C.J. and Flatt, P.R. (1986), Animal models of diabetes in Nattrass (ed.): Recent Advances in Diabetes, Edinburgh, UK, Churchill Livingstone, 2: 71-89.

Baker, H., Frank, O., Angelis, B. and Feingold, S. (1951), Plasma tocopherol in man at various times after ingesting free or acetylated tocopherol, Nutr Rep. Inv., 21, 531-536.

Barlow-Walden, L.R., Reiter, R.J., Abe, M., Pablos, M.I., Chen, L. and Doeggeler, B. (1995), Melatonin stimulates brain glutathione peroxidase activity, Neurochem Int., 266: 497.

Baskaran, K., Ahamath,B.K., Shanmugasundaram.K.R. and Shanmugasundram,E.R.B. (1990), Antidiabetic effect of a leaf extract from *Gymnema sylvestre* in non-isulin dependent diabetes mellitus patients, J Ethnopharmacol, 30: 295-305.

Beutler, E. (1984), Red Cell Metabolism- A Manual of Biochemical Methods, 3rd edition, Grune and Stralton Oriando, 21: 11-20.

Bolkent, S., Akev, N., Ozsoy, N., Inceli, M.S., Can, A., Okyar, A. and Ya ardag, R. (2004), Effect of *Aloe vera* (L.) Burn Fil. Leaf gel and pulp extracts on kidney in type II diabetic rat models, INDIAN J EXP BIOL, 42: 48-52

Bordia, A. (1997), Effect of ginger (*Zingiber officinale* Rosc) and fenugreek (*Trigonella foenum graecum* L) on blood lipids, blood sugar and platelet aggregation in patients with coronary artery disease, Prost. Leuko. EFA., 56: 379-384.

Boynes, J.W. (1991), Role of oxidative stress in development of complication in diabetes, Diabetes, 40: 405-411.

Brandstrup, N., Kirk, J.E. and Bruni, C. (1957), Determination of hexokinase in tissues, J. Gerontol., 12: 166-171.

Brown, K.K. (1999), Diabetes, 48:1415.

Bucolobe, B. and David, H. (1973), Clin. Chem., 19: 476

Bukan, N., Sancak, B., Yayuz, O., Koca, C., Tutkun, F., Ozcelikay, T.A. and Altan, N. (2003), Lipid peroxidation and scavenging enzyme levels in the liver of streptozotocin- induced diabetic rats, INDIAN J. BIOCHEM. BIOPHYS., 40: 447-450.

Cameron, G.R., Milton, R.F., and Allen, J.W. (1943), Estimation of flavonoids, Lancet, 179.

Cartmell, M.T. and Kingsworth, A.N. (2000), Acute pancreatitis, Hosp. Med., 61: 383-385.

Chen, C.Y., Holtzman, G.I. and Bakhit, R.M. (2002), High genistein isoflavone supplementation modulated erythrocyte antioxidant enzymes and increased running pakistan endurance in rats undergoing one session of exhausting exercise, A pilot study, J. Nutr., 1:1.

Cherrington, A.D. (1999), Control of glucose uptake and release by the liver *in vivo*, Diabetes, 18: 1198-1214.

Chiu, C.K., Martinez, S.D., Yoon, C. and Chuang, L. (2002), Relative contribution of insulin sensitivity and β-cell function to plasma glucose and insulin concentration during the oral glucose tolerance test, Metabolism, 51 (1): 115-120.

Chowdhury, S, and Bhattacharya, B. (2003), Role of chromium in human metabolism, with special reference to type 2 diabetes, Science and Culture, 69, (3-4): 135-138.

Clark, E.P. and Collip, J.B. (1925), Biochem, 63: 461-463.

Clermont, A. C. (1998), Invest Opthamol. Vis Sci., 39 (suppl4): S1000.

Clore, N.J., Alred, J., White, D., Li, J. and Stillman, J. (2002), The role of plasma fatty acid composition in endogenous glucose production in patients with type 2 diabetes mellitus, Metabolism, 51(11): 1471-1477.

Cogan, D.G., Kinoshita, J.H. and Kador, P.F. (1984), Aldose reductase and complications of diabetes, Ann Int Med., 101: 82-91.

Coppey, J.L., Gellett, S.J., Davidson, P.E., Dunlap, A.J., Lund, D.D.and Yorek, A.M. (2000), Effect of antioxidant treatment of STZ induced diabetic rats on endoneural blood flow, motor nerve conduction velocity and vascular reactivity of epineural arterioles of the sciatic nerve, Diabetes, 50: 1927-1937

Culling, C.F.A. (1979), Handbook of Histopathological and Histochemical Techniques, III edition, New York, Butterworth and Co. Ltd., 7.

Cunningham, J. (1991), Reduced mononuclear leucocyte ascorbic acid content in adults with insulin-dependent diabetes mellitus consuming adequate dietary Vitamin C, Metabolism, 40: 149-9.

David, M.N. (1996), and The pathophysiology of diabetic complication: How much does the glucose hypothesis explain? Ann Intern Medicine, 174 (1pt): 286-289.

Davie, S.J., Gould, B.J. and Yudkim, J.S. (1992), Effect of Vitamin C on glycosylation of proteins, Diabetes, 41: 167-73.

Devasagayam, T.P.A. and Sainis, K.B. (2002), Immune system and antioxidants, especially those derived from Indian medicinal plants, INDIAN J EXP BIOL, 40:639-655.

Devasagayam, T.P.A., Boloor, K.K. and Ramasarma, T. (2003), Methods for estimating lipid peroxidation: Analysis of merits and demerits, INDIAN J. BIOCHEM. BIOPHYS., 40: 300-308.

Dominguez, J.H. (2000), Kidney International, 57:92-104

Drabkin, D.L. and Austin, J.M. (1932), Spectrophotometric constants for common haemoglobin derivatives in human, dog and rabbit blood, J. Bio. Chem., 98: 719-733.

Eddouks, M., Lemhadri, A. and Michel, J.B. (2004), Caraway and Caper: Potential antihyperglycemic plants in diabetic rats, J Ethnopharmacol, 93 (2-3): 261-264.

Eddouks, M. and Maghrani, M. (2004), Phlorizin like effect of *Fraxinus excelsior* in normal and diabetic rats, J Ethnopharmacol, 94 (1): 149-159.

Erikksson, U.J. and Borg, L.A.H. (1991), Protection by free oxygen radical scavenging enzymes against glucose-induced embryonic malformation *in vitro*, Diabetes, 34: 1014.

Eshrat, H.M. (2003), Effect of *Coccinia indica* (L) and *Abroma augusta* (L) on glycemia, lipid profile and indicators of end organ damage in streptozotocin induced diabetic rats, Indian Journal of Clinical Biochemistry, 18 (2): 54-63.

Esterbauer, H., Dieber-rotheneder, M., Patel, H., Waeg, G. and Striegl, G. (1977), Effect of oral supplementation with D alpha- tocopherol on the vitamin E content of human low density lipoproteins and resistance to oxidation, J. Lipid Res., 32: 1325-1232.

Evans, P. and Halliwell, B. (1999), Free radicals and Hearing, Ann NY Acad Sci., 884: 19.

Flegg, H.M. (1972), Ann. Clin. Biochem., 10: 79.

Fong, S.D., Alello, L.,\ Gardner, W.T., King, L.G., Blankenship, G., Cavallerano, D.J., Ferris, L.F. and Klein, R. (2004), Retinopathy in diabetes, Diabetes Care, 27: S84-S87.

Frei, B. (1994), Reactive oxygen species and antioxidant vitamins : mechanism of action, Am J Med., 97(suppl 3A): 5S

Fossati, P. and Principe, L. (1982), Clin. Chem., 28: 2077.

Garg, M.C., Ojha, S. and Bansal, D.D. (1996), Antioxidant status of streptozotocin diabetic rats, Indian J Exp Biol., 34 (3); 264 – 266.

Geiss, L. (1997), Diabetes surveillance, Atlanta Ga, U.S. Department of health and human services, 1997-1998.

Gerich, J.E. (1998), The genetic basis of type 2 diabetes mellitus: Impaired insulin secretion versus impaired insulin sensitivity, Endocr Rev., 19: 491-503.

Ghosal, S. (2002), Herbal health-products in global perspective, Science and culture, 68: 33-40.

Good, C.A., Krames, H. and Somogy, M. (1933), Chemical procedure for analysis of polysaccharides, Meth. Enzymol., 8: 34.

Govindarajan, R., Vijayakumar, M., Rawart, A.K.S. and Mehrotra, S. (2003), Free radical scavenging potential of *Picorrhiza kurrooa Royle ex Benth*, INDIAN J EXP BIOL,41: 875-79.

Green, M.J. and Hill, H.A.O. (1984), Chemistry of dioxygen, Methods Enzymol., 105

Grover, J.K. and Yadav, S.P. (2004), Pharmacological actions and potential uses of *Momordica charantia*: A review, J Ethnopharmacol, 93: 123-132.

Grover, J.K., Yadav, S. and Vats, V. (2002), Medicinal plants of India with antidiabetic potential, J Ethnopharmacol, 81: 81-100.

Guillgusseau, P.J. (1994), Diabetes metabolism, 20: 219-228.

Gutteridge, J.M.C. and Halliwell, B. (1990), Role of oxygen free radicals and catalytic metal ions in human disease: An overview, Methods in Enzymology, 186: 1.

Ha, H. and Kim, K.H. (1999), Diabetes Res. Clin. Pract., 45: 147-151.

Haddad, S.P., Depot, M., Seltaf, A. and Cherrah, Y. (2001), Use of antidiabetic plants in Morocco and Quebec, Diabetes Care, 24: 608-609.

Halleux, C.M., Servais, I. and Reul, B.A. (1998), Multi hormonal control on gene expression and their secretion from cultured human visceral adipose tissue, increased responsiveness to glucocorticoids in obesity, Clinical Endocrinol. Meta., 83: 902-910.

Halim, E.M. (2003), Lowering of blood sugar by water extract of *Azadirachta indica* and *Abroma augusta* in diabetic rats, INDIAN J EXP BIOL, 41:636-640.

Halliwell, B. and Aruoma, O.I. (1991), DNA damage by oxygen derived species. Its mechanisms and measurement in mammalian system, FEBS Lett., 281: 9.

Hamdan, I. and Afifi, F.U. (2004), Studies on the *in vitro* and *in vivo* hypoglycemic activity of some medicinal plants used in treatment of diabetes in Jordanian traditional medicine, J Ethnopharmacol, 92 (2-3): 251-256.

Hamilton,C.A. (2004), Medicinal plants, Conservation and livelihoods, Biodiversity and Conservation, 13: 1477-1517.

Herbert, V. (1996), Symposium: Prooxidant effects of antioxidant vitamins, Introduction, J. Nutr., 126: 1197s.

Hedge, J.E. and Hofreiter,B.T. (1962), Carbohydrate Chemistry 17 (Eds. Whistler, R.L and Be Miller, J.N), Academic press, New york, 34-40.

Hemalatha, S., Wahi, A.K., Singh, P.N. and Chansouria, J.P.N. (2004), Hypoglycemic activity of *Withania coagulans* dunal in streptozotocin induced diabetic rats, J Ethnopharmacol, 93 (203): 261-264.

Hirose, H., Kawai, J., Yamamoto, Y., Taniyama, M., Tomita, M., Matsubra, K., Okazaki, Y., Ishii, T., Oguma, Y., Takei, I. and Saruta, J. (2002), Effects of Proglitazone on metabolic parameters, body fat distribution and serum adiponectin levels in Japanese male patients with type 2 diabetes metabolism, 51 (3): 314-317.

Holl,R.W., Heinze,E. and Seifert,M. (1994), Longitudinal analysis of somatic development in pediatric patients with IDDM genetic influence of height and weight, Diabetologia, 37: 92-99.

Hussain, H.E.A. (2002), Hypoglycemic, hypolipidemic and antioxidant properties of combination of curcumin from *Curcuma longa*, Linn. and partially purified product from *Abroma augusta*, Linn. in streptozotocin induced diabetes, Ind. J. Clin. Biochem., 17: 33-43.

Hussain, H.E.A. (2002), Reversal of diabetic retinopathy in STZ-induced diabetic rats using traditional Indian antidiabetic plant *Azadirachta indica* (L), Ind. J. Clin. Biochem., 17: 115-123.

Hussain, M.E.H., Jamil, K. and Rao, M. (2001), Preliminary studies on the hypoglycemic effect of *Abroma augusta* in alloxan diabetic rats, Indian J. Clin. Biochem., 16: 77.

Illing, E.K.BB., Gray, C.H. and Lawrence, R.D. (1991), Blood glutathione and non-glucose reducing substances in diabetes, Biochem. Journal., 48: 637.

Irshad, M. and Chaudhuri, P.S. (2002), Oxidant-antioxidant system: Role and significance in human body, INDIAN J EXP BIOL, 40: 1233-1239.

Ivorra, M.D., Paya, M. and Villard, A. (1989), A review of natural products and plants as potential antidiabetic drugs, J Ethnopharmacol, 27: 243-275.

Jakus,V. (2000), The role of free radicals, oxidative stress and antioxidant systems in diabetic vascular disease,Bratisl.lek.listy.,101:541-551.

Jeong, J., Young, H., Yang, W.J., Shin, S.J., Kwon, H.J. and Kim, W.C. (2003), Characterization of streptozotocin induced diabetic rats and pharmacodynamics of insulin formulation, Biotechnol Biochem., 67 (11): 2398-2.

Kaczmar, D.M.D.T. (1998), Herbal support for Diabetes management, Clinical Nutrition insights, Advanced Nutrition Publication, 6: 8.

Kakkar, P., Das, B. and Viswanathan, P.N. (1984), A modified spectrophotometric assay of SOD, Indian J. Biochem. Biophysics., 21; 130-132.

Kameswararao, B., Kesavulu, M.M. and Apparao, C. (2003), Evaluation of antidiabetic effect of *Momordia cymbalaria* fruit in alloxan-diabetic rats, Fitoterapia, 74: 7-13.

Kaplowitz, N., Ookhtens, M. and Aw, T.V. (1985), The regulation of hepatic glutathione, Annu. Rev. Pharmacol. Toxicol., 25: 715-744.

Kiho, T., Watanabe, T., Nagai, K. and Ukai, S. (1992), Hypoglycemic activity of polysaccharides fraction from rhizome of *Rehmannia glutinosa* LIBOSC *F.hueichingensis* HSIAO and the effect on carbohydrate metabolism in normal mouse liver, Yokugaku zasshi, 112: 393-400.

Kinalski, M., Sledziewski, A., Jelejko, B., Zoerzycki, W. and Kinalski, I. (2000), Acta Diabetol., 37: 179-183.

King, K.J. and Wootton, I.D.P. (1959), Micromethods in Medical Biochemistry, 3rd edition, Churchill, London, 61.

Koida, H. and Oda, T. (1959), Pathological occurrence of glucose-6-phosphatase in liver disease, Clin. Chem. Acta., 4: 554-561.

Kornberg, A. and Horecker, B.L. (1955), Methods of enzymology, Volume 1, Academic Press, New York, 323.

Kose, K., Dogan, P. and Ozesmi, C. (1993), Contraceptive steroids increase erythrocyte lipid peroxidation in female rats, Contraception, 47: 421.

Kotlar, M., Rodriguez, C., Sainz, R.M., Antolin, I. and Menendezpelaez, A. (1998), Melatonin increase gene expression for antioxidant enzymes in rat brain cortex, J. Pineal. Res., 24: 83.

Kowluru, A.R., Tang, J. and Kern, S.T. (2001), Abnormalities of retinal metabolism in diabetes and experimental galactosemia VII- effect of long-term administration of antioxidants on the development of retinopathy, Diabetes, 50: 1936-1942.

Krishna, G. and Ranjan, S. K. (1991), Special Analytical Techniques in Nutritional Biochemistry, Kalyani publishers, New Delhi, 1: 61-81.

Krishnakumar, K., Augusti, K.T. and Vijayammal, P.L. (1999), Hypoglycaemic and antioxidant activity of *Salacia oblonga* wall extract in streptozotocin-induced diabetic rats, Indian J Physiol Pharmacol., 43 (3): 510.

Kumari, K. and Augusti, K.T. (2002), Antidiabetic and antioxidant effects of S-methyl cysteine sulfoxide isolated from onions (*Allium cepa* Linn.) as compared to standard drugs in alloxan diabetic rats, INDIAN J EXP BIOL, 40 : 1005-1009.

Laakso, M., Lehto, S. and Renttila, I. (1993), Lipids and lipoproteins predicting coronary heart disease mortality and morbidity in patients with non-insulin dependent diabetes, Circulation, 88, 1421-1430.

Laviola, L., Belsanti, G., Devalli, M.A., Napali, R., Perrini, S., Weir, C.W., Giorgino, R. and Giorgino, F. (2001), Effect of streptozotocin diabetes and diabetes treatment by islet transplantation on *in vivo* insulin signaling in rat heart, Diabetes, 50: 2709-2720.

Lemhadri, A., Zeggwagh, A.N., Maghrani, M., Jouad, H. and Eddouks, M. (2004), Anti-hyperglycemic activity of the aqueous extract of *Origanum vulgare* growing wild in Tafilalet region, J Ethnopharmacol, 92 (23): 251-256.

Lernmark, A. (1999), Clinical Chemistry, 45 (8PH2): 1331-1338.

Li, W.L., Zheng, H.C., Bukuru, J. and Kimp, D.N. (2004), Natural medicines used in the traditional Chinese medical system for therapy of diabetes mellitus, J Ethnopharmacol, 92; 108-117.

Lowry, O. H., Rosenbrough, N.J., Farr, A.L. and Randall, R. J. (1951), Protein measurment with Folin phenol reagent, J. Biol. Chem., 193: 265.

Luck, H. (1974), In: Methods in Enzymatic Analysis-2 (Ed. Bergmeyer), Academic Press, New York, 885.

Lwata, K., Shibuga, H., Ohkawa, Y. and Inui, N. (1984), Chromosomal aberrations in V79 cells induced by superoxide radical generated by the hypoxanthine-xanthine oxidase systems, Toxicol. Lett., 22, 75.

Maiti, R., Jana, D., Das, U.K. and Ghosh, D. (2004), Antidiabetic effect of aqueous extract of seed of *Tamarindus indica* in streptozotocin-induced diabetic rats, J Ethnopharmacol, 91 (2-3): 209-213.

Malhotra, R.P. (1991), Compendium of Indian Medicinal plants, Central Drug Research Institute, Lucknow, Publication and Information Directorate (CSIR), New Delhi, 2: 137-138.

Malick, C.P. and Singh, M.B. (1980), In: Plant Enzymology and Histoenzymology, Kalyani Publishers, New Delhi, 286.

Malloy, H.T. and Evelyn, K.A. (1937), J. Biol. Chem., 119: 481.

Mannisto, S., Alfthan, G., Virtanen, M., Kataja, V., Usitupa, M. and Pietinen, P. (2000), Toenail selenium and breast cancer – A case control study in Finland, Eur. J. Clin. Nutr., 54: 98.

Manonmani, G., Anbarasi, K., Balakrishnan, K., Veluchamy, G. and Devi, C.S.S. (2002), Effect of *Terminalia arjuna* on the antioxidant defense system in alloxan induced diabetes in rats, Biomedicine, 22: 52-61.

Mansour, H.A., Newairy, A.S., Yousef, M. and Shewers, S.A. (2002), Toxicology, 170: 221-228.

Marks, J.B. and Raskin, P. (1998), Nephropathy and hypertension in diabetes, Med Clin North Am., 82: 877-907.

Maroo, J., Vasu, V.T. and Gupta, S. (2003), Dose dependent hypoglycemic effect of aqueous extract of *Enicostemma littorale* blume in alloxan induced diabetic rats, Phytomedicine, 10 (2-3): 196-9.

Mary, N.K., Shylesh, B.S., Babu, B.H. and Padikkal, J. (2002), Antioxidant and hypolipidemic activity of a herbal formulation-Liposem, INDIAN J EXP BIOL, 40: 901-904.

Matsuda, M., Morikawa,T.and Yoshikawa.M. (2002), Antidiabetogenic constituents from several natural medicines, Pure & Appl Chem, 74: 1301.

Meister,A. (1983), Selective modification of glutathione metabolism, Science, 20: 472-477.

Metin, G., Atukeren, P., Gumustas, K.M., Belce, A. and Kayserilioglu, A. (2002), The effect of vitamin E treatment on oxidative stress generated in trained rats, J. Exp. Med., 198: 47-53.

Misra, H.P. and Fridovich, A. (1972), Assay of superoxide dismutase, J. Biol. Chem.., 247:3170-3171.

Mooradian, A.D. (1994), Selected vitamins and minerals in diabetes, Diabetes Care, 17: 464-79.

Mori, M.D., Baviera, M.A., Ramalho, O.T.L., Vendramini, C.R., Brunetti, L.I. and Pepubo, T.M. (2003), Temporal response pattern of biochemical analytes in experimental diabetes, Biotechnol. Appl. Biochem., 38:183-191.

Moron, M.S., De Pierre, J.N. and Mannervik, V. (1979), Levels of glutathione, glutathione reductase, glutathione-S-transferase activities in rat lung and liver, Biochem. Biophys. Acta., 582:67-68.

Mukai, K., Morimoto, H., Okaiuchi,Y. and Nagaoka, S. (1993), Kinetic study of reactions between tocopheroxyl radicals and fatty acid, Lipids, 28: 753.

Mutalik,S., Sulochana, B., Chetana, M., Udupa, N. and Devi, V.P. (2003), Preliminary studies on acute and subacute toxicity of an antidiabetic herbal preparation- Dianex, 41: 316-320.

Nagao, M., Wakabayashi, K., Suwa, Y. and Kobayashi, T. (1986), Alteration of mutagenic potentials by peroxidase, catalase and superoxide dismutase in antimutagenesis and anticarcinogenesis mechanism, edited by APE Hartman, T. Kada and A. Hollaender (Plenum Press, New York), 73.

Nathan, D.M. (1993), Long term complication of diabetes mellitus, N Engl J Med., 328: 1676.

Netlson, S. (1957), Microtechniques of clinical chemistry for the routine laboratory, C.C. Thomas, Springfield, Illinios, 381.

Nichans, W.J. Jr. and Samuelson, B. (1968), Eur J Biochem., 6: 126.

Niki, E. and Noguchi, N. (2002), Effect of antioxidant against atherosclerosis, Molecular and Cellular Biochemistry, 234/235:19-25.

Norbiato, G., Bevilacque, M. and Merino, R. (1984), Effects of potassium supplementation on insulin binding and insulin action in human obesity: Protein modified fast and refeeding, European J. Clin. Invest., 44: 414-9.

O'Ruhilly, S. (1997), BMJ, 314: 955.

Oberley, L.W. (1988), Free radicals and diabetes, Free Radio Bio med., 5: 113-124.

Offer, T., Russo, T. and Samuni, A. (2000), The proxidative activity of SOD and nitro oxide SOD mimes, FASEB, 14: 1215.

Okhawa, H., Ohisi, N. and Yagi, K. (1979), Assay for lipid peroxidation in animal tissues by thiobarbituric acid reactions, Anal. Biochem., 95:351.

Okyar, A., Can, A., Baktir, G. and Situpinar, N. (2001), Effect of *Aloe vera* leaves on blood glucose level in type 1 and type 2 diabetic rats models, Phytother. Res., 15: 157.

Omaye, S.T., Turbul, T.D. and Sauberlich, H.C. (1979), Selected method for the determination of ascorbic acid in animal cells, tissues and fluids, Mc. Cormic, D.B, Wright, D.L. (eds) Methods of Enzymology, Academic Press, New York, 62: 3-11.

Oser, B.L. (1971), Food Analysis, Hawks Physiological Chemistry, 14th Ed. Mc Graw Hill, New York, 1094-1098.

Owen, J.A., Iggo, B., Scandrett, F.J. and Stewart, C.P. (1954), Biochem J., 58: 428.

Packer, L. (1994), Vitamin E is nature's master antioxidant, Science American Journal, 54: 100.

Palada, M.C. and Crossman, S.M.A. (1999), Evaluation of tropical leaf vegetables in the Virgin Islands: 388-393. In: J. Janick (ed), Perspectives on new crops and new uses, ASHs Press, Alexandria, VA.

Palanivel, R., Thangavel, M., Selvendiran, K. and Sakthisekaran, D. (2001), Insulino-mimetic effect of ammonium paratungstate on protein metabolism in streptozotocin-induced diabetic rats, Biomedicine, 21 (2& 3): 23-30.

Palozza, P., Caluiello, G., Serini, S., Maggiano, N., Lanza, P., Ranelletti, F.O. and Bartoli, G.M. (2001), β-carotene at high concentrations induces apoptosis by enhancing oxy-radical production in human adenocarcinoma cells, Free radic. Biol. Med., 30: 1000.

Pari, L. and Satheesh, M.A. (2004), Antidiabetic activity of *Boerhaavia diffusa* L. effect on hepatic key enzymes in experimental diabetes, Journal of Ethnopharmacology, 91 (1): 109-13.

Pei, S. (2001), Ethnobotanical approaches of traditional medicine studies: Some experience from Asia, Pharmaceutical Botany, 39: 74-79.

Petrolonis, A.J., Yang, Q., Tummino, P.J., Fish, S.M., Pruck, A.E., Jain, S., Parsons, T.F., Dales, N.A., Ge, L., Langston, S.P., Schuller, A.G., Tartaglia, L.A., Chen, H. and Hong, S.B. (2004), Enzymatic characterization of the pancreatic islet-specific glucose-6-phosphatase related protein (IGRP), J. Biol.Chem., 279 (14): 13976-83.

Peveir, R.C., Fair, C.G. and Boller, I. (1992), Eating disorder in adolescents with IDDM : A controlled study, Diabetes Care, 15: 1356-1360.

Piegeolet, E. and Corbisier, P. (1990), GPx, SOD and catalase inactivation by A.O., Mech. Age Dev., 51: 283.

Ponnachan, P.T.C., Paulose, C.S. and Panikkar, K.P. (1993), Effect of leaf extract of *Aegle marmelos* in diabetic rats, Indian J Exp. Biol., 31: 345.

Pontrelli, L., Parris, W., Adeli, K. and Cheung, C. (2002), Atrovastatin treatment beneficially alters the lipoprotein profile and increase low density lipoprotein particle diameter in patients with combined dyslipidemia and impaired fasting glucose / type 2 diabetes , Metabolism ,51(3):334-342.

Prakasam, A., Sethupathy, S. and Pugalendi, K.V. (2002), Antihyperglycemic effect of *Casearia esculenta* root extracts in streptozotocin-induced diabetic rats, Pharmazie, 57 (11): 758-60.

Radin, N. (1981), In: Methods in Enzymology, Lowenstein, J.M. (ed.), Academic Press, New York, 72: 5

Raghuramulu, N., Nair, M.K. and Kalyanasundaram, S. (1983), A Manual of Laboratory Techniques, I edition, National Institute of Nutrition, KMR, Hyderabad, 31-32.

Raju, J., Gupta, D., Rao, A.R., Yadava, P.K. and Bayuer, N.Z. (2001), *Trigonella foenum graecum* (fenugreek) seed powder improves glucose homeostasis in alloxan diabetic rat tissues by reversing the altered glycolytic, gluconeogenic and lipogenic enzymes, Mol..Cell Biochem., 224 (1-2) : 45-51.

Ramanathan, M., Jaiswal, A.K. and Bhattacharya, S.K. (1999), INDIAN J EXP BIOL, 37: 182-183.

Rana, S.V.S., Janu, A. and Singh, R. (2002), Inevitable glutathione, then and now, INDIAN J EXP BIOL, 40: 706-716.

Rasch, R. and Mogensen, C. E. (1980), Urinary excretion of albumin and total protein in normal and streptozotocin diabetic rats, Acta Endocrinol., 95(3):376-381.

Ray, G. and Husain, A.S. (2002), Oxidants, antioxidants and carcinogenesis, INDIAN J EXP BIOL, 40: 1213-1232.

Reddy, K.P., Subhani, K.H., Chan, P.A. and Kumar, K.B. (1995), Effect of light and benzyl adenine on dark treated growing rice leaves, II changes in peroxidase activity, Plant Cell Physiol., 26, 987-994.

Reitman, S. and Frankel, S. (1957), In: Varley, H., Gowenlock, H.A. and Bell, M. (1980), Practical Clinical Biochemistry, V edition, London, William Heinmann Medical Books Ltd., 1088.

Reynolds, J.E.F. (1997), Martindale - The Extra Pharmacopoeia, 30th edition, (The Pharmaceutical Press, London), 276.

Roe J.H. and Kuether, C. A. (1953), The determination of ascorbic acid in whole blood and urine through 2,4 dinitro phenylhydrazine derivative of dehydro ascorbic acid, J. Biol. Chem., 147:399-407.

Rosenberg, H.R. (1992), Chemistry and Physiology of the Vitamins, Inter science publishers Inc, New York, 452-453.

Rotruck, J.T., Pope, A.L., Ganther, H.E. and Swanson, A.B. (1984), Selenium: Biochemical roles as a component of glutathione peroxidase, Science, 179:588-590.

Ryan, E.M., Lee, H.M., Liu, Y., Comanducci, R., Ramamurthy.N. and Golub, L. (2002), Non-antimicrobial tetracycline reduces systemic biochemical markers associated with diabetic complication, Periodontal Research –therapy programe:6-9.

Sabu, C.M. and Kuttan, R. (2004), Antidiabetic activity of *Aegle marmelos* and its relationship with its antioxidant properties, Indian J. Physiol Pharmacol., 48 (1): 81-88.

Sabu, M.C.and Kuttan, R. (2000), Antidiabetic activity of some medicinal plants-relation with their antioxidant property, Amala research Bulletin, 20:81-86.

Sachdewa, A., Nigam, R. and Khemani, L.D. (2001), Hypoglycemic effect of *Hibiscus rosasinensis* L. leaf extract in glucose and streptozotocin induced hypoglycemic rats, INDIAN J EXP BIOL, 39: 284-286.

Salmuddin, N.C., Bequer,U. and Beauen, N. (1996), Biochem.Mol.Biol.Int.,40:853-860.

Sanderson, J.H. and Philips, C.E. (1981), An Atlas of Laboratory, Animal Haematology, Clarendon Press, Oxford, 473.

Shanthakumari, P., Prakasam, A. and Pugalendi, K.V. (2003), Modulation of oxidative stress parameters by treatment with *Piper betle* leaf in streptozotocin induced diabetic rats, Indian Journal of Pharmacology, 35: 373-378.

Schanderl, S.H. (1970), Methods in Food Analysis, Academic Press, New York, 709.

Sekar, N. and Govindasamy, S. (1990), Insulin mimetic role of vanadate on plasma membrane insulin receptor, J Biosci., 15: 115.

Shanmugasundaram, E.R.B., Rajeshwari, G., Baskaran, K., Kumar, B.R.R., Shanmugasundaram, K.R., and Ahmath, K.B., (1990). Use of *Gymnema sylvestre* leaf extract in the control of blood glucose in insulin dependent diabetes mellitus, J Ethnopharmacol, 30:281-294.

Shorr, R.I., Ray, W.A., Daughtery, J.R., and Griffin, M.R. (1996), Individual sulfonylureas and serious hypoglycemia in older people, J.Am. Geriatric Soc., 44:751-755.

Shukla, R., Sharma, S.B., Puri, D., Prabhu, K.M. and Murthy, P.S. (2000), Medicinal plants for treatment of diabetes mellitus, Indian J. Clin. Biochem., 15 (Supp1): 169-177.

Sies, H. (1985), Oxidative stress introductory remarks in oxidative stress, Edited by Sies, H , Academic Press, London: 147.

Sinclair, A.J., Taylor, P.B. and Girling, A.J. (1994), Low plasma ascorbate levels in patients with type 2 diabetes mellitus consuming adequate dietary vitamin C, Diabet. Med., 11: 898-901.

Smith,S.R. (1996), The endocrinology of obesity, Endocrinol.Metab.Clin. North.Am, 25:921-942.

Soliman, T.A., Omar, M., Assem, M.A., Nasar, S.I., Rizk, M.M., Matary, E. and Alaily, K.R. (2002), Serum leptin concentrations in children with type I diabetes mellitus: Relationship to body mass index, insulin dose and glycemic control, Metabolism, 51 (3): 292-296.

Sridhar,G.R. (2002), Diabetes in India: Snapshot of Panorama, Current Science, 80 (7): 791.

Sridhar, G.R. and Nagamani,R. (2002), In: Ann NY Acad. Sci., 958: 390-393.

Sridhar,G.R., Rao,P.V. and Ahuja, M. M. S. (2002), In RSSDI textbook of Diabetes(eds Ahuja,M.M.S.*et al), RSSDI,* Hyderabad, *95-112.*

Srivastava, R. (2000), Studying the information needs of medicinal plant stakeholders in Europe, TRAFFIC Dispatches, 15: 5.

Stamler, J., Vaccaro, O. and Neaton, J.D. (1993), Diabetes, other risk factors and 12 year cardiovascular mortality for men screened in the multiple risk factor intervention trial, Diabetes Care, 16: 434-444.

Tomlinson, D.R. (1998), Diabet. Metab., 24: 79-83.

The Expert Committee on the Diagnosis and Classification of Diabetes mellitus (2001), Report of the Expert Committee on the Diagnosis and Classification of diabetes mellitus, Diabetes Care, 24: S5-S20.

Twaij, H.A. and Badr, A. A. (1988), Hypoglycemic activity of *Artemisia herba alba*, Journal of Ethnopharmacology., 24: 123.

Ugochukwu, N.H. and Babady, N.E. (2003), Antihyperglycemic effect of aqueous and ethanolic extracts of *Gangronema latifolium* leaves on glucose and glycogen metabolism in livers of normal and streptozotocin-induced diabetic rats, Life Science, 73 (15): 1925-38.

United Nations Population Division (1999), Department of Economic and Social Affairs: World Urbanization Prospects, Revision, NewYork.

Upadhyay,O.P., Singh,R.M. and Dutta,K. (1996), Studies on antidiabetic medicinal plants used in Indian Folklore, Aryavaidyan ,6:159.

Ursine, F., Maeorino, M. and Flone, R.B. (1995), The diversity of glutathione peroxidase, Methods in Enzymol., 252: 38.

Varley, H. (1969), Clinical Chemistry, Fourth edition, CSS publishers and distributors, New Delhi, India, 446.

Watkins, J.B. and Sherman, S.B. (1991), Pharmacol., 260: 1337-343.

Wichtl,M. (1994), Herbal Drugs and Phytopharmaceuticals, Boca Raton, FL: CRC Press, 104.

Wild, S., Roglic, G., Green, A., Sicree, R. and King, H. (2004), Global prevalence of Diabetes, Diabetes care, 27: 1047-1053.

Wilson,R.L. (1998), Free radical and tissue damage, Mechanistic evidence from radiation studies. In: Biochemical mechanisms of liver injury, New York, Academic Press, 123-125.

Wolf, S.P. and Dean, R.T. (1987), Biochem J., 245: 45-250.

Wolf, S.P., Jiang, Z.Y. and Hunt, J.U. (1991), Free radio Biol Med., 10: 339-352.

World Health Organization (1994), Prevention of diabetes mellitus, Technical Report series: WHO, Geneva: 844-848.

Wyngaarden, J.B., Smith, L.H. and Bennett, J.C. (1992), Cecil Textbook of Medicine, W.B. Saunders, Philadelphia, PA.

Yoshikawa, M., Murakami, T., Kishi, A., Kageura, T. and Matsuda, H. (2001), Antidiabetogenic constituents from several natural medicines, Chem. Pharm. Bull., 49: 863.

Yue, D.K., Hanvell, M.A. and Satshell, P.M. (1984), The effects of aldose reductase inhibition on nerve sorbitol and myoinositol concentrations in diabetic and galactosemic rats, Metabolism, 33: 1119-1122.

Zakaria, H., Simpson, K., Brown, P.R. and Krotulovic,A. (1979), Use of reversed phase HPLC analysis for the determination of provitamin A carotenes in tomatoes, J. chromatography, 176: 109-117.

Zhang, R., Zhou, J., Jia, Z., Zhang, Z. and Gu, G. (2003), Hypoglycemic effect of *Rehmannia glutinosa* oligosaccharide in hyperglycemic and alloxan-induced diabetic rats and its mechanism, J Ethnopharmacol, 90: 109.

Zilversmit, D.B. and Davis, A.K. (1950), Microdetermination of phospholipids by TCA precipitation, J. Lab. Clin. Med., 35: 155-159.

Zimmet, P. (2000), Coca-colonization and the chronic disease epidemic: can the doomsday scenario be averted?, J. Intern. Med., 247: 301-310.

Zimmet, P., Alberti, K.G.M.M. and Shaw, J. (2001), Global and societal implication of the diabetes epidemic, Nature, 414: 782-787.

APPENDICES

APPENDICES

APPENDIX-I
ESTIMATION OF TOTAL CARBOHYDRATE
(Hedge and Hofreiter, 1962)

Principle

Carbohydrates are first hydrolysed into simple sugars using dilute hydrochloric acid. In hot acidic medium glucose is dehydrated to hydroxyl furfural. This compounds forms with anthrone a green coloured product with absorption maximum at 630nm.

Reagents

1. 2.5 N HCl
2. Anthrone reagent: Dissolved 200mg anthrone in 100ml of ice cold 95%H_2SO_4. Prepared fresh before use.
3. Stock standard Glucose: Dissolved100mg in 100ml water.
4. Working Standard: 10ml of stock diluted to 100ml with distilled water. Stored, refrigerated after adding a few drops of toluene.

Procedure

Weighed 100mg of the sample in a boiling tube. Hydrolysed by keeping it in boiling water bath for three hours with 5 ml of 2.5N HCl and cooled it room temperature. Neutralized it with solid sodium carbonate until the effervescence ceased. Made up the volume to 100 ml and centrifuged. Collected the supernatant and took 0.5 ml and 1.0 ml aliquots for analysis. Prepared the standards by taking 0, 0.2, 0.4, 0.6, 0.8 and 1.0 ml of the working standard. '0' served as the blank. Made up the volume to 1 ml in all the tubes including the sample tubes by adding distilled water. Then added 4.0 ml of anthrone reagent. Heated for eight minutes in a boiling water bath. Cooled rapidly and read the green to dark green colour at 630 nm. Constructed a standard graph by plotting concentration of the standard on the x-axis versus absorbance on the y-axis. From the standard graph calculated the amount of carbohydrate present in the sample.

Calculation

$$\text{Amount of carbohydrate present in the sample} = \frac{\text{mg of glucose}}{\text{Volume of test sample}} \times 100$$

APPENDIX -II
ESTIMATION OF PROTEIN
(Lowry *et al.*, 1951)

Reagents

1. Alkaline copper reagent
   Solution A: 2% sodium carbonate in 0.1N NaOH
   Solution B: 0.5% copper sulphate in water
   Solution C: 1% sodium potassium tartarate in water
   50ml of solution A was mixed with 0.5ml of Solution B and 1ml of solution C just before use.

2. Folin's-phenol reagent: into a 1500ml of round bottomed flask, 100mg sodium tungstate, 25g of sodium molybdate, 700ml of water, 50ml of o-phosphoric acid and 100ml of concentrated hydrochloric acid were added and refluxed for 10 hours. Then 150g of lithium sulphate, 50ml of distilled water and a few drops of bromine were added. The mixture was boiled to remove excess bromine. It was then cooled and diluted to one litre with water. The reagent was diluted in 1:2 ratio with distilled water just before use.
3. Standard Bovine Serum Albumin: 10mg of crystalline BSA was dissolved in 100ml of distilled water.

Procedure

Aliquot of the suitably diluted serum and tissue (0.1ml to 10ml by two serial dilutions) was made upto 1ml with water and 4.5ml of alkaline copper reagent was added to all the tubes including blank. Blank containing 1.0ml of water and standard containing aliquots of BSA were also treated similarly. The content was left to stand for 10min at room temperature. Then 0.5ml of diluted Folin's-phenol reagent was added. The blue colour developed was read at 640nm after 20min in a Shimadzu UV-spectrophotometer. The values were expressed as mg/dl.

APPENDIX-III
ESTIMATION OF THIAMINE
(Raghuramulu *et al*, 1983)

Principle

Alkaline potassium ferricyanide oxidizes thiamine to thiochrome, which is fluorescent compound. The thiochrome is extracted in isobutyl alcohol and measured in fluorimeter.

Reagents

1. 15% NaOH
2. 1% potassium ferrricyanide
3. Sodium sulphate
4. Isobuytl alcohol
5. 0.1N H_2SO_4
6. Stock standard thiamine hydrochloride: Dissolved 50 mg thiamine hydrochloride in 500 ml of 0.1N H_2SO_4 containing 25% alcohol.
   Working standard solution: Diluted 5 ml of the stock solution to 100 ml with 0.1N H_2SO_4 and again diluted to 100 ml with 0.1N H_2SO_4 and then used.

Procedure

Weighed accurately 2.5 g of finely ground sample in a 250 ml conical flask. 100 ml of 0.1N H_2SO_4 was slowly added. The flask was stoppered and allowed to stand overnight. Then it was shaken vigorously and filtered through Whatman No.1 filter paper. First 10-15 ml of filtrate was discarded.

Pipetted out 10 ml of the extract in duplicate into 100 ml separating funnels. Pipetted out 10 ml of working standard (in 4-5 replicates) into separate separating

2. Folin's-phenol reagent: into a 1500ml of round bottomed flask, 100mg sodium tungstate, 25g of sodium molybdate, 700ml of water, 50ml of o-phosphoric acid and 100ml of concentrated hydrochloric acid were added and refluxed for 10 hours. Then 150g of lithium sulphate, 50ml of distilled water and a few drops of bromine were added. The mixture was boiled to remove excess bromine. It was then cooled and diluted to one litre with water. The reagent was diluted in 1:2 ratio with distilled water just before use.
3. Standard Bovine Serum Albumin: 10mg of crystalline BSA was dissolved in 100ml of distilled water.

Procedure

Aliquot of the suitably diluted serum and tissue (0.1ml to 10ml by two serial dilutions) was made upto 1ml with water and 4.5ml of alkaline copper reagent was added to all the tubes including blank. Blank containing 1.0ml of water and standard containing aliquots of BSA were also treated similarly. The content was left to stand for 10min at room temperature. Then 0.5ml of diluted Folin's-phenol reagent was added. The blue colour developed was read at 640nm after 20min in a Shimadzu UV-spectrophotometer. The values were expressed as mg/dl.

APPENDIX-III
ESTIMATION OF THIAMINE
(Raghuramulu *et al*, 1983)

Principle

Alkaline potassium ferricyanide oxidizes thiamine to thiochrome, which is fluorescent compound. The thiochrome is extracted in isobutyl alcohol and measured in fluorimeter.

Reagents

1. 15% NaOH
2. 1% potassium ferrricyanide
3. Sodium sulphate
4. Isobuytl alcohol
5. 0.1N H_2SO_4
6. Stock standard thiamine hydrochloride: Dissolved 50 mg thiamine hydrochloride in 500 ml of 0.1N H_2SO_4 containing 25% alcohol.
   Working standard solution: Diluted 5 ml of the stock solution to 100 ml with 0.1N H_2SO_4 and again diluted to 100 ml with 0.1N H_2SO_4 and then used.

Procedure

Weighed accurately 2.5 g of finely ground sample in a 250 ml conical flask. 100 ml of 0.1N H_2SO_4 was slowly added. The flask was stoppered and allowed to stand overnight. Then it was shaken vigorously and filtered through Whatman No.1 filter paper. First 10-15 ml of filtrate was discarded.

Pipetted out 10 ml of the extract in duplicate into 100 ml separating funnels. Pipetted out 10 ml of working standard (in 4-5 replicates) into separate separating

funnels. 3 ml of 15% NaOH was added into each separating funnel immediately followed by 4 drops of potassium ferricyanide solution. It was shaken gently for 30 seconds. Then added 15 ml of isobutanol rapidly from a quick delivery burette. Stoppered immediately and shaken vigorously for 60 seconds and the layers were allowed to separate. The bottom layer was drained off carefully and added one spatula full of sodium sulphate. The clear extract from the top layer was collected. Similarly pipetting out 10 ml of the extract set a blank and the above procedure was followed except for the addition of ferricyanide. A blank was prepared for the standard separately. The primary and secondary filters were selected and adjusting the standard blank to 0 reading (a) and standard to 100 (a^1), set the fluorimeter. Then the sample blank (x^1) and sample readings (x) were read.

$$\text{Microgram thiamine in 100 g sample} = \frac{0.25\times10}{a - a^1} \times \frac{(x - x^1)\times 100}{10} \times \frac{10}{5}$$

APPENDIX- IV
ESTIMATION OF RIBOFLAVIN
(Raghuramulu *et al.*, 1983)

The native fluorescence of riboflavin in neutral pH is used in the chemical estimation of this vitamin.

Reagents

1. Riboflavin standard: 25 mg of riboflavin is dissolved in 300 to 400 ml of water, adding 1.2 ml of glacial acetic acid and warming at a low temperature to aid solution. After the riboflavin is dissolved, the solution is cooled and made upto 1 litre. This stock solution has a concentration of 25 µg per ml. 2 ml of this stock solution is diluted to 5 ml to give a working standard of 1 µg per ml.
2. 4% potassium permanganate
3. 1:1 hydrogen peroxide – water mixture

Procedure

To about 25 ml of the vitamin extract, one or two drops of caprylic alcohol was added, followed by 3 ml of freshly prepared 4% potassium permanganate solution. The mixture was stirred well and within 2 min 3 ml of 1:1 H_2O_2 water solution was added to discharge the permanganate colour and the pH adjusted to 7.0 with NaOH. The volume was made upto 35 ml and the solution was filtered and the fluorescence of the filtrate was measures in a fluorimeter using appropriate filter.

Calculation

The fluorescence of a known aliquot of the filtrate was taken as A. 1µg of riboflavin was added and the reading noted as B. A small pinch of sodium hydrosulfite was added to destroy the riboflavin only and the reading recorded as C.

$$\text{The quantity of riboflavin in the solution taken for fluorimetry} = \frac{A - C}{B - A} \times 1\mu g$$

From this, the riboflavin content of the sample was calculated making due allowance for dilution and enzyme blanks.

APPENDIX -V
ESTIMATION OF MOISTURE CONTENT
(Raghuramulu *et al.*, 1983)

Principle

Estimation of moisture is one of the most often performed determinations in food analysis. Moisture is lost when food is heated not much higher than the temperature of boiling water or by allowing standing overnight over dehydrating agent or by heating over vacuum.

Procedure

Heated a pair of weighed crucibles at 100°C in an oven and labeled A and B. Placed on an asbestos sheet for 2 min and then transferred them to a desiccator where they remained for ½ h. Recorded their weights in an analytical balance. Repeated this procedure till two successive constant weights were obtained (with maximum difference of 0.002 g).

Weighed definite amounts of sample (2 g) in each dish and placed in an electric oven thermostatically controlled at 100-105°C. Heated for a stipulated time (2 h) and cooled in a desiccator for ½ h and weighed. This was also weighed till successive weighing showed no further loss.

The loss of weight equals the moisture present in the sample. The loss of weight divided by the weight of the original sample multiplied by 100 gives the percentage of moisture.

APPENDIX-VI
ESTIMATION OF FIBRE CONTENT
(Raghuramulu *et al.*, 1983)

Reagents

1. 0.255 N Sulphuric acid: 0.7 ml of H_2SO_4 in 99.3 ml of water
2. 0.313 N NaOH: 1.25 g of NaOH in 100 ml water.
3. Ether

Procedure

5 g of the sample was weighed into a 500 ml beaker and 200 ml of boiling 0.255N H_2SO_4 was added. The mixture was boiled for 30 min keeping the volume constant by adding water at frequent intervals (a glass rod inserted in the beaker helps smooth stirring and boiling). At the end of the period the mixture was filtered and then transferred the residue to a beaker containing 200 ml of boiling 0.313N NaOH. After boiling for 30 min (keeping the volume constant as before) the mixture was filtered through muslin cloth. The residue was washed with some hot water till free from alkali followed by washing

with some alcohol and ether. It was then transferred into a crucible, dried overnight at 80-100°C and weighed. The crucible was heated in a muffle furnace at 600°C for 2-3 h . Cooled and weighed again. The difference in weight represents the weight of the fibre.

APPENDIX-VII
ESTIMATION OF CALCIUM
(Clark and Collip, 1925)

Principle

Calcium is precipitated from the ash solution as calcium oxalate. The precipitate is dissolved in acid and the amount of oxalate is determined titrimetrically by titrating with potassium permanganate.

Reagents

1. 4% ammonium oxalate solution
2. 2% ammonia solution
3. 0.01N $KMnO_4$
4. 2N sulphuric acid

Procedure

2.0ml of ash solution was taken in a centrifuge tube, added 1.0ml of 4 per cent ammonium oxalate. Mixed well and allowed it to stand overnight. The next day it was centrifuged. The precipitate was washed thoroughly with 3.0ml of 2% ammonia solution. Centrifuged and discarded the supernatant. This process was repeated till the supernatant gave no precipitate with calcium chloride. Added 2.0ml of 2N sulphuric acid mixed and warmed in a beaker containing almost boiling water to complete the solution of oxalate. Removed and titrated with 0.01N potassium permanganate required to titrate the calcium oxalate.

APPENDIX-VIII
ESTIMATION OF PHOSPHORUS
(Fiske and Subbarow (Oser, 1971))

Principle

Phosphate solution is treated with an acid molybdate reagent, which reacts with inorganic phosphates to form phosphomolybdic acid. The hexavalent molybdenum of the phosphomolybdic acid is reduced by the addition of 1, 2, 4-amino naphthol sulponic acid to produce a blue color, which is probably a mixture of lower oxides of molybdenum. The blue color produced is proportional to the amount of phosphorus present, which is estimated colorimetrically at 660nm.

Reagents

1. Molybdate I solution: 25 g of reagent grade ammonium molybdate in 200 ml of distilled water and transferred to a litre volumetric flask containing 500 ml of

10N H_2SO_4 and then made upto the mark and mixed.

2. Molybdate II solution: Dissolved 25 g of reagent grade ammonium molybdate in 200 ml of distilled water and transferred to a litre volumetric flask containing 300 ml of 10N H_2SO_4 and then made up to the mark and mixed.
3. 15% sodium bisulfite: 30g / 200ml of distilled water. Stirred to dissolve. Allowed to stand well. Stored for several days and filtered.
4. 20% sodium bisulfite: 20 g in 100 ml distilled water.
5. ANSA reagent: 97.5 ml of 15% sodium bisulfite + 0.25 g of 1,2,4 ANSA + 2.5 ml of 20% sodium bisulfite, stoppered.
6. Standard phosphate solution: Weighed accurately 35.1 mg of potassium dihydrogen phosphate. Dissolved in water, added 1.0 ml of 10N H_2SO_4. Made up the volume to 100 ml with distilled water in a standard flask. 1.0 ml of this contains 8µg of phosphorus.

Procedure

Into a series of test tubes, pipetted out 0.5, 1.0, 1.5, 2.0 and 2.5 ml of working standard solution corresponding to 8-24µg. Made up the volume to 4.3 ml with water and added 0.5 ml molybdate I solution and 0.2 ml of ANSA. 0.1 ml of ash solution was taken and treated similarly with molybdate II. The colour developed was read after 20 minutes in a colorimeter using red filter against a reagent blank at 660 nm.

By plotting the concentration of phosphorus on the X-axis and the colorimeter readings on the Y-axis, the concentration of phosphorus present in the sample was calculated.

APPENDIX-IX
ESTIMATION OF TOTAL IRON
(Oser, 1971)

Principle

Iron reacts with potassium thiocyanate in the presence of saturated potassium persulphate and concentrated sulphuric acid to give red coloured solution. The intensity of the colour developed is proportional to the concentration of iron, which is measured colorimetrically at 540 nm.

Reagents

1. 3N potassium thiocyanate (Prepared fresh)
2. Saturated potassium persulfate (prepared fresh): 5 g / 100 ml water
3. Con. Sulphuric acid
4. Stock standard iron solution: 70.2 mg of ferrous ammonium sulphate was dissolved in distilled water and added 1.0 ml of concentrated sulphuric acid. Made up the volume to 100 ml with distilled water.

Procedure

Into a series of test tubes added 0.5-2.5ml of working standard from solution corresponding to 35-75g of iron. Pipetted out 0.5 and 1.0 ml of ash solution in duplicates. The volume in each tube was made upto 3.85ml with distilled water. Then added 0.2ml of saturated potassium persulphate solution.0.15ml of concentrated sulphuric acid and 0.3ml

of 3ml potassium thiocyanate. The colour developed was read in a colorimeter against a reagent blank at 540nm within 10 minutes.

Plotting the concentration of iron on X-axis and the colorimeter reading on Y-axis constructed a graph. From the graph, calculated the amount of total iron present in 100 ml of the ash solution.

APPENDIX-X
ESTIMATION OF SODIUM AND POTASSIUM
(Raghuramulu *et al.*, 1983)

Principle

The sample in solution is introduced in the form of a fine continuous spray into a nonluminous gas flame. The emitted light, characteristic for the ion being analysed, is isolated and focused on a photoelectric cell and the current intensity is measured on a suitable meter.

Reagents

1. Standard sodium solution: Prepared a stock standard solution containing 100 meq of sodium/l by dissolving 5.85 g of dried NaCl in glass distilled water and diluting it to 1 l. working standards are prepared by taking 10-16 ml of the stock solution and making up the volume to 1 l. these standards represent 100-160 meq of Na per l at 1:100 dilution. A further 1:5 dilution was employed for all the working standard solutions so that the dilution factor for Na is 500.
2. Standard potassium solution: Prepared a stock standard solution containing 100 meq of K per l by dissolving 0.746 g of dried KCl in glass distilled water and diluting it to 1 l. Working standards were prepared by taking 15-35 ml of stock standard and diluting it to 1 l. these standards represent 3-7 meq K, at 1 in 20 dilution.

Procedure

The sodium and potassium content were estimated using flame photometer. For this standard sodium solution (0-100 ppm) was first injected and the readings were recorded. A standard curve relating ppm of the standard and flame photometer readings was drawn. A sample extract was then fed and the reading was noted. The ppm of the extract was then deduced with the use of standard curve. In the same way potassium was also estimated. The ppm was converted to mg/100 g sample.

APPENDIX -XI
ESTIMATION OF CHROMIUM
(Krishna and Ranjan, 1991)

A known volume of sample suspension was digested with 25 ml of triple acid mix (3:2:1 Conc.HNO_3: $HClO_4$: Conc.H_2SO_4) and left aside for 3-4 hours in a fume cupboard.

Then heated for 4 h. , cooled, washed 3-4 times with deionised water and made up to 50 ml in a volumetric flask. The standard chromium solution and the sample extractswere first aspirated through an air-C_2H_2 flame into atomic absorption spectroscopy and readings were recorded at 358 nm.

APPENDIX -XII
ESTIMATION OF CATALASE
(Luck, 1974)

Catalase has a double function as it catalyses the following reaction:

i. Decomposition of Hydrogen peroxide to give water and oxygen

$$2H_2O_2 \longrightarrow 2H_2O + O_2$$

ii. Oxidation of H donors, For example methanol, formic acid phenol with consumption of one mole of peroxide

$$ROOH + AH_2 \longrightarrow H_2O_2 + ROH + A$$

Principle

The UV light absorption of hydrogen peroxide solution can be easily measured between 230nm and 250nm. On decomposition of hydrogen peroxide by catalase, the absorption decreases with time. The enzyme activity could be arrived at from this decrease.

Reagents

1. Phosphate buffer: 0.067M (pH7.0)

Dissolved 3.522g of KH_2PO_4 and 7.628g of $NaHPO_4.2H_2O$ in distilled water and made up the volume to one litre.

2. Hydrogen peroxide – phosphate buffer

Diluted 0.16mlof H_2O_2 (10% w\v) to 100ml with phosphate buffer. Prepared fresh. The absorbance of the solution should be about 0.5 at 240nm with a 1cm path light.

Procedure

Enzyme extract

Homogenized the liver tissue in mortar and pestle with M\150 phosphate buffer (assay buffer diluted 10 times) at 1-4°C and centrifuged. The supernatant was used for the assay.

Assay

Read against control cuvette containing the enzyme solution as in experimental cuvette, but containing hydrogen peroxide free phosphate buffer (M\15).

Pipetted into experimental cuvette, 3ml of hydrogen peroxide-phosphate buffer. 0.01-0.04ml of sample added. Noted the t required for a decrease in absorbance from 0.45 to 0.4. This value was used for the calculations. If 't' was more than 60 seconds, then repeated the measurements with more concentrated solution of the sample.

Calculations

Calculated the concentration of H_2O_2 using extinction coefficient 0.036 per µmole per ml.

PPENDIX -XIII
ESTIMATION OF PEROXIDASE
(Reddy *et al.*, 1995)

Principle

In the presence of hydrogen donor (pyrogallol or diansidine) peroxidase converts hydrogen peroxide to water and oxygen. The oxidation of pyrogallol or diansidine to a coloured product called purpurogalli is formed colorimetrically.

Pyrogallol + H_2O_2 ⟶ Oxidised pyrogallol + H_2O + O_2

Reagents

1. Pyrogallol – 0.05M phosphate buffer (pH 6.5)
2. 1% H_2O_2
3. Enzyme extract: Macerated one part of the sample with 5 parts (w/v) of 0.1 m phosphate buffer (pH 6.5) in a homogeniser. Centrifuged the homogenate at 300g for 15 min. Used the supernatant as the enzyme source. All procedures were carried out at 0-5°C.

Procedure

Pipetted out 3ml of 0.05M pyrogallol solution and 0.05 to 1ml of enzyme extract in a test tube. Adjusted the spectrophotometer to read '0' at 430 nm. Added 0.5ml of 1% H_2O_2 in a test cuvette. Recorded the change in absorbance every 30 seconds upto 3 minutes.

Calculation

Change in absorbance/min at 430nm = X
Weight of plant material/liver taken = A mg
Volume of the extract taken for assay = 0.1ml
Change in absorbance for 0.1ml = X
Change in absorbance for 5ml extract = (X / 0.1) x 5 =Y
Peroxidase activity/g of plant / liver tissue = Y x (1000 / A) units

APPPENDIX -XIV
ASSAY OF SUPEROXIDE DISMUTASE
(Misra and Fridovich, 1972)

Principle

Superoxide dismutase uses the photochemical reduction of riboflavin as oxygen generating system and catalyses the inhibition of NBT reduction, the extent of which can be assayed spectrophotometrically.

Reagents

1. 50 mM potassium phosphate buffer, pH 7.8
2. 45 µM methionine
3. 5.3 mM riboflavin

4. 84 µM nitro blue tetrazolium (NBT)
5. 20 µM potassium cyanide

Procedure

The incubation medium contained in a final volume of 3 ml, 50 mM potassium phosphate buffer (pH 7.8), 45µM methionine, 5.3 mM riboflavin, 84µM NBT and 20 µM potassium cyanide. The tubes were placed in an aluminium foil-lined box maintained at 25°C and equipped with 15W fluorescent lamps. Reduced NBT was measured spectrophotometrically at 600 nm after exposure to light for 10 min. the maximum reduction was evaluated in the absence of the enzyme.

One unit of enzyme activity is defined as the enzyme reaction, which gave 50% inhibition of NBT reduction in one minute under the assay conditions and expressed as specific activity in units.

APPENDIX- XV
ESTIMATION OF POLYPHENOL OXIDASE
(Esterbauer *et al.*, 1977)

Principle

Phenol oxidases are copper proteins of wide occurrence in nature, which catalyse the aerobic oxidation of certain phenolic substrates to quinines, which are autooxidised to dark brown pigments generally known as melanins. The polyphenol oxidase comprise of catechol oxidase and laccase. The activities of these enzymes are important with regard to

a) Plant defense mechanisms against pest and diseases
b) Appearance, palatability and use of plant products.

1 Unit of either catechol oxidase or laccase is defined as the amount of enzyme that transforms 1µM of dihydrophenol to 1µM of quinone / min under the assay conditions.

Enzyme Extract

The enzyme extract may be prepared by grinding 5 g leaves with a mortar and pestle in about 20 ml medium containing 50 mM Tris-HCl, pH 7.2, 0.4M sorbitol and 10 mM NaCl. Centrifuged at 20000 g for 10 min, and used the supernatant for the assay.

Procedure

Added 2.5 ml of 0.1M phosphate buffer pH 6.8 and 0.3ml of catechol solution (0.01M) into cuvette and set the spectrophotometer at 495 nm. Now added 0.2 ml of enzyme extract and started recording the change in absorbance for every 30 seconds upto 5 minutes.

The enzyme activity was calculated as follows

Enzyme units in the test = (ΔA/min)
K for catechol oxidase = 0.272
K for laccase = 0.242
ΔA = Final reading – initial reading

APPENDIX - XVI
ASSAY OF GLUTATHIONE PEROXIDASE
(Rotruck *et al.*, 1984)

A known amount of enzyme preparation was allowed to react with H_2O_2 in the presence of GSH for a specified time period. Then the remaining GSH was measured by the method of Ellman.

$$2GSH + H_2O_2 \xrightarrow{\text{Se-GPx}} GSSG + 2\,H_2O$$

Reagents

1. 0.4M Tris buffer, pH 7.0
2. 10mM Sodium azide solution
3. 10% Trichloro acetic acid
4. 0.4mM EDTA
5. 20mM Hydrogen peroxide solution
6. 2mM Glutathione solution

Procedure

To 2ml of Tris buffer, 0.2ml of EDTA, 0.1ml of sodium azide and 0.5ml of tissue homogenate/0.1ml of plasma were added to the mixture; 0.2ml of glutathione followed by 0.1ml of hydrogen peroxide were added. The contents were mixed well and incubated at 37°C for 10 min along with a tube containing all the reagents except sample. After 10min the reaction was arrested by the addition of 0.5ml of 10% TCA, centrifuged and the supernatant was assayed for glutathione by the method of Ellman.

The activities were expressed as µg of GSH consumed/min/mg protein.

APPENDIX- XVII
ESTIMATION OF GLUTATHIONE–S-TRANSFERASE
(Beutler, 1984)

Principle

Glutathione-S-transferase catalyses the reaction of 1-chloro-2,4 dinitrobenzene (CDNB) with the SH group of glutathione.

$$CDNB + GSH \longrightarrow CDNB\text{-}S\text{-}glutathione$$

The activity of the enzyme was measured by following the increase in absorbance at 340 nm.

Reagents

1. K_2HPO_4 – 0.5 M (pH 6.5)
2. 20 mM glutathione
3. 25 mM CDNB in 95% ethanol
4. Enzyme extract: Homogenised the sample with Tris-HCl buffer (pH 7.2). The homogenate was filtered and the filtrate was centrifuged at 4°C for 30 min at 8500 rpm. The supernatant was used as enzyme source.

Procedure

Preparation of enzyme extract

Ground about 5 g of the sample in a medium and made up to 20 ml with the medium containing 50 mM Tris HCl (pH 7.2, 0.4 M sorbitol and 10 mM NaCl. Centrifuged the homogenate at 2000g for 10 min and used the supernatant for the assay.

Assay

0.5 ml of K_2HPO4 buffer was taken in a test tube, and 0.1 ml of CDNB was added. Added 8.8 ml of glass distilled water. Incubated the tubes at 37°C for 10 min. Then 0.5 ml of glutathione was added to the reaction mixture. Added 0.2 ml of enzyme extratct to the reaction mixture. Run a blank like test except by adding enzyme. Measured the absorbance at 340 nm.

Glutathione-s-transferase activity in the extract is expressed as µmoles of CDNB-GSH conjugate / min / mg protein.

APPENDIX -XVIII
ESTIMATION OF ASCORBIC ACID
(Roe and Kuether, 1953)

Principle

Ascorbate is converted to dehydroascorbate by treatment with activated charcoal or bromine. Dehydro ascorbic acid then reacts with 2,4-dinitrophenyl hydrazine to form osazones, which dissolves in sulphuric acid to give an orange-coloured solution whose absorbance can be measured spectrophotometrically at 540 nm.

Reagents

1. 4% TCA
2. 9N H_2SO_4
3. 2% 2,4-dinitrophenyl hydrazine – dissolved 2g of DNPH in 100 m of 9N H_2SO_4.
4. 10% thiourea
5. 80% sulphuric acid
6. Stock standard: Dissolved 100 mg of ascorbic acid in 100 ml with 4% TCA.
7. Working standard: Diluted 10 ml of the stock solution to 100 ml with 4% TCA.

Procedure

1 g of the sample was homogenized in 4% TCA upto 10 ml. Centrifuged at 2000rpm for 10 minutes. The supernatants obtained were treated with a pinch of activated charcoal. Shaken well and kept for 10 minutes. Centrifuged and.0.5 and 1.0 ml aliquots of this supernatant were taken for the assay. The assay volumes were made upto 2.0 ml with 4% TCA. The working standard solution containing 20 – 100 µg of ascorbate were pipetted out, the volumes of which were also made upto 2.0 ml with 4% TCA. Added 0.5 ml of 2% DNPH reagent to all the tubes, followed by 2 drops of 10% thiourea solution. Incubated at 37°C for 3 h . The osazones formed were dissolved in 2.5 ml of 85% sulphuric acid, in cold, drop by drop, with no appreciable rise in temperature. To the blank alone, DNPH reagent and thiourea were added after the addition of sulphuric acid. After incubation for 30 minutes at room temperature, the absorbance was read spectrophotometrically at 540 nm.

APPENDIX- XIX
ESTIMATION OF TOCOPHEROL
(Emmerie-Engel method, 1938 as described by Rosenberg, 1992)

Principle

Tocopherols can be estimated using Emmerie- Engel reaction which is based on reduction of ferric to ferrous ions by tocopherols, which then forms a red colour with 22'dipyridyl tocopherols and carotenes are first extracted with xylene and the extinction read at 460 nm to measure carotenes. Correlation is made for these after adding ferric chloride and reading at 520 nm.

Reagents

1. Absolute alcohol
2. Xylene
3. 2,2' dipyridyl: 1.2g of $FeCl_36H_2O_2$ in one litre of ethanol stored in brown bottle.
4. Standard solution of D, L - tocopherol 10 mg/l in absolute alcohol. 91 mg of tocopherol is equivalent to 100 mg of tocopherol acetate.

Procedure

Into 3 stoppered centrifuge tubes (test, standard and blank), pipetted out 1.5 ml of each tissue extract, 1.5 ml of standard and 1.5 ml of water respectively. To the test and blank added 1.5 ml of the ethanol and to the standard, added 1.5 ml of water. Added 1.5 ml of xylene to all the tubes, stoppered, mixed well and centrifuged.

Transferred 1.5 ml of xylene layer, into another stoppered tube, taking care not to include any ethanol or protein. Added 1.0 ml of 2,2' dipyridyl reagent to each tube, stoppered and mixed. Pipetted out 1.5 ml of the mixtures into colorimeter cuvettes and read the extinction of test and standard against blank at 460 nm. Then in turn beginning with the blank, added 0.33 ml of $FeCl_3$ solution. Mixed well and after exactly 15 minutes read test and standard against the blank at 520 nm.

The value of vitamin E can be calculated using the formulae:

$$\text{Amount of tocopherols} = \frac{(\text{Reading at 520nm} - \text{Reading at 460 nm}) \times 0.29 \times 15}{\text{Reading of standard at 520 nm}}$$

APPENDIX -XX
ESTIMATION OF TOTAL CAROTENOIDS
(Zakaria *et al.*, 1979)

Principle

The total carotenoids in the sample were extracted in petroleum ether. The total carotenoids were estimated in UV/visible spectrophotometer at 450 nm.

Reagents

1. Petroleum ether
2. Anhydrous sodium sulphate
3. Calcium carbonate
4. 12% alcoholic potassium hydroxide

Procedure

Weighed 5-10g (w) of the sample and saponified for about 30 minutes in a shaking water bath at 37°C after extracting the sample in 12% alcoholic KOH. Transferred the saponified extract into a separating funnel (packed with glass wool and $CaCO_3$) containing 10 to 15 ml of petroleum ether and mixed gently. Taken up the carotenoid pigments into the petroleum ether layer. Transferred the lower aqueous phase to another separating funnel, and the petroleum ether extract containing the carotenoid pigments to an amber-coloured bottle. Repeated the extraction of the aqueous phase similarly with petroleum ether, until it is colourless. Discarded the aqueous phase. To the petroleum ether extract added a small quantity of anhydrous Na_2SO_4 to remove turbidity. Noted the final volume of the petroleum ether extract, and diluted by a known dilution factor (v). The absorbance of the extract at 450nm (P) was noted in a spectrophotometer using petroleum ether as a blank.

$$\text{Amount of total carotenoids present} = \frac{P \times 4 \times V \times 100}{W} \ \mu g$$

APPENDIX- XXI
EXTRACTION AND ESTIMATION OF FLAVONOIDS
(Cameron *et al.*, 1943)

Extraction

A portion of the ground plant material was weighed out and extraction was carried out in two steps, firstly with MeOH: H_2O (9:1) and secondly with MeOH: H_2O (1:1). At each step, sufficient solvent was added to make a liquid slurry and the mixture was left for 6-12 h. filtration to separate the extract from the plant material was carried out rapidly by using a glass wool or cotton wool plug in the neck of a filter funnel. The two extracts were then combined and evaporated to about $1/3^{rd}$ of the original volume or until most of the MeOH had been removed. The resultant aqueous extract was cleared of low polarity contaminants such as fats, terpenes, chlorophylls and xanthophylls by extraction (in a separating funnel) with hexane or chloroform. This was repeated several times and the extracts combined. The solvent-extracted aqueous layer containing the bulk of the flavonoids was then concentrated.

Reagents

1. Vanillin reagent - 1% vanillin in 70% conc. H_2SO_4
2. Catechin standard - 110 µg/ml

Procedure

An aliquot of the extract was pipetted into a test tube and evaporated to dryness. Then added 4ml of vanillin reagent and heated for 15 minutes in a boiling water bath. A standard was also treated in the same manner. Then the optical density was read at 340 or 360nm.

APPENDIX -XXII
ESTIMATION OF POLYPHENOLS
(Malick and Singh, 1980)

Phenol reacts with phospho molybdic acid in Folin — Ciocalteau reagent in alkaline medium and produce blue coloured complex (Molybdenum blue), which is read in a spectrophotometer at 650 nm.

Reagents

1. 80 % ethanol
2. Diluted Folin-Ciocalteau reagent
3. 20 % sodium carbonate
4. Stock standard: 100 mg of catechol in 100 ml of water
5. Working standard: 10 ml of the stock standard was diluted to 100 ml. 1 ml of this contains 100 µg of catechol

Procedure

1.0 g of the sample was homogenized using 20 ml of 80 % ethanol. The homogenate was centrifuged at 10,000 rpm, for 20 minutes. The supernatant was saved. The residue was re-extracted with 10 ml of 80 % ethanol. Centrifuged and collected the supernatant. Pooled the supernatant and evaporated to dryness. The residue was dissolved in a known volume of distilled water (5 ml) and 2.0 ml was taken for the experiment.

0.5-2.5 ml of working standard catechol solution corresponding to 50-250 µg of catechol, were pipetted out into a series of test tubes. The volume was made upto 2.5 ml with water. To all the tubes, added 0.5 ml of diluted Folin-Ciocalteau reagent. After 3 minutes added 2.0 ml of 20 % sodium carbonate solution to each tube and mixed thoroughly. The tubes were placed in a boiling water bath for exactly 1 minute. Cooled and measured at 650 nm against a reagent blank.

Plotting the concentrations of catechol on X-axis and absorbance on Y-axis. From the standard graph, the amount of polyphenols present in the samples were estimated and expressed as mg of polyphenols per g of the sample.

APPENDIX- XXIII
ESTIMATION OF REDUCED GLUTATHIONE
(Moron *et al.*, 1979)

Principle

Reduced glutathione is measured by its reaction with 55'-dithio 2-nitro benzoic acid (DTNB) to give a compound that absorbs at 412 nm.

Reagents

1. 5% TCA
2. Sodium phosphate buffer (0.2M) pH 8.0
3. DTNB solution – 0.6mM in 0.2M phosphate buffer
4. Standard glutathione – Dissolved 10 mg of reduced glutathione in 100 ml of 5% TCA

Procedure

1 g of the sample was homogenized in 5% TCA to give a 20% homogenate. The precipitated protein was centrifuged down at 100 rpm for 10 minutes. The homogenate was cooled on ice and 0.1 ml of supernatant was taken for the estimation. The volume of

aliquot was made up to 1 ml with 0.2 M sodium phosphate buffer (pH 8.0). 2 ml of freshly prepared DTNB solutions was added to the tubes and the intensity of yellow color formed was read at 412nm in a spectrophotometer after 10 minutes.

A standard curve of GSH was prepared using concentrations ranging from 2 to 10 nanomoles of GSH in 5% TCA.

APPENDIX -XXIV
ESTIMATION OF TANNINS
(Schanderl, 1970)

Tannin-like compounds reduce phosphotungstomolybdic acid in alkaline solution to produce a highly coloured blue solution, the intensity of which is measured in a spectrophotometer at 700nm.

Reagents

1. Folin-Denis reagent

Dissolved 100g of sodium tungstate and 20g of phosphomolybdic acid in 750ml distilled water in a suitable flask and added 50ml phosphoric acid. Refluxed the mixture for 2h and made up to one litre with distilled water. Protected the reagent from exposure to light.

2. Sodium carbonate solution

Dissolved 350 g sodium carbonate in one litre of distilled water at 70-80°C. Filtered through glasswool after allowing it to stand overnight.

3. Standard tannic acid: Dissolved 100mg tannic acid in 100ml of distilled water.

4. Working standard solution: Diluted 5ml of stock solution to 100ml with distilled water. One ml contains 50µg tannic acid.

Procedure

Extraction of tannins

Weighed 0.5g of the powdered material and transferred to a 250ml conical flask. Added 75ml alcohol. Heated the flask gently and boiled for 30min. centrifuged at 2000 rpm for 20min and collected the supernatant in 100ml volumetric flask and made up the volume. Transferred 1ml of the sample extract to a 100ml volumetric flask containing 75ml alcohol. Added 5ml of Folin-Denis reagent, 10mlof sodium carbonate and diluted to 100ml with water. Mixed well. Read the absorbance at 700nm after 30min. If absorbance is greater than 0.7 made a 1+4 dilution of the sample. Prepared a blank with water instead of the sample. Prepared a standard graph by using 0-100µg of tannic acid.

Calculation

Calculated the tannin content of the sample as tannic acid equivalents from the standard graph.

APPENDIX- XXV
EXTENT OF INHIBITION OF *IN VITRO* LIPID PEROXIDATON
(Okhawa *et al.*, 1979)

An *in vitro* model of goat liver homogenate was used for induction of lipid peroxidation, mediated by $FeSO_4$ as a pro-oxidant. Application of the relevant plant

tissue extract in the medium was tried with an objective of assessing the extent of inhibition of *in vitro* lipid peroxidation by the measurement of Thio Barbituric Acid Reactive Substances (TBARS) in the experimental mixtures. TBARS were measured spectrophotometrically at 535nm.

Reagents

1. Tris Buffered Saline (TBS): 10mM Tris, 0.15 M NaCl, pH 7.4
2. Ferrous sulphate: $FeSO_4$ was prepared fresh in TBS and added at 10 µmoles final concentration in the assay medium.
3. 1% Thio Barbituric Acid- 1g TBS in 100ml of hot water or TBS
4. 70% alcohol
5. Acetone
6. 5% Goat liver homogenate prepared in TBS (cold)

Procedure

A 5% liver homogenate was prepared in TBS (cold). 50µl of it was used in the assay. 0.5g of fresh plant tissue was weighed accurately and homogenized in 1ml of cold TBS. 50µl of it was used in the assay. Ferrous sulphate at a final concentration of 10µmoles was added in the assay medium to induce oxidation. The final volumes in the test tubes were made up to 500 µl with cold TBS.

Controls were prepared for each sample, containing the respective plant extract (50µl), liver homogenate (50µl) and TBS to make up the final volume to 500µl. Pro-oxidant was not added to the control tubes.

A blank containing no plant extract, no liver homogenate, but only $FeSO_4$ and TBS to make a final volume of 500µl, was also prepared. An assay medium corresponding to 100% oxidation was prepared by adding all the other constituents except the relevant plant extracts, and the volume made up to 500µl with cold TBS. The experimental medium corresponding to auto-oxidation contained only the liver homogenate, and TBS to make up the final volume to 500µl. All the tubes were incubated at 37°C for 1 hour.

Following the incubation period, 500µl of 70% alcohol was added to all the tubes to stop the reaction. 1ml of 1% TBA was added to all the tubes, followed by boiling in a hot water bath for 20 min. After cooling to room temperature, the tubes were centrifuged. To the clear supernatants collected *in toto* added 500µl of acetone and measured the TBARS at 535nm in a spectrophotometer.

APPENDIX -XXVI
DETERMINATION OF INHIBITION OF SUPEROXIDE GENERATION (Varley, 1969)

The extent of superoxide generation was studied on the basis of inhibition in the production of nitroblue tetrazolium formazon of the superoxide ion by the plant sample measured colorimetrically at 560 nm.

Reagents

1. EDTA (0.1 M containing 1.5 mg NaCN/ 100 ml)

2. NBT (1.5 mM)
3. 0.12 mM riboflavin
4. 0.067M phosphate buffer, pH 7.8
5. Dimethyl sulfoxide

Procedure

Herbal extracts (20 mg concentration) was employed as sample. The assay tubes contained test sample with 0.2ml of EDTA, 0.1 ml NBT, 0.05 ml riboflavin and 2.55 ml of phosphate buffer. The control tubes were also set up where in DMSO was added instead of sample.

All the tubes were vortexed and measured the initial optical density at 560 nm. After that, these tubes were placed in an area where they received uniform illumination for 30 minutes. Again the optical density was measured at 560 nm. The difference in optical density before and after illumination is the quantum of superoxide production and the percentage of inhibition by the test sample was calculated by comparing with the optical density of control.

APPENDIX- XXVII
DETERMINATION OF INHIBITION OF NITRIC OXIDE GENERATION
(Green and Hill 1982)

Aqueous solution of sodium nitro prusside spontaneously generates nitric oxide (NO) at physiological pH, which interacts with oxygen to produce nitrite ion, which is measured colorimetrically.

Reagents

1. Phosphate buffered saline
2. Sodium nitroprusside (100 mM)
3. Griess reagent (1% sulfanilamide, 2% H_3PO_4, 0.01% Naphthalene diamine dihydrochloride)

Procedure

Herbal extracts served as sample. 3 ml of reaction mixture containing sodium nitroprusside in PBS and extract was incubated at 25°C for 150 minutes. Controls were kept without test compound in an identical manner. After incubation, 0.5 ml of reaction mixture was removed and 0.5 ml of Griess reagent was added. The absorbance of the chromophore formed was read at 546 nm.

The percentage inhibition of nitric oxide generation was measured by comparing the absorbance values of control and those of test compounds.

APPENDIX - XXVIII
ESTIMATION OF HAEMOGLOBIN
(Drabkin and Austin, 1932)

Blood is diluted with an alkaline solution of potassium cyanide and potassium ferricyanide, haemoglobin is oxidized to methaemoglobin which then combines with cyanide to form cyanmethaemoglobin which is measured colorimetrically at 540nm.

Reagents

1. Drabkin's reagent: this reagent contains 0.05g of potassium cyanide, 0.20g of potassium ferricyanide and 1g of sodium bicarbonate in 1litre of distilled water (pH 9.6).
2. Cyanmethaemoglobin standard: This was obtained commercially and had a concentration of 16g/dl.

Procedure

The reaction mixture in a volume of 5.02ml contained 5.0ml of Drabkin's reagent and 0.02ml of blood. The reaction mixture was kept at room temperature for 5 minutes to ensure the completion of the reaction and read at 540nm against a reagent blank containing reagent alone.

The haemoglobin content was expressed as g/dl blood.

APPENDIX-XXIX
ENUMERATION OF RED BLOOD CORPUSCLES
(Sanderson and Phillips, 1981)

The total erythrocyte count was determined accurately by diluting a measured quantity of blood with a fluid isotonic solution by the method of Huxtable.

Reagents

Red blood cell dilution fluid (Hayem's fluid) – 5g of sodium sulphate, 1g of sodium chloride, 0.5g of mercuric chloride were dissolved in 200ml of distilled water.

Procedure

Blood was sucked exactly upto the 0.5ml mark in the RBC pipette and the diluting fluid was drawn immediately upto the mark and the blood mixed thoroughly with the diluting fluid. It was left for 2-3 min for proper mixing. The Neubauer counting chamber was placed along with its cover glass in position. The capillary stem of the pipette was emptied which contains only the diluting fluid. This was done by discarding first 3-5 drops.

Charging of the counting chambers

One drop of dilute blood was released into the groove of the Neubauer counting chamber. It was left for cells to settle for 2-3 min. the counting chamber was put under the microscope and the ruled area was located.

Erythrocytes were counted in the 5 squares of the counting area of 1mm square. The number of cells in the 4-corner square was counted.

Calculation

The total number of cells found in 5 groups of 16 squares is multiplied by 10,000 to give the number of cells in millions/mm of blood.

APPENDIX-XXX
ENUMERATION OF WHITE BLOOD CORPUSCLES
(Sanderson and Phillips, 1981)

WBC diluting fluid or Truk's fluid was used as the diluent which can destroy RBC's.

Reagents

WBC diluting fluid was prepared by mixing

1. Glacial acetic acid

2. Gentian violet 1%
3. Water 95ml

Procedure

The method of counting is similarly to RBC counting except that the count is made in 4 large (1mm) cover squares of the Neubauer counting chamber.

Calculation

The total number of cells in 4 squares is multiplied by a factor of 2500 to give the count/mm of blood.

APPENDIX-XXXI
DETERMINATION OF DIFFERENTIAL LEUCOCYTE COUNT
(Sanderson and Phillips, 1981)

The film was prepared by Leishman's method.

Leishman's method

A Romanowosky type stain designed to differentiate leucocyte was used.

Reagents

1. Leishman's stain powder – 0.15g
2. Methyl alcohol, acetone free – 100ml

Procedure

Leishman's stain powder was taken in a glass mortar and few ml of methyl alcohol was added. The stain was ground to dissolve completely. The stain was then transferred to a 100 ml measuring cylinder. Few ml of methyl alcohol was added into mortar and ground. The same was repeated till the Leishman's stain powder is completely dissolved. The volume was made upto 100ml with methyl alcohol. The stain was poured into a clean dry bottle and closed well and used after 2-3 weeks.

The blood film was placed in a level position and the dry blood film was covered with the stain, which should be evenly distributed over the entire slide. At the end of one minute, the quantity of buffer solution or distilled water was doubled carefully and mixed with the stain by means of the clean pipette. The film was allowed to stain for 7 or 8 minutes and the excess stain was removed by washing with the distilled water for 2 min. The water was then washed off with fresh distilled water. The film was dried in air. When the film was dried, it was then examined microscopically.

APPENDIX-XXXII
PLATELET COUNT
(Sanderson and Phillips, 1981)

Reagents

Dacies fluid: This was prepared by dissolving 5.0g of sodium citrate and 1ml of 40% formaldehyde and made upto 100ml with distilled water. To 19ml of this solution 1ml of 0.2% brilliant cresyl blue solution was added just before use. This solution was filtered and used.

Procedure

Venous blood collected with EDTA was used for platelet count. 0.05ml of blood was diluted with 0.95ml of Dacies fluid and mixed well using a narrow bore Pasteur

pipette, the counting chamber was filled with the diluted blood. The cells were allowed to settle to the bottom of the chamber for 15min. To prevent from drying, the chamber was placed in a petridish, which contained a piece of wet filter paper.

Using the 40X objective with reduced condenser aperture the platelets were counted in 1/5 sq. mm^{-5} of the small squares of the large center square. From this the number of platelets in cu.mm of blood was calculated as,

$$\frac{\text{Cells x Blood dilution x Chamber depth}}{\text{Area of chamber counted}}$$

Platelet count is expressed as number of cells/mm.

APPENDIX- XXXIII
DETERMINATION OF SERUM BILIRUBIN
(Malloy and Evelyn, 1937)

Reagents

1. 0.7ml of Diazo reagent (0.3ml of solution A (0.5% of sodium nitrate) +10ml of solution B (1g of sulphanilic acit in 15ml of conc. HCl and made up to one litre with water)
2. 0.7ml of 1% Sulphanilic acid
3. 3.5ml of Methanol
4. Standard bilirubin (20-100µg/ml of chloroform)

Procedure

To 0.2 ml of serum added 5.4ml of water. Pipetted out 2.8ml of this in to second tube for blank. To the test added 0.7ml of diazo reagent and to the blank 0.7ml of 1%sulphanilic acid solution. Mixed, allowed to stand for 5 minutes and added 3.5 ml of methanol to each tube. To the standard bilirubin added 3.5ml of methanol, 0.7ml of diazo reagent and 1.8ml of water, read at 540nm.

APPENDIX- XXXIV
ESTIMATION OF BLOOD UREA
(Netlson, 1957)

Blood urea was estimated by DAM-TSC method.
Urea reacts directly with diacetyl monoxime in the presence of thiosemicarbazide to form a red colour product, which is measured at 540nm.

Reagents

1. Reagent A: 50mg of ferric chloride, 0.2ml of water, 1ml of o-phosphoric acid and 2.5ml of water.
2. Reagent B: 50ml of con. sulphuric acid and 450ml of water
3. Reagent C: 1g of diacetyl monoxime in 50ml water.
4. Reagent D: 250mg of thiosemicarbazide in 50ml of water.
5. Reagent I: Mixed 0.25ml of Reagent A with 500ml of Reagent B (prepared freshly)

6. Reagent II: Mixed 33.5ml of reagent C with 33.5ml of Reagent D. Diluted freshly to 500ml.
7. Stock standard: Dissolved 100mg of urea in 100ml of distilled water. This solution was prepared in a saturated solution of benzoic acid for long use.
8. Working standard: 2ml of stock standard was diluted to 100ml with distilled water. 1ml of this solution contains 20µg of urea.

Procedure

To 0.2ml of blood 1.8ml of 10% TCA was added, mixed well and after 10min centrifuged. 0.5ml of the supernatant was taken and made up the volume to 3ml with water and then added 2ml of reagent I followed by 2ml of reagent II. Mixed well, stoppered with marbles and heated vigorously in a boiling water bath for 20min. Blank and standards (10-50µg) were treated similarly. Removed the tubes, cooled and read against the blank.

The amount of urea was expressed as mg/dl.

APPENDIX -XXXV
ESTIMATION OF CREATININE
(Owen et al., 1954)

Reagents

1. 1ml of 10% sodium tungstate
2. 2ml of 2/3N sulphuric acid
3. 1ml of 0.04M picric acid
4. 1ml of 0.75N sodium hydroxide
5. Standard: 10-50µg

Procedure

0.2ml of serum was taken and added 3ml of water, 1ml of 10% sodium tungstate and 2ml of 2/3N sulphuric acid. Kept for 10minutes and centrifuged. 3ml of supernatant was taken and added 1ml of 0.04 M picric acid and 1ml of 0.75N sodium hydroxide and allowed to stand for 20 minutes. Blank and standard were treated similarly. The colour developed was read at 500nm.

APPENDIX- XXXVI
ESTIMATION OF ALKALINE PHOSPHATASE (ALP)
(Raghuramulu *et al.*, 1983)

Reagents

1. Disodium phenyl phosphate 100mmole/l
2. Sodium carbonate-bicarbonate 100mmole/l
3. Buffer-substrate-equal volumes of solution 1 and 2.
4. Phenol reagent of Folin-Ciocalteau
5. Sodium carbonate solution-150g of anhydrous sodium carbonate per litre.
6. Stock standard phenol-1g/l (1g of phenol in 100mmole per litre hydrochloric acid).
7. Working standard-100ml of dilute phenol reagent. 5ml of stock standard diluted to 500ml with water. This contains 10µg phenol/ml.

Procedure

Pipetted out 4ml of buffer substrate in a test tube and placed in a water bath at 37°C for a few minutes, added 2ml of serum, mixed, stoppered and left in a bath exactly for 15 minutes. Removed, added 1.8ml of diluted phenol reagent. Set up control containing 4ml buffer substrate and 0.2ml of serum to which added 1.8ml of dilute phenol reagent. Mixed and centrifuged. Took 4ml of supernatant from each and added 2ml of sodium carbonate. Standard was prepared by adding 2ml of sodium carbonate to 4ml of working standard containing phenol reagent. Placed the three tubes in 37°C bath for 15 minutes and read at 700nm against a blank.

The King-Armstrong unit corresponds to the liberation of 1mg of phenol by 100ml of serum under the assay condition and is calculated from the difference between the test and the control readings.

APPENDIX -XXXVII
ESTIMATION OF SERUM ASPARTATE TRANSAMINASE (AST) AND ALANINE TRANSAMINASE (ALT)
(Reitman and Frankel, 1957)

The serum is treated with the substrate and the liberated oxaloacetic acid/pyruvic acid is allowed to react with 2,4-dinitro phenyl hydrazine and then with sodium hydroxide. The colour developed is read at 540nm and compared with pyruvate standard readings which is also treated similarly at 37°C.

Reagents

1. Buffer substrate: 100 mmole/l phosphate buffer and 2 mmole/l 2-oxoglutarate with 100mmole/l L-aspartate included for AST or 200 mmole/l DL-alanine for ALT. Dissolved 15g K_2HPO_4, 2g KH_2PO_4 and 300mg 2-oxoglutaric acid in 700-800ml of water and (a) for AST added 15.7g L-aspartate, monosodium salt or (b) for ALT added 17.8g DL-alanine. In both cases checked the pH, adjusted to 7.4- with sodium hydroxide.
2. 4-Dinitro phenyl hydrazine (DNPH), 1 mmole (200mg)/l in 1 mole/l hydrochloric acid.
3. Sodium hydroxide solution 400 mmole (16g/l).
4. Pyruvate solution 2mmole/l (22mg sodium pyruvate/100ml).

Procedure

Added 2mlof serum to 1ml of buffer substrate, mixed and incubated for 60min for AST or 30 min for ALT at 37°C in a water bath. Removed, added 1ml of DNPH, allowed to 20 min at room temperature, added 10ml of sodium hydroxide solution, mixed and after 5 min, read at 500-550nm. For the blank put up 0.2ml of serum, 1ml of buffer substrate and 1ml of DNPH, mixed and completed as the test.

A standard curve is prepared by putting up several control sera of known AST and ALT values covering the range up to 50-70U/l.

APPENDIX -XXXVIII
ESTIMATION OF BLOOD GLUCOSE
(Raghuramulu *et al.*, 1983)

Reagents

Orthotoluidine=30ml

Thiourea=750mg

Made up to 500ml with glacial acetic acid. Kept at room temperature for 24h and then used.

Standard=100mg of glucose in 100ml of water.

Procedure

To 0.2ml of serum added 1.8ml of distilled water. Mixed well, from this took 0.5ml and then added 5.0ml of orthotoluidine. Kept it in a boiling water bath for 10 minutes, cooled and took the readings at 620nm(red filter). For standard, took 0.2ml of the 100mg% of glucose and treated as above.

APPENDIX -XXXIX
ESTIMATION OF GLYCOGEN
(Good *et al*, 1933)

Reagents

1.30%KOH
2.95% Ethanol
3.2N H_2S0_4
4.NaOH

Procedure

The liver was taken out rapidly from the animal and the excess blood removed by blotting between folds of filter paper and weighed quickly to the nearest 0.1g. Minced the liver and a portion of it was immediately put into a weighed stoppered test tube containing 30% KOH and weighed again. The difference between this weight and the original weight of the tube plus the KOH solution gives the weight of the liver sample used. It was digested in a boiling water bath for 1½ hour. Cooled in ice-cold water. Two volumes of 95% ethanol were then added and the mixture heated just to boiling. Spurting was avoided. It was left to stand overnight in the cold, then homogenized and centrifuged; the precipitate was dissolved in5-10ml warm water. The glycogen was reprecipitated with 2 volumes of 95% ethanol. It was centrifuged and washed several times with 60% ethanol. 2ml of 2N H_2S0_4 per gram of liver was added and hydrolyzed in a boiling water bath for 3-4 hours. The solution was neutralized with NaOH using phenol red as indicator, then made up to a known volume and filtered. Glucose was determined in aliquot. The factor 0.93 was used to convert glucose to glycogen.

APPENDIX -XL
ESTIMATION OF FRUCTOSAMINE
(Armbruster, 1987)

Reagents

1.50mM potassium phosphate buffer (pH 7)
2.100 µm nitro blue tetrazolium.
3.Standard = 40µg-200µg

Procedure

The reaction mixture contained 1.5ml of 50mM potassium phosphate buffer, 1.4ml of 100µm nitroblue tetrazolium and 0.1ml of serum in a final volume of 3 ml.

Incubated at 25°C for 30 minutes. The standards were also treated as above and read the colour developed at 530nm.

APPENDIX XLI
ESTIMATION OF GLUCOKINASE
(ATP: D-Hexose-6- β-phosphotransferase)
(Brandstrup *et al.*, 1957)

Reagents

1. 5mM glucose solution; 90mg of glucose was dissolved in 100ml of distilled water.
2. 72mM ATP solution: 36.6g of ATP was dissolved in 100ml of distilled water.
3. 50mM magnesium chloride solution: 596mg of magnesium chloride was dissolved in 100ml of distilled water.
4. 12.5mM potassium dihydrogen phosphate: 170mg of potassium dihydrogen phosphate was dissolved in 100ml of distilled water.
5. 0.1M potassium chloride solutions: 746mg of potassium chloride was dissolved in 100ml of distilled water.
6. 0.5M potassium fluorides: 2.1g of sodium fluoride was dissolved in 100ml of distilled water.
7. Tris-HCl buffer- 0.01M, pH 8.0: 121mg of Tris (hydroxyl methyl) methylamine was dissolved in 100ml of distilled water and the pH was adjusted to 8.0 by using 0.01 M hydrochloric acid.
8. 10% trichloroacetic acid

Procedure

The reaction mixture in a total volume of 5.0ml contained the following viz., 1.0ml of glucose solution, 0.5ml of ATP solution, 0.1ml of magnesium chloride solution, 0.4mlof potassium dihydrogen phosphate, 0.4mlof potassium chloride, 0.4ml of sodium fluoride and 2.5ml of Tris-HCl buffer (pH 8.0). The mixture was pre-incubated at 37°C for 5 min. the reaction was initiated by the addition of 2ml of tissue homogenate. 1 ml of the reaction mixture was immediately removed to the tubes containing 1ml of 10% TCA which was considered as zero time. A second aliquot was removed after 30min incubation at 37°C. The protein precipitate was removed by centrifugation and residual glucose in the supernatant is estimated by the O-toluidine method.

The enzyme activity was expressed as μmoles of glucose phosphorylated/min/mg protein

APPENDIX-XLII
ESTIMATION OF LIVER GLUCOSE-6-PHOSPHATASE
(Glucose-6-Phosphate Phospho Hydrolase)
(Koida and Oda, 1959)

Reagents

1. 0.1M citrate buffer pH 6.5
2. Substrate: Glucose-6-phosphate–0.01M: 15mg of glucose-6-phosphate was dissolved in 10ml of distilled water.
3. 10% Trichloro aceticacid

4. Ammonium molybdate solution: 2.5g of ammonium molybdate was dissolved in 100ml of 3N H_2SO_4.
5. Amino Naphthol Sulfonic Acid (ANSA): 500mg of 2-amino-4-naphthol sulfonic acid was dissolved in 195ml of 15% sodium bisulfite and 5ml of 20% sodium sulfite solution was added for complete solubilisation. The solution was filtered and stored in the brown bottle.

Procedure

Incubation mixture contains 0.7ml of citrated buffer, 0.3ml of substrate and 0.3ml of tissue homogenate. The reaction mixture was incubated at 37°C. for 1h addition of 1ml of 10% TCA to the reaction tubes terminated the reaction of the enzyme. The suspension was centrifuged and the phosphorous content of the supernatant was estimated by the method of Fiske and Subbarow. The enzyme activity was expressed as μmoles of inorganic phosphorus liberated/min/mg protein.

APPENDIX- XLIII
ESTIMATION OF LIVER GLUCOSE-6-PHOSPHATE DEHYDROGENASE
(Kornberg and Horecker, 1955)

Reagent

1.2ml of 0.05 m triethanolamine buffer (pH 7.6)
2.0.1ml of 0.01M NADP
3.0.05ml of 0.031 M glucose-6-phosphate

Procedure

Measured into a test tube 2ml of triethanolamine buffer, 0.1ml of NADP and 1ml of liver homogenate. Mixed and allowed to stand for 5 minutes at 25°C.Added 0.05ml of glucose-6-phosphate and after about 2 minutes, read the extinction at 340nm every minute for 5 minutes. Used a blank with tissue homogenate plus buffer but without NADP and glucose-6-phosphate. The glucose-6-phosphate dehydrogenase activity was measured by the initial rate of reduction of $NADP^+$ at 25°C by following the increase in absorption at 340nm.

APPENDIX-XLIV
EXTRACTION OF LIVER LIPIDS
(Radin, 1981)

Reagents

Extraction solvent (hexane: isopropanol 3:2 v/v)

Procedure

For each gram of tissue added 18 ml extraction solvent and homogenized thoroughly. After 30-60 seconds of mixing, centrifuged and transferred the supernatant into a 25 ml graduated flask. The insoluble residue was resuspended in 3 ml extraction solvent and centrifuged after 5 minutes. Repeated with another 3 ml of solvent and finally made upto 25 ml. Aliquots were used for the estimation of phospholipids.

APPENDIX-XLV
ESTIMATION OF TOTAL CHOLESTEROL
(Flegg, 1972)

Principle

Cholesterol esters in serum are hydrolysed by esterase. The free cholesterol produced is oxidized to form hydrogen peroxide which further reacts with phenol and 4-amino antipyrene by the catalytic action of peroxidase to form a red coloured quinoneimine dye complex. Intensity of the colour formed is directly proportional to the amount of the cholesterol present in the sample.

$$\text{Cholesterol ester} + H_2O \xrightarrow{\text{Cholesterol esterase}} \text{Cholesterol} + \text{Fatty acids}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{Cholesterol oxidase}} \text{Cholestermone} + H_2O$$

$$H_2O_2 + \text{4-AAP} + \text{Phenol} \xrightarrow{\text{Peroxidase}} \text{Red quinoneimine dye} + H_2O$$

Reagents

R1 Enzyme reagent	-	2 x 20 ml / 4 x 20ml
R2 Enzyme reagent	-	2 x 5ml / 4 x 5l
R3 Cholesterol std	-	3ml (200mg)
R4 Precipitating reagent	-	1x 5ml / 2x 5 ml
R5 HDL standard	-	3ml (25 mg/dl)

Reagent Preparation

Mix 4 parts of R1 (enzyme reagent1) and one part of R2 (enzyme reagent 2) (1 ml working reagent - 0.8 ml of R1 and 0.2 ml of R2). Working reagent is stable for atleast 8 weeks when stored at 2-8°C.

Procedure

Took dry test tubes and labelled as Blank (B), Standard (S) and test (T). Pipetted out 1 ml of working reagent in all the 3 tubes. Then added 10µl of distilled water in Blank, 10µl of standard solution in (S) tube, 10µl of sample in (T) tube. All the tubes were mixed well, and incubated at 37°C for 5 minutes or at room temperature (25°C) for 15 minutes. Measured the absorbance of the standard and test against blank within 60 minutes at 505 nm.

Calculation

$$\text{Cholesterol (mg/dl)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times 200$$

APPENDIX-XLVI
ESTIMATION OF HDL CHOLESTEROL
(Flegg, 1972)

Reagents

R1 Enzyme reagent	-	2 x 20 ml / 4 x 20ml
R2Enzyme reagent	-	2 x 5ml / 4 x 5l

R3 Cholesterol standard	-	3ml (200mg)
R4 Precipitating reagent	-	1x 5ml / 2x 5 ml
R5 HDL standard	-	3ml (25 mg/dl)

Procedure

Taken100µl of precipitating reagent (R4) and 100µl of sample in a test tube. Mixed well and incubated at room temperature for 5 minutes. Centrifuged at 3000-4000 rpm for 8-10 minutes to obtain clean supernatant. Pipette out 1 ml of working reagent in (B), (S) and (T) tubes. Then added 50µl of distilled water in blank, 50µl of HDL standard was added in the standard tube. Separated supernatant 50µl was added in the (T) tubes. Mixed well and incubated at 37°C for 5 minutes or at room temperature (25°C) for 15 minutes. Measured the absorbance of the standard and test against blank within 60 minutes at 505 nm.

Calculation

$$\text{HDL cholesterol (mg/dl)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times 25 \times 2$$

APPENDIX-XLVII
ESTIMATION OF TRIGLYCERIDES
(Bucolobe and David, 1973 Fossati and Principe, 1982)

Principle

$$\text{Triglyceride} + H_2O \xrightarrow{\text{LPL}} \text{Glycerol} + \text{Fatty acid}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{Glycerol kinase}} \text{Glycerol-3-phosphate} + \text{ADP}$$

$$\text{Glycerol-3-phosphate} + O_2 \xrightarrow{\text{GPO}} \text{DHAP} + H_2O_2$$

$$H_2O_2 + \text{4-amino antipyrine} + \text{phenol} \longrightarrow \text{Red colored complex} + H_2O$$

Reagents

Reagent	Code numbers	
	LS-8-50 (ml)	**LS-8-100 (ml)**
R_1 : Reagent 1	2x20	2x40
R_2 : Reagent 2	2x5	2x10
R_3 : Triglycerides Std (200 mg /dl)	1x3	1x3

Preparation of reagents

Working reagents preparation

Mixed 20 ml of Reagent 1, and 5 ml of reagent 2 (stable for 8 weeks at 2-8°C). For obtaining good results, working reagent may be made as and when required (0.8 ml of R1 and 0.2 ml of R2).

Procedure

Taken dry test tubes and labeled as Blank (B), standard (S) and Test (T). Pipetted out 1 ml of working reagent in all the 3 tubes. Distilled water 0.01 ml added in B, standard 0.01 ml in S and 0.01 ml of sample in T was added. Mixed well and incubated for 5 minutes at 37° or at room temperature for 15 minutes. Recorded the absorbance of the standard and test against blank.

Calculation

$$\text{Triglycerides in mg/ dl} = \frac{\text{Absorbance of T}}{\text{Absorbance of S}} \times 200$$

APPENDIX XLVIII
ESTIMATION OF PHOSPHOLIPIDS
(Zilversmit and Davis, 1950)

Reagents

1. 1ml of 5 N H_2SO_4
2. 2 drops of 2N HNO_3
3. 1ml of 2.5% ammonium molybdate
4. 0.1 ml of ANSA

Procedure

An aliquot of the liver lipid extract was pipetted out into a Kjeldahl flask and evaporated to dryness. 1 ml of 5 N H_2SO_4 was added and digested in a digestion rack till it becomes light brown. It was then cooled to room temperature. One or two drops of 2 N HNO_3 were added, digested again till it become colorless. The Kjeldahl flask was cooled. 1 ml water was added and heated in a boiling water bath for 5 minutes. 1 ml of 2.5% ammonium molybdate and 0.1 ml ANSA (0.02 g ANSA + 0.12 g sodium bisulphate = 0.12 g sodium sulphite dissolved in 10 ml of water) were added and the volume made up to 10 ml with distilled water. The absorbance was measured at 660 nm within 10 minutes.

APPENDIX-XLIX
DETERMINATION OF ALBUMIN TO GLOBULIN RATIO
(King and Wootton, 1959)

Reagents

1. 5.8 ml of 28% Sodium sulphate
2. Ether span reagent

Procedure

0.2 ml of plasma was added to 5.8 ml of 28% sodium sulphate in glass stoppered tube and mixed by inversion .1 ml of ether span reagent was added and the stoppered tube gently inverted 20 times, and centrifuged for 10 minutes. The globulin forms a

eater-water interface. A pipette was inserted; 3 ml of supernatant was withdrawn and added to 3 ml of Biuret reagent, after mixing, kept at 37° for 10 minutes. The solution was cooled and the colour read at 540 nm.

Globulin = total protein –albumin

A/G ratio = Albumin reading/(total protein –albumin)

APPENDIX -L
THIOBARBITURIC ACID REACTIVE SUBSTANCES
(Nichans and Samuelson, 1968)

Reagents

1. 0.1 M phosphate buffer (Ph 7.0)
2. 2 ml of TCA-TBA-HCl reagent (15% w/v TCA and 0.37% w/v 2- thiobarbituric acid in 0.25 N HCl)
3. n-butanol

Procedure

One gram of tissue was homogenized with 0.1 M phosphate buffer (pH 7.0). 2 ml of TCA-TBA-HCl reagent (15% w/v TCA and 0.37% w/v 2- thiobarbituric acid in 0.25NHCl) was added to 1 ml of tissue homogenate (or 0.2 ml serum +0.8 ml buffer) and mixed thoroughly. The contents were heated in a boiling water bath for 15 minutes. After cooling, the flocculent precipitate was removed by centrifugation at 100 x g for 10 minutes. The supernatant was shaken with n-butanol to extract the colored complex into the organic phase. The absorbance of the butanol layer was read at 535 nm against a blank that does not contain the sample. The concentration of MDA (TBARS) can be calculated using an extinction coefficient of $1.56 \times 10^5 M^{-1}cm^{-1}$.

APPENDIX-LI
ASSAY OF SUPEROXIDE DISMUTASE (SOD)
(Kakkar *et al.*, 1984)

Reagents

1. 0.025M Sodium pyrophosphate buffer; pH 8.3
2. 186µM Phenazine methosulfate
3. 300µM Nitro Blue Tetrazolium (NBT)
4. 780µM NADH
5. Glacial acetic acid
6. 6N butanol
7. Chloroform
8. Ethanol

Procedure

0.5ml of tissue homogenate was diluted to 1ml with water. Then 2.5ml of ethanol and 1.5ml of chloroform (all reagents chilled) were added. This mixture was shaken for 1min at 4°C and then centrifuged. The enzyme activity in the supernatant was determined.

The assay mixture contained 1.2ml of sodium pyrophosphate (0.025M, pH 8.3), 0.1ml of 186µM phenazine methosulfate, 0.3ml of 300µM NBT, and 0.2ml of 780µM NADH, approximately diluted the enzyme preparation and water in a total volume of 3ml. Reaction was started by the addition of NADH. After incubation at 30°C for 90seconds the reaction was stopped by the addition of 1ml glacial acetic acid. The

reaction mixture was stirred vigorously and shaken with 4ml of n-butanol. The intensity of the chromogen in the butanol layer was measured at 560nm against butanol blank. A system devoid of enzyme served as control.

One unit of enzyme activity is defined as the enzyme reaction, which gave 50% inhibition of NBT reduction in 1min under the assay conditions and expressed as specific activity in units/mg protein.

APPENDIX- LII
ESTIMATION OF ASCORBIC ACID
(Omaye *et al.*, 1979)

Copper to form dehydroascorbic acid diketoglutaric acid oxidized the ascorbic acid. These products when treated with 2,4- dinitrophenylhydrazine (DNPH) form the derivative, bis-2,4 dinitrophenylhydrazone, which undergoes rearrangement to form a product with absorption maximum at 520nm. Thiourea provides a mild reducing medium, which helps to prevent interference from ascorbic acid chromogens.

Reagents

1. 2,4-DNPH reagent: 2.0 g of DNPH was dissolved in 100 ml of 9N H_2SO_4. To this 4.0g of thiourea was added and mixed.
2. Trichloroacetic acid 6%
3. Sulphuric acid 85%
4. Standard ascorbic acid: 10 mg of L- ascorbic acid was dissolved in 100 ml of 4% TCA. This was diluted to prepare a working standard of concentration 100µg/ml.
5. Activated charcoal

Procedure

0.5 ml of tissue homogenate was mixed thoroughly with 1.5 ml of 6%TCA and centrifuged for 20 minutes at 3500g. To 0.5 ml of the supernatant, 0.5 ml of DNPH reagent was added and mixed well. The tubes were allowed to stand at room temperature for an additional 3 hours. Removed, placed in ice-cold water and added 2.5 ml of 85% sulphuric acid and allowed to stand for 30 minutes.

A set of standards containing 10-50 µg of ascorbic acid were taken and processed similarly along with a blank, containing 0.5 ml of 4%TCA. The colour developed was read at 530 nm.

APPENDIX-LIII
ESTIMATION OF α-TOCOPHEROL
(Baker *et al*, 1951)

This method involves a reduction of ferric ion to ferrous ion by α-tocopherol and the formation of red coloured complex with 2,2'-dipyridyl. Absorbance of the chromophore was measured at 520nm.

Reagents

1. Petroleum ether 60-80°C.
2. Double distilled ethanol
3. 0.2%-2,2'-dipyridyl in ethanol
4. 0.5% Ferrric chloride in ethanol
5. Stock standard: 100mg of α-tocopherol in 100ml of distilled ethanol.
6. Working standard: Stock solution was diluted to a concentration of 10µg/ml in distilled ethanol.

Procedure

To 0.1ml of sample 1.5ml of ethanol and 2ml of petroleum ether were added, mixed and centrifuged. The supernatant was evaporated to dryness at 80°C. to this was added 0.2ml of 2,2' dipyridyl solution and 2ml of ferric chloride solution. Mixed well and kept in dark for 5 min and added 2ml of butanol. The intense red colour developed was read at 520nm. Standard tocopherol in the range of 10-100µg were taken and treated similarly along with blank containing only the reagent.

The amount of α-tocopherol was expressed as mg/dl plasma and µM/mg tissue.

APPENDIX-LIV
HISTOPATHOLOGICAL STUDIES
(Culling, 1979)

Reagents

1. 10% Formalin
2. Acetone
3. Xylene
4. Praffin wax
5. Hematoxylin and eosin stain

Procedure

Tissue samples were taken and preserved in 10% formalin solution for a minimum one hour. Formalin was removed from the tissue samples with running water. Dehydration of the fixed tissue was done by giving three changes of acetone (each 100ml). Cleaning of tissue from acetone was effected by three changes of xylene (each 500ml) in total duration of three hours. Incubation of processed tissue in melted paraffin was done by two changes for 3-4 hours in an incubator maintained at 58-60°C. Embedding of the tissue in paraffin wax was then done by immersing the tissue in molten paraffin and then cooling it to harden the paraffin. Section of the paraffin embedded tissue was done using a microtome adjusted to 1-3µ thickness. The paraffin sections were carefully taken on glass slides.The sections were then cleaned by immersing in xylene. The sections were stained with hematoxylene and eosine stain and screened to evaluate the morphology and cellular composition.