Studies on the beneficial effects of dietary fiber on experimentally induced diabetic rats with particular emphasis on glomerular filtration matrix

Thesis submitted to the University of Mysore for the award of

Doctor of Philosophy In Biochemistry

By

G. Suresh kumar M.Sc.,

Department of Biochemistry and Nutrition

Central Food Technological Research Institute

Mysore-570 020, India

July 2005

To my Parents - who gave me everything – that l possess - for their strong support – love, care, and endless prayers – I humbly and respectfully place this effort of mine at their feet.

DECLARATION

I hereby declare that the thesis entitled "**Studies on the beneficial effects of dietary fiber on experimentally induced diabetic rats with particular emphasis on glomerular filtration matrix**" embodies the results of research work done by me, under the guidance of Dr. P.V Salimath, Scientist, Department of Biochemistry and Nutrition Central Food Technological Research Institute. Mysore.

I further declare that the results are not submitted for the award of any other degree or fellowship.

Place: Mysore

Date : (**G. Suresh kumar**)

Dr . Paramahans V. Salimath
Scientist
Department of Biochemistry and Nutrition

CERTIFICATE

I certify that the thesis entitled "**Studies on the beneficial effects of dietary fiber on experimentally induced diabetic rats with particular emphasis on glomerular filtration matrix**" submitted to the University of Mysore, Mysore for award of the Degree of Doctor of Philosophy in Biochemistry by G. Suresh kumar is the result of research work carried out by him under my guidance, at the Department of Biochemistry and Nutrition, Central Food Technological Research Institute, Mysore, during the period 2000-2005.

Paramahans V. Salimath

ACKNOWLEDGEMENT

In the first place, I am thankful to **Dr. P. V. Salimath**, for his excellent guidance and co-operation during the course of this investigation and preparation of thesis.

I am grateful to **Dr. V. Prakash**, Director, CFTRI for providing me an opportunity to work in this Institute. My thanks are due to **Dr. S.G. Bhat**, Head, Department of Biochemistry and Nutrition, and **Dr. R. N. Tharanathan** for the interest shown in the investigation and the valuable suggestions given during the preparation of the thesis. My sincere thanks are due to **Dr. K. Sambaiah** and **Dr. Shylaja M. Dharmesh** for the guidance and help rendered during the course of nutritional studies and in the preparation of the thesis. I extend my heartfelt gratitude to **Dr. G. Muralikrishna** for encouragement given during the course of this investigation. I thank **Dr. P. Saibaba** for help rendered with Animal House Facility.

I must also thank Mr. Madesh and Mr. Rudraiah, Deputy Registrars of the University of Mysore, Mysore for the award of fellowship of University of Mysore.

I thank J. L Urs, Mrs. Rita Das and other members of Animal House for the help rendered during animal experiments. My thanks are due to Asha, Mukund, Shivaswamy and other staff of Central Instruments Facility and Services (CIFS) of CFTRI for the technical assistance rendered in the usage of various equipments. Mr Shantakumar and

Seetaramaiah of Glass Blowing Section and Members of the Library for the prompt help rendered whenever required.

I am thankful to Dr. Mahadevamma, Mrs. Revathi Baskaran and my other colleagues Subba Rao, Nandini, Nirmala, Harish Prashanth, Harish Nayaka, Rachappaji, Srinivas, Kazi, Vijayalakshmi, Mrs. Hemalatha, Revanappa, Rashmi, Siddaraju, Manjunath and my other labmates who have helped me at various times during this investigation.

I am grateful to the Monozyme Co. India Ltd and Mr. Kumar for timely help.

I am deeply indebted to all my family members, friends, relatives and those who continuously encouraged directly or indirectly to enable me to carryout this work successfully.

CONTENTS

	Page No
List of Abbreviations	
Synopsis	**I - IX**
CHAPTER I - Introduction	**1 - 41**
CHAPTER II - Materials and Methods	**42 - 87**
CHAPTER III - Results and Discussion	**88 - 203**
3.1 Studies on the role of spent turmeric on diabetic status in streptozotocin induced diabetic rats	**88 - 145**
3.1.1 Summary and Conclusions	**146 - 148**
3.2.a Studies on the role of *Emblica officinalis* on diabetic status in streptozotocin induced diabetic rats	**149 - 155**
3.2.b Chemical and biological studies on the polysaccharides of *Emblica officinalis*	**156 - 185**
3.2.1 Summary and Conclusions	**186 - 188**
3.3 Phenolic acids of *Curcuma longa* and *Emblica officinalis*	**189 - 201**
3.3.1 Summary and Conclusions	**202 - 203**
CHAPTER IV - Bibliography	**204 - 226**

List of Abbreviations

Abbreviations

μg	Microgram(s)	NMR	Nuclear magnetic resonance spectroscopy
mg	Milligram(s)		
g	Gram(s)	SEM	Scanning electron microscopy
mm	Millimeter(s)	$[\alpha]_D$	Specific rotation
μL	Microlitre(s)	cm	Centimeter(s)
mL	Millilitre(s)	L	Litre(s)
D_2O	Deuterium oxide	min	Minute(s)
h	Hour(s)	N	Normal
^oC	Degree Celsius	M	Molar
mM	Millimolar	Rha	Rhamnose
nM	Nanometer	Fuc	Fucose
eV	Electron volts	Ara	Arabinose
meq	Milliequivalents	Xyl	Xylose
V	Volts(s)	Man	Mannose
v/v	Volume/volume	Gal	Galactose
w/v	Weight/volume	Glc	Glucose
rpm	Revolutions per minute	GalA	Galacturonic acid
PC	Paper chromatography	DF	Dietary fibre
TFA	Trifluoroacetic acid	SCFA	Short chain fatty acid
HPLC	High performance liquid Chromatography	DM	Diabetes mellitus
		SEM	Standard error of means
HPSEC	High performance size Exclusion chromatography	BuA	Butyric acid
		GLC	Gas liquid chromatography
GC-MS	Gas liquid chromatography mass spectrometry	FTIR	Fourier transform infrared spectroscopy

Synopsis

Diabetes is a disease of great concern to many all over the world and is characterized by its complications such as diabetic neuropathy, retinopathy and nephropathy. Diabetic nephropathy leading to kidney damage is one of the serious concerns and has aroused lot of research interest. During diabetic nephropathy changes in glomerular basement membrane (GBM) are known to take place making the glomeruli thicker. The structure and composition of GBM is of importance for its function as filtration barrier in the glomerulus. The glomerular filtration matrix, which is essential for normal filtration to take place is formed of heparan sulphate, type IV collagen and laminin. During diabetes thickening of GBM is known to take place due to reduction in heparan sulfate and laminin and accumulation of type IV collagen leading to abnormal filtration.

Dietary management of diabetes is now well established to be one of the means in the management of diabetes. In this category, dietary fibers and foods rich in dietary fibers are well accepted to have beneficial functions in the management of diabetes. But there are not many scientific investigations on the role of these dietary fibers in minimizing complications of diabetes, such as diabetic nephropathy. In recent years, fermentation of dietary fibers to short chain fatty acids (SCFA), such as acetate, propionate and butyrate is being increasingly recognized to play beneficial functions in normal physiological condition and also in alleviating pathological conditions such as cancer, diabetes, etc. Our laboratory has shown that butyric acid ameliorates diabetic and diabetic nephropathy state.

Among the foods that possess beneficial effects on diabetes, dietary fiber rich foods are one of the important classes. Spent turmeric, a by-product of turmeric industry is one of the rich sources of dietary fibers. Hence, one of the objectives was to study the effects of dietary fiber rich food, spent turmeric, a by-product of curcumin industry, on diabetic and diabetic nephropathy state with particular emphasis on glycoconjugate metabolism in glomerular basement membrane.

The thesis contains four chapters. Chapter I; Introduction, II; Materials and Methods, III; Results and Discussion along with Summary and Conclusions; IV Common Bibliography. Results and Discussion of the thesis presented in Chapter II is divided into three subsections. Section 3.1 deals with studies on the role of spent turmeric on diabetic status in streptozotocin induced diabetic rats and summary and conclusions of this section are presented in 3.1.1. The section 3.2 is divided into two subsections. Section 3.2.a deals with studies on the role of *Emblica officinalis* on diabetic status in streptozotocin induced diabetic rats and section 3.2.b. deals with chemical and biological studies on the polysaccharides of *Emblica officinalis*. Summary and conclusions of these two subsections (3.2.a and 3.2.b) are presented in section 3.2.1. The studies carried out on phenolic acids of *Curcuma longa* and *Emblica officinalis* is presented in section 3.3 and its summary and conclusions are presented in sections 3.3.1.

Chapter I: Consists of general introduction on diabetes, complications of diabetes such as diabetic nephropathy, nutritional management of

diabetes and biological activity of polysaccharides are provided with proper literature citation. At the end, scope of the present study is given.

Chapter II: This chapter deals with Materials and Methods that are employed in this investigation. Colorimetric, spectroscopic, microscopic, analytical methods, isolation procedures, methods employed in structural analysis and histopathological methods are detailed.

Chapter III: In this chapter results obtained are discussed with appropriate literature citations. Section 3.1 deals with effect of spent turmeric in streptozotocin induced diabetic rats. Spent turmeric is used as a rich source of dietary fiber. Water intake, urine sugar, urine volume, fasting blood sugar were increased during diabetes in the starch fed diabetic group (SFD) and these were alleviated to different extents by feeding spent turmeric (TFD). Diet intake in the spent turmeric fed group was more and could be due to caloric dilution and palatability. Decreased body weight in the starch fed diabetic group was augmented by spent turmeric (TFD) feeding.

Activities of disaccharidases such as maltase, sucrase and lactase were increased in the intestine and decreased in the kidney during diabetes when compared to controls and these enzyme activities were alleviated in spent turmeric fed diabetic group.

Glomerular filtration rate (GFR) was tested in terms of creatinine clearance. Weight of the kidney and size of the glomeruli were

increased in the starch fed diabetic group (SFD) and the feeding of spent turmeric ameliorated them.

Weights of various organs (expressed as g/100g body weight) such as liver, spleen, lungs, brain, heart and testis were increased to different extents during diabetes and was prevented by feeding spent turmeric in the diet. Effect of spent turmeric on glycoconjugate constituents in different tissues were analysed in terms of total sugar, uronic acid, amino sugar, sulfate and proteins and these were ameliorated to different extents by the feeding of spent turmeric. Not much effect was observed in the sulfate content in kidney. Protein content was decreased during diabetes and was prevented by the feeding of spent turmeric to some extent.

Glycosaminoglycans (GAGs) were isolated from different organs and their content was decreased in the starch fed diabetic group (SFD) and was ameliorated by the feeding of spent turmeric in the diet. Constituents of GAGs such as total sugar, uronic acid, aminosugars and sulfate were decreased during diabetes and they were alleviated in the spent turmeric fed diabetic group (TFD).

Effect of spent turmeric on some of the enzymes of glycoconjugate metabolism like L-glutamine fructose-6-phosphate amino transferase (GFAT), N-acetyl-β-glucosamindase (NAG) and β-glucuronidase in kidney were studied. The activities of GFAT, N-acetyl-β-glucosaminidase increased during diabetes and was ameliorated by the

feeding of spent turmeric. There was no significant change in β-glucuronidase activity during diabetes.

Since the major emphasis was to study changes in glomerular basement membrane during diabetic nephropathy state, an attempt was made to study in depth, the role of spent turmeric in minimizing the complications of diabetic nephropathy state with particular emphasis on glycoconjugate metabolism. Hence GAGs were isolated from the kidney and the contents of GAGs were analysed. In the starch fed diabetic group (SFD) the content of GAGs was decreased and was ameliorated by feeding spent turmeric (TFD). Total sugar, uronic acid and amino sugar contents decreased during diabetes and spent turmeric feeding prevented the decreased contents. The decrease in sulfate content indicated decrease in sulfated GAGs and it was ameliorated by the feeding of spent turmeric diet. Further total GAGs were fractionated into heparan sulfate and chondroitin sulfate using chondroitinase ABC. Analysis of this fraction clearly indicated that heparan sulfate was the major GAG in GBM. Results indicated that during diabetes, heparan sulfate content decreased and the feeding of spent turmeric in the diet ameliorated it. This was further confirmed by agarose gel electrophoresis. The electrophoretic pattern revealed the presence of heparan sulfate as the predominant GAG and chondroitin sulfate was in minor amounts. The results clearly indicated a decrease in heparan sulfate content during diabetes and was alleviated in spent turmeric fed diabetic group.

Histopathological studies of kidney sections was carried-out by a combination of alcian blue and **P**eriodic **A**cid **S**chiffs (PAS) staining. Alcian blue stains glycosaminoglycans, which is blue in color and was positive in control kidney sections. Trace intensity of pink color was observed due to PAS stain. PAS positive glycoproteins were elevated during diabetes and there was alleviation in spent turmeric fed diabetic group. The observed alterations due to Alcian blue-PAS stain during diabetes was alleviated by the feeding of spent turmeric during diabetes.

Immunohistochemsitry was done with primary antibody for type IV collagen and secondary antibody tagged with FITC. The results clearly indicated increased content of type IV collagen during diabetes, which was ameliorated during diabetes by the feeding of spent turmeric. The changes in type IV collagen were further confirmed by estimating the content of hydroxyproline and was estimated as a measure of type IV collagen. Our studies clearly showed that the content of type IV collagen increased during diabetes (SFD) and was modulated by the feeding of spent turmeric (TFD). This clearly depicted that spent turmeric has beneficial effect in countering diabetic nephropathy status that occurs in the glomerular basement membrane with particular emphasis on heparan sulfate and type IV collagen. Section 3.1.1 contains summary and conclusion of this section.

The other food material studied for the effect on diabetes and diabetic nephropathy status was *Emblica officinalis.* Many Ayurvedic preparations use *Emblica officinalis* in many of the ailments such as

cancer, immunomodulatory disorders, atherosclerotic, including diabetes.

Section 3.2 is subdivided into "a" and "b" sub-sections. 3.2.a deals with Results and Discussion of the studies on the role of *Emblica officinalis* on diabetic status in streptozotocin induced diabetic rats.

Diabetes was induced in rats using streptozotocin and the rats were grouped into starch fed control and diabetic (SFC/SFD), *Emblica officinalis* fed control and diabetic (EFC/EFD). The rats fed with *Emblica officinalis* at 1.0% level developed diarrhea with in 3 days and hence the experiment was terminated. The 0.5% *Emblica officinalis* fed diabetic group (SFD) showed increased diet intake and loss of body weight when compared to starch fed diabetic group (SFD). Urine sugar, urine volume, fasting blood glucose and glomerular filtration rate were increased in *Emblica officinalis* fed diabetic group (EFD) when compared to starch fed diabetic group (SFD) and hence *Emblica officinalis* did not shown beneficial effects at 0.5% concentration on diabetic and diabetic nephropathy status. At lower concentration of *Emblica officinalis* i.e. 0.1% and 0.025% levels also no significant alleviations in the diet intake and body weight were observed when compared to control and diabetic groups. No beneficial effects were observed in terms of urine sugar, urine volume, water intake, fasting blood glucose and glomerular filtration rate by the feeding of *Emblica officinalis* during diabetes when compared to starch fed diabetic group.

Sections 3.2.b deals with chemical and biological studies on the polysaccharides of *Emblica officinalis*.

Since no beneficial effects were observed by *Emblica officinalis* at 1.0, 0.5, 0.1, and 0.025% concentrations on diabetic and diabetic nephropathy state, an attempt was made to study the polysaccharides present in *Emblica officinalis*, if they have any biological functions and then study chemical nature of such polysaccharides. Various polysaccharides were isolated from *Emblica officinalis* such as water soluble, pectic polysaccharide, hemicellulose A and hemicellulose B and were analyzed for the carbohydrate composition, antioxidant activity and wound healing activity. Pectic polysaccharide showed potential antioxidant activity and wound healing property.

The pectic polysaccharides isolated from *Emblica officinalis* was fractionated on DEAE-cellulose and the column was eluted with water, ammonium carbonate (0.1 to 0.5 M) and alkali (0.1,0.2 M) and checked for the antioxidant activity. The fraction eluted with 0.2 M ammonium carbonate showed good antioxidant activity. Carbohydrate composition of DEAE eluted fractions was studied. The 0.2 M ammonium carbonate eluted fraction had 37% of uronic acid and 51% was galactose and contained trace amounts of arabinose and xylose. Since, 0.2 M Ammonium Carbonate eluted Fractions (ACF) showed good antioxidant activity it was taken for detailed investigation. Homogeneity criteria and molecular weight determinations were done with cellulose acetate electrophoresis, HPLC and Sepharose CL-4B column chromatography. Purity and molecular weight were found to be 96% and 55 Kda,

respectively. The purified pectic polysaccharide (ACF) was subjected to structural analysis using methylation anlysis, GC-MS, Smith degradation, periodate oxidation, formic acid release, optical rotation, FT-IR and NMR. By combination of these structural studies, the ACF having potential antioxidant activity was found to have galacturonic acid backbone in α-1,4 linkages with branches through O-6. The branches had galactose, arabinose and rhamnose. Galactose was the terminal sugar. Section 3.2.1 deals with summary and conclusions of 3.2.a and 3.2.b.

Section 3.3 deals with content of free and bound phenolics of *Emblica officinalis* and C*urcuma longa* and their antioxidant activity was studied using reducing power ability, free radical scavenging activity and DNA damage. Results clearly showed that phenolics of *Emblica officinalis* have better antioxidant activity when compared to phenolics of C*urcuma longa*.

Separation and identification of free and bound phenolics of *Emblica officinalis* and C*urcuma longa* was done using HPLC.

Section 3.3.1 deals with summary and conclusions of 3.3.

Chapter IV: Finally the thesis concludes with a collective bibliography citation for all the chapters.

Chapter I

Introduction

Definition

Diabetes mellitus is a syndrome characterized by chronic hyperglycaemia, which affects large number of people around the globe. The Roman Aretares meaning 'to run through a siphon' gave the term 'diabetes'. The sweetness of the diabetic urine was first mentioned in Ayurveda of Susruta. The term 'mellitus' was given by Cullen, which means 'honeyed'[1]. The global figure of people with diabetes is set to rise from the current estimate of 150 million in 2010 to 300 million in 2025 [2]. Diabetes occurs due to lack of insulin or dysfunction of insulin. Insulin allows glucose (sugar) to enter body cells to convert it into energy, synthesize protein and store as fats (Fig. I). During diabetes there is a decrease in the anabolism and an increase in the catabolism (Fig. II). Diabetes mellitus may be diagnosed clinically by the presence of characteristic symptoms such as glucosuria, excessive thirst, polyuria, unexplained weight loss and the consequences lead to one or more complication associated with the disease.

Causes for diabetes

Factors involved in the onset of diabetes mainly are, 1. genetic 2. environmental factors (virus, diet, stress, life style, malnutrition in utero, etc.) 3. immunological factors 4. pancreatic pathology 5. age, 6. pregnancy, etc.

Diabetes is generally classified into two main forms: Type-I diabetes is primarily due to autoimmune-mediated destruction of pancreatic β-cells, resulting in absolute insulin deficiency. People with Type-I diabetes must take exogenous insulin for survival and hence is called

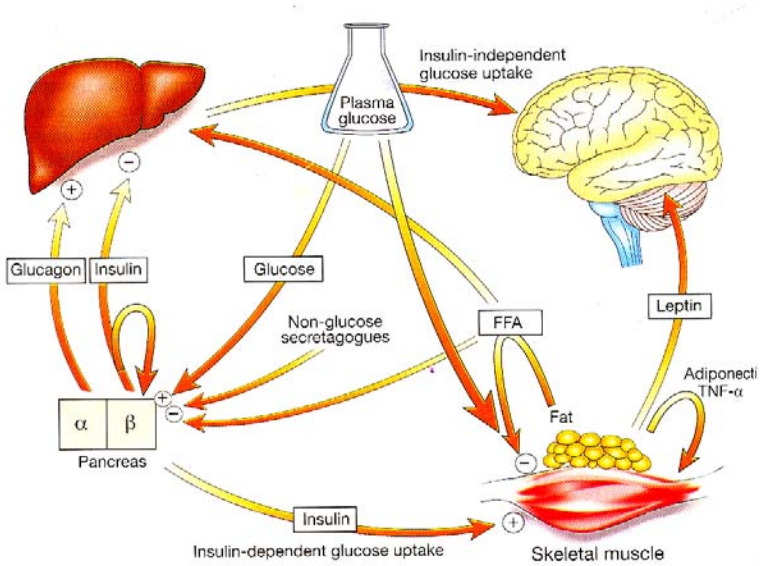


Fig. I. Cross talk in the regulation of glucose metabolism.

Source*:* Alan RS, Ronald KC. *Nature* **414** (2001) 804.

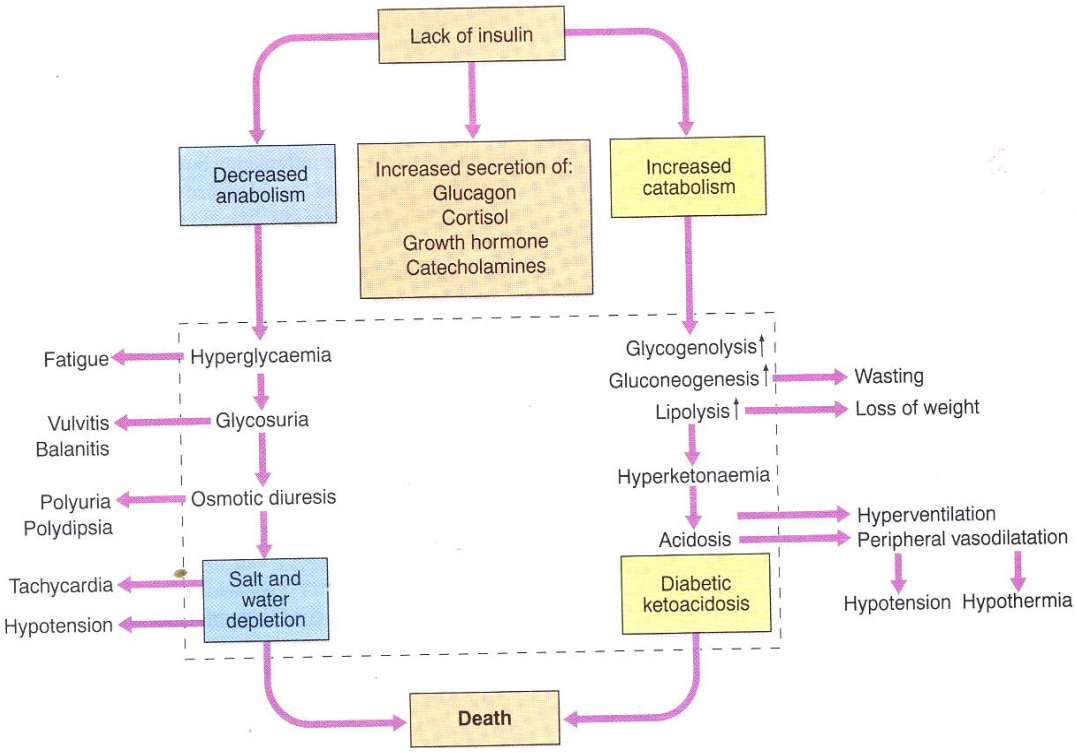


Fig. II. Metabolic consequences of lack of insulin and their symptoms.

Source: Davidson, **1999**. Principles and Practice of Medicine: New York, 18th Edn. pp 479.

insulin dependent diabetes mellitus (IDDM). Type - II diabetes is characterized by insulin resistance and/or abnormal insulin secretion either of which may predominate. People with Type II diabetes are not dependent on exogenous insulin and hence belong to non-insulin dependent diabetes mellitus (NIDDM). The other types are listed in Table I.

Metabolism during diabetes

Carbohydrate metabolism

Body needs glucose as major energy source. In normal metabolism, complex carbohydrates are converted in to simpler form like glucose, which is absorbed by intestine and is stored as glycogen in the liver and muscle. Insulin promotes utilization of glucose by tissues and lowers the blood glucose. During diabetes decreased synthesis of insulin or abnormal functions of cells towards the insulin leads decreased insulin level or abnormal functions of cells towards the insulin and leads to changes in carbohydrate metabolism in different tissues. In liver and kidney there is an increase in gluconeogensis resulting in the formation of glucose leading to acidosis[3]. In retina and nerves there is an increase in enzymatic conversion of glucose from glycolytic pathway to the polyol pathway, where glucose is converted to sorbitol and is oxidized to fructose by sorbitol dehydrogenase, which may complicate microvascular diseases [4].

Protein metabolism

The protein after digestion is converted into amino acids which are absorbed into the blood from the gastrointestinal tract, and utilized for

the biosynthesis of proteins, enzymes etc. Any amino acid that is not required is stored as nitrogen in liver, but the carbon skeleton is used for the synthesis of glucose and fat, which become available for energy or

Table I. Classification of diabetes and its cause

Types of diabetes	Causes
Type I (IDDM)	IA classical IB Autoimmune
Type II (NIDDM)	Non obese NIDDM obese NIDDM
Malnutrition related diabetes mellitus	Maturaity onset diabetes of the young fibrocalculous pancreatic diabetes Protein deficient pancreatic diabetes
Other types	Pancreatic diseases Hormonal etiology Drug or chemical induced Certain genetic syndromes Insulin receptor antibodies Other conditions
Impaired glucose tolerance (IGT)	IGT in non-obese and obese IGT in maturity onset diabetes of the young (MODY) IGT in other conditions
Gestational diabetes (GDM)	-Do-

Source: Anderson JW, Geil GB, **1994**. In: Shills. M.E. Olson. J.A. Shike. M.A. (Ed.) Modern Nutrition in Health and Disease. 8th Edn. Philadelphia. USA. pp 1259.

storage. In diabetic condition, there is increased protein catabolism and an increase in ketone bodies in the blood and urine and decreased utility of amino acids for protein synthesis resulting in weight loss and muscle wasting[3].

Fat metabolism

Fats are nothing but esterified fatty acids with glycerol. Before absorption they are broken down and they are reformed after absorption for storage. Metabolism of fats is similar to carbohydrates during diabetes. The concentration of free fatty acids rises in diabetic individuals due to the mobilization of fatty acids from adipose tissue by lipolysis of triglycerides [5]. Fatty acids in the liver are converted in to acetyl Co-A that gets converted into ketone bodies. During diabetic condition there is an increase in the production of these ketone bodies viz., acetoacetate and 3-hydroxybutyrate resulting in hyperketonuria [6].

Biochemistry of diabetic complication

Development of diabetes-specific microvascular and macrovascular pathology lead to diabetic neuropathy, retinopathy and nephropathy.

Mechanism of hyperglycaemia-induced damage[7]

Four main hypotheses about how hyperglycaemia causes diabetic complications have generated a large amount of data. These hypotheses are (Fig III): (i) increased polyol pathway flux; (ii) increased advanced glycation end-products (AGEs) formation; (iii) activation of protein kinase C (PKC) isoforms; and (iv) increased hexosamine pathway flux.

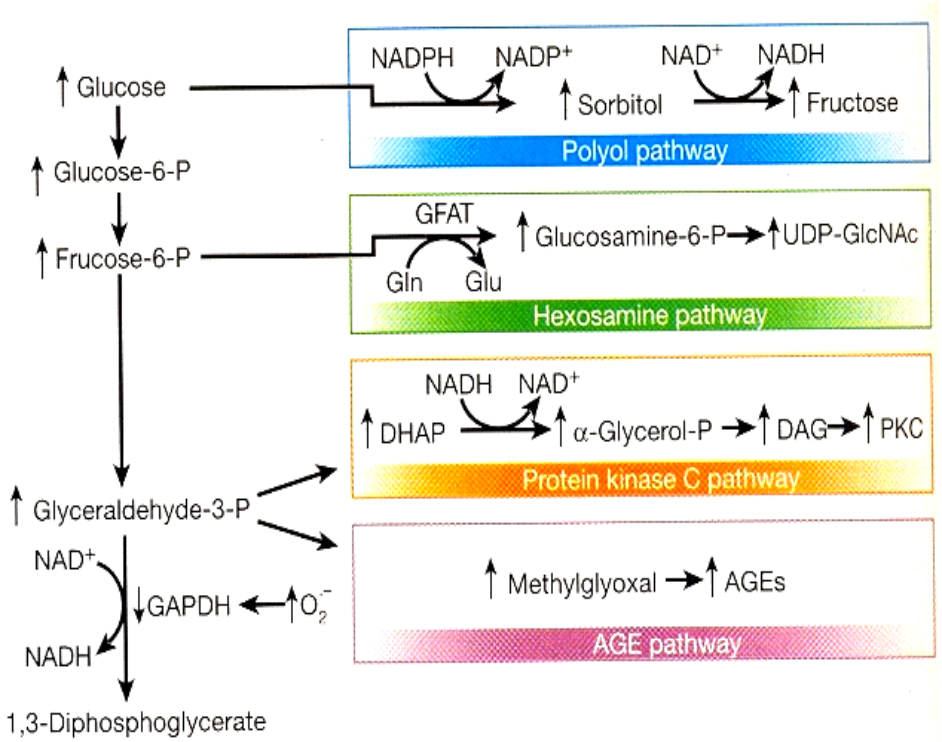


Fig. III. Potential mechanism by which hyperglycaemia induced mitochondrial superoxide overproduction activates four pathways during diabetic condition.

Source: Alan RS, Ronald KC. *Nature*, **414** (2001) 804.

Increased polyol pathway flux

Aldose reductase (present in brain, nerves, aorta, muscle, erythrocytes and ocular lens) has low affinity for glucose [8], but in a hyperglycaemic environment, increased metabolic intermediates of glucose such as sorbitol are formed due to increased enzymatic conversion. Sorbitol is oxidized to fructose by the enzyme sorbital dehydrogenase and accumulates in high concentration in tissues such as nerve and lens. This accumulation could lead to changes in osmotic pressure, causing the cells to swell, thereby damaging the tissues. Contribution of this pathway to diabetic complications may be very much species, site and tissue specific.

Increased intracellular formation of Advanced Glycation End Products (AGEs)

AGEs are formed due to increased intracellular auto-oxidation of glucose to glyoxal. Decomposition of the Amadori products (glucose derived 1-amino-1-deoxyfructose lysine) leads to 3-deoxyglucosone (perhaps accelerated by amadoriase). Fragmentation of glyceraldehyde 3-phosphate and dihydroxyacetone phospahate results in methylglyoxal and 3-deoxyglucosone. These products react with amino groups of intracellular and extracellular proteins to form AGEs, which are detoxified by the glyoxalase system [9] under normal condition. The AGE precursor 3-deoxymethyl glyoxalate is substrate for other reductases [10]. Production of intracellular AGE precursors damage target cells by three general mechanisms.

a) Intracellular proteins modified by AGEs have altered function.

b) Extracellular matrix components modified by AGEs precursors interact abnormally with other matrix components and with the receptors for matrix proteins on cells [7].

c) Plasma proteins modified by AGEs precursors bind to AGEs receptors on endothelial cells, mesangial cells and macrophages, inducing receptor-mediated production or reactive oxygen species. These AGEs receptor ligation activates the pleiotropic transcription factor NF-kB, causing phathological changes [11].

Activation of protein kinase C (PKC)

The PKC family comprises at least eleven isoforms, nine of which are activated by the lipid second messenger – diacyl glycerol (DAG). Hyperglycaemia increases the amount of DAG[12], in microvascular cells, retina and renal glomeruli. Hyperglycemia may also activate PKC isoforms indirectly through AGEs receptors [13] and increased activity of polyol pathway [14]. Activation of PKC-β isoforms has been shown to mediate retinal and renal blood flow abnormalities [15] .

In addition to affecting hyperglycaemia-induced abnormalities of blood flow and permeability, studies have shown that activation of PKC contributes to the increased microvascular matrix protein accumulation by inducing expression of TGF-β1 due to which there is increased synthesis of fibronectin and type IV collagen both in cultured mesangial cells [16] and in glomeruli of diabetic rats [17].

Increased flux through hexosamine pathway

Shunting of excess intracellular glucose into the hexosamine pathway might also cause several manifestations of diabetic complications. In this pathway, fructose-6-phosphate is diverted from glycolysis to provide substrates for reactions that require UDP-N-acetylglucosamine for proteoglycan synthesis and the formation of O-linked glycoproteins. In hyperglycaemia, the rate-limiting enzyme glutamine: fructose-6-phosphate aminotransferase (GFAT) is involved in the conversion of glucose to glucosamine. This further induces increase in the transcription of TGF-α, TGF-β and PAI-1[18]. This pathway has an important role in the diabetic nephropathy as shown in Fig. IV.

Complications of diabetes

The causes of the long-term complications of diabetes are even more perplexing. One of the many complications is the thickening of the basement membrane that surround walls of the capillaries. Complications are not a single disease but rather a heterogeneous group of diseases. Diabetic people are two times more likely to have heart attacks or strokes than normal. Complications of diabetes are mainly diabetic retinopathy, neuropathy and nephropathy[19, 20] .

Retinopathy

This is one of the most common complications. In diabetes the chances of becoming blind is almost 25 times higher than normal. Venous dilation, loss of pericytes, endothelial cells proliferation with

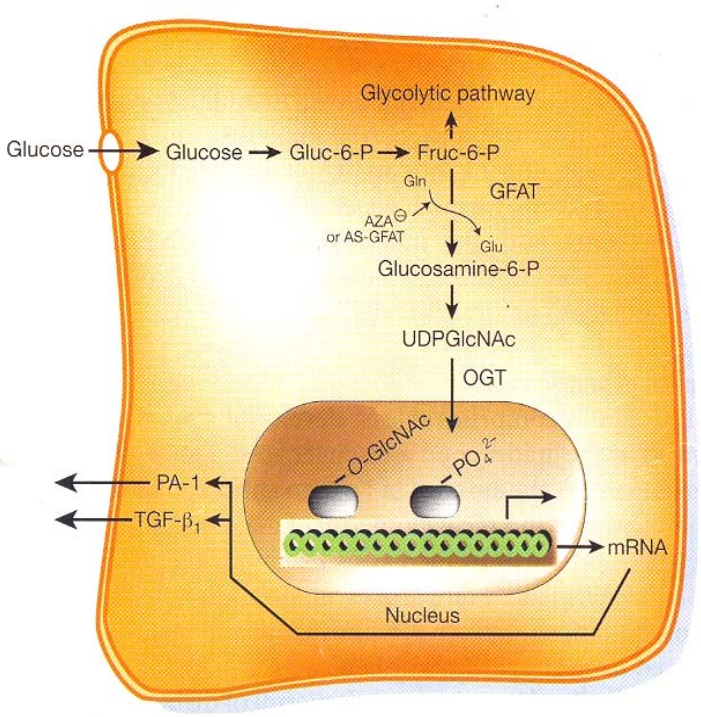


Fig. IV. The hexosamine pathway.

Source: Alan RS, Ronald KC. *Nature,* **414** (2001) 815.

hyalinization, wrinkling of Descemet's membrane and increased refractive power of lens[21] are some of the symptoms associated with the retinopathy.

Neuropathy

The reported frequency of neuropathy among diabetic patients ranges from 5-60%. The frequency increases with age and duration. The symptoms associated with neuropathy are morphological abnormality of nerve fibres, non-painful numbness of the toes and the front of the foot, burning pain, fullness of skin of the toes, feet, legs, etc[22] .

Nephropathy

Nephropathy in Type-I diabetic patients is well defined and progresses through five stages [23]. The complications due to diabetic nephropathy in type II diabetic patients are less well characterized due to high cardiovascular risk. The development and progression of diabetic nephropathy is influenced or modulated by several factors such as, age, gender, susceptibility to nephropathy, glycaemic control, hypertension, protein intake, dyslipoproteinemia, smoking and proteinuria [24] .The kidney is the target organ for clinically important manifestations of the secondary microvascular complications of diabetes mellitus[25]. Whole kidney enlargement is an early feature of experimental and human diabetes [26, 27]. In animals, nephromegaly occurs within 4 days of diabetes onset and most IDDM patients have large kidney at diagnosis. This enlargement is due to a combination of tubular hypertrophy and hyperplasia and interstitial expansion [28, 29], and is probably a response to increased glucose and fluid filtration and their active reabsorption. The

effects of disturbed insulin production in streptozotocin treated rats showed similarities with diabetic nephropathy [30]. It is also established that there is no direct toxic effect of streptozotocin (STZ) on the kidney [31].This suggests that the STZ diabetic rat model may be considered as a useful model for studying glomerular filtration matrix comparable to human diabetic nephropathy.

Normally there are 350-1050×10^6 glomeruli and it accounts for 1% of total kidney volume [32]. Their contribution to whole organ enlargement is insignificant and each comprises a convoluted knot of capillaries supported by a scaffold of mesangium, made up of cellular and matrix components. Blood enters the glomerulus via one or more afferent arterioles and leaves via usually one efferent vessel (Fig. V3). Each capillary comprises a basement membrane (Fig. V6), which is continuous with the mesangium and is lined by a fenestrated endothelium. The outer surface of the basement membrane is covered by an epithelium of interdigitating foot processes (Fig. V5). No two adjacent foot processes arise from the same epithelial cell, and they are separated by a narrow filtration slit and membrane. The glomerular tuft is surrounded by Bowman's capsule, which is continuous with the basement membrane of the proximal convoluted tubule. Haemofiltration occurs from within the capillary, through the endothelial fenestrae across the basement membrane, through the filtration slits and across the filtration membrane, into the urinary space defined by Bowman's capsule and thus into the tubular part of the nephron (Fig. V2). Glomerular enlargement occurs within days of onset of experimental diabetes [33]. Animal studies suggest that most of the enlargment is due to

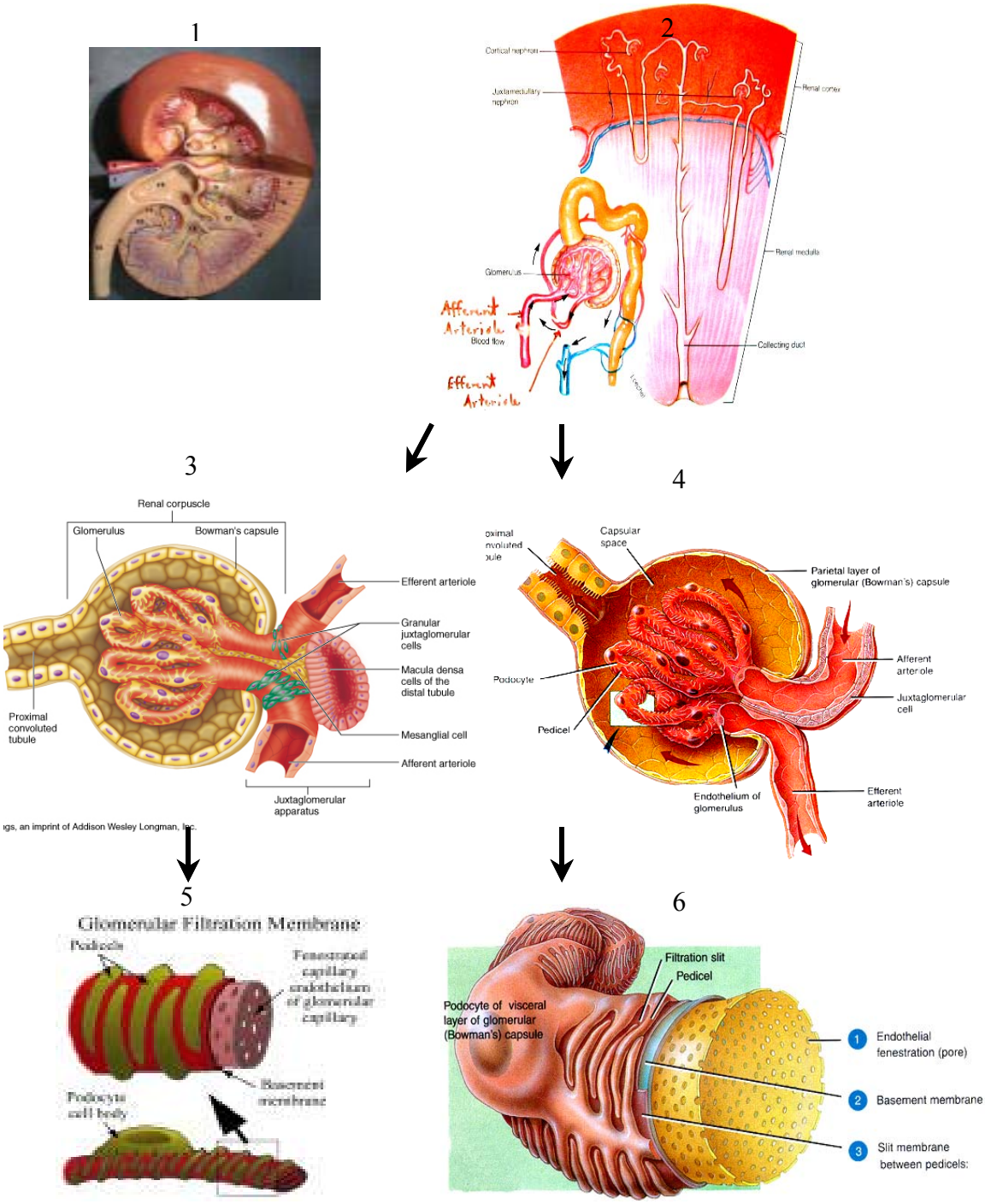


Fig. V. 1 - Kidney, 2 - Nephron, 3 & 4 - Glomeruli, 5&6 - Basement membrane.

Source: Internet collections of Google.

an increase in capillary length and diameter.

Normally, mesangial matrix comprises predominatly type IV collagen, with smaller proportions of laminin, fibronectin and entactin, and the proteoglycans chondroitin and dermatan sulphate. This matrix is arranged around dense mesh of microfibrils, which forms a plexus around the mesangial cells. At the mesangial-endothelial cell interface and the attachment points of the capillary basement membrane, the microfibrils are more tightly organized [34]. This arrangement lends itself to various functions of the mesangium, such as providing a support for the capillaries that is contractile and thus able to adjust the tension in the basement membrane, and forming a mesh that can entrap, neutralize and dispose of circulating macromolecules and pathogens. Matrix is produced by epithelial and endothelial cells on the surface of the Glomerular Basement Membrane (GBM), and also by the mesangial cells themselves[35]. Degradation and recycling is a function of the mesangium [36] and also possibly macrophages.

The GBM represents size and charge-selective barrier of the glomerulus. It is composed of a backbone of type IV collagen which makes up the lamina densa, flanked on either side by layers with different composition, the lamina rara interna and externa. The lamina rara externa contains heparan sulphate porteoglycan (HSPG) and attachment proteins such as laminin, entactin and other glycoproteins. The structure and composition of the GBM is of importance for its function as a filtration barrier in the glomerulus. Mesangial cells (MC) are embedded in an Extra Cellular Matrix (ECM) between the

capillaries and play a critical role in the modulation of glomerular blood flow and filtration by contraction and relaxation. Experiments with electrically neutral dextran molecules of differing sizes has shown that there is unrestricted permselectvity of the GBM for molecules < 60,000 molecular weight, which have an effective radius[37] of 24 A^o . The physical characteristics of albumin (radius 36A^o) therefore, albuminuria is a sensitive marker of disordered permselectivity.

There is probably little effective prevention of protein filtration by the endothelial fenestrae. However, GBM provides both a size restriction in the form of collagen meshwork and a negatively charged electrostatic barrier of proteoglycan molecules [38]. The negative charge sites are concentrated in the lamina rara interna (Fig. VI) and externa in normal kidney. The course of diabetic nephropathy is mainly characterized by changes of urinary albumin excreted and glomerular filtration rate. As mentioned above, the major pathological changes in diabetic nephropathy are thickening of the GBM (Fig. VIII) and mesangial matrix expansions. Most data have been reported on abnormalities in glomerular type IV collagen, laminin, fibronectin and heparan sulfate content[39, 40].

Glycoproteins and proteoglycans in glomerular basement membrane during diabetes

Glycoproteins

Collagen is one of the first known constituents of the extracellular matrix glycoproteins. Type IV collagen provides a scaffold for other ECM components, due to its network-like structure. During diabetes,

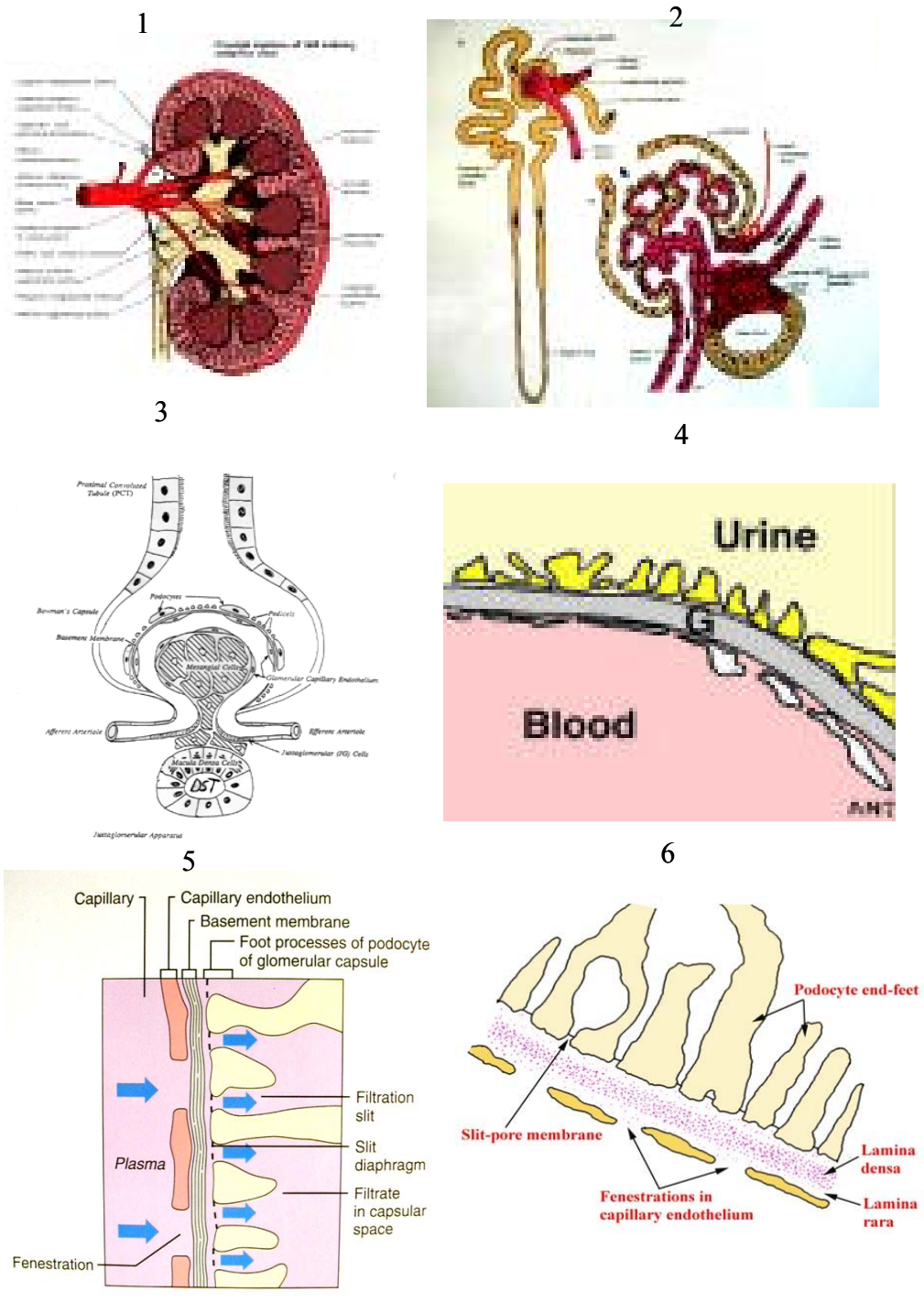


Fig. VI. 1 - Kidney, 2 - Nephron, 3 - Glomeruli, 4 - Glomerlar basement membrane, 5&6 - Structure of GBM.

Source: Internet collections from Google.

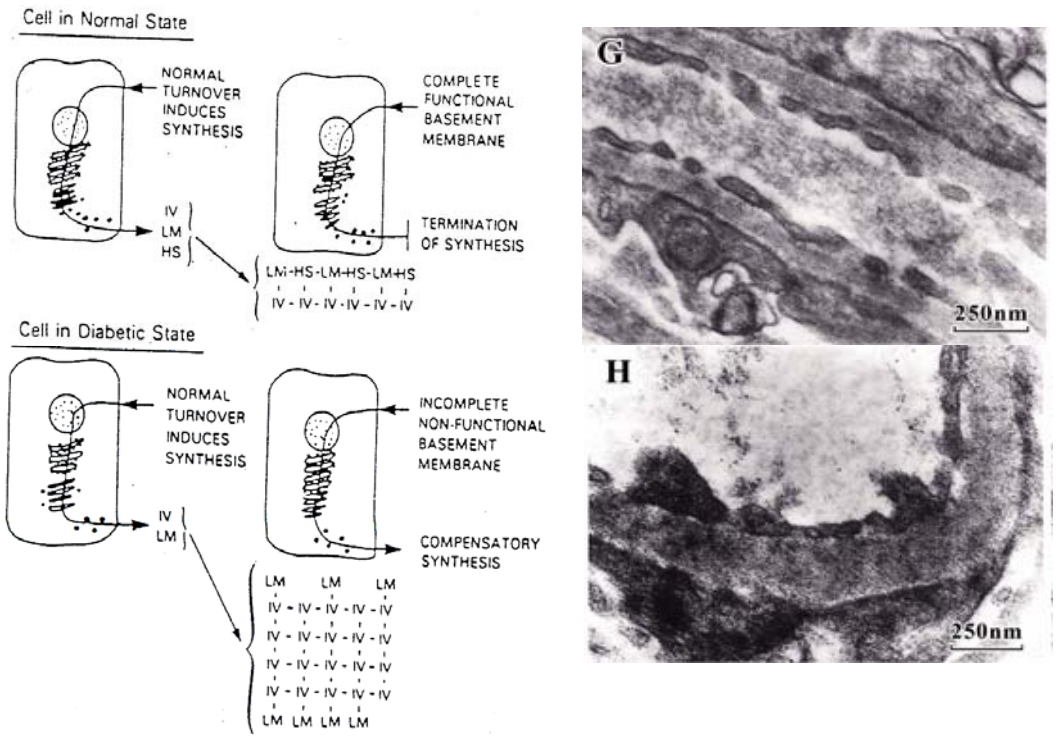


Fig. VII. Model proposed during the thickening of basement membranes in normal and diabetes condition.

Source: Rochrbach DH, Martin GR. *Ann New York Acad Sci*, **401**(1982)203.

type IV collagen increases in content as shown in Fig. VIII. This is rich in hydroxyproline and hydroxylysine. Klein *et al* [41] reported that diabetic glomeruli had greater hydroxyproline content than non-diabetic glomeruli. Kin et al [42] showed that during the course of the disease, α3 (IV) collagen segregates from that of α1 (IV). In diabetic kidneys, antibodies to α3 (IV) collagen reacted intensely with the thickened GBM but not with the mesangium. In contrast, the reactivity of antibody to various components of α1 (1V) was prominent within the expanded mesangial matrix. Attempts to study the interaction between high glucose concentrations and collagen synthesis have been more successful. Progressive glomerular sclerosis is often accompanied by an accumulation of mesangial matrix components especially the interstitial collagen[43,44]. Mesangial cells grown in high glucose concentration produce more type I and IV collagen. It has been shown that nonenzymatic glycation of type IV collagen decreases its susceptibility to be degraded by the matrix metalloproteinase - stromelysin 1 (MMP-3) and gelatinase B (MMP-9) [45]. Several investigations carried out in this regard have concluded that laminin and fibronectin in the mesangial matrix and the GBM are increased in diabetic nephropathy [46,47] . Urinary excretion of these components could be used as early markers in diabetic nephropathy [48,49]. The increased presence of fibronectin is thought to result from autocrine stimulation by TGF-β of mesangial cell[50] and podocytes[51]. In the mesangial cells, glycated albumin stimulates TGF-β release, while in the podocytes, glucose is effective [52, 53].

Proteoglycans

Proteoglycans are widely distributed in connective tissues and on the cell surface of mammalian tissues and are functional materials influencing cell differentiation and morphogenesis[54]. Proteoglycans consist of core proteins linked to glycosaminoglycan (GAG) chains and that the GAG chains interact with number of growth factors [55] and with important functional proteins[56,57]. There are six classes of glycosaminoglycans (GAGs), namely chondroitin sulfate (CS), dermatan sulfate (DS), heparan sulfate (HS), keratin sulfate (KS), heparin, and hyaluronic acid (HA) and have some common features. The long unbranched heteropolysaccharide chains are made largely of disaccharide repeating units, in which one sugar is a hexosamine (D-glucosamine or D-galactosamine) and the other is either a neutral sugar (D-galactose, in keratin sulfate) or a uronic acid (D-glucuronic or L-iduronic acid). Other constituents of GAGs are sulfate groups, linked either by ester bonds to sugar residues or by amide bonds to the amino group of glucosamine. The carboxyl groups of the uronic acid and the sulfate groups contribute to the highly polyanionic nature of glycosaminoglycans. With the exception of hyaluronic acid, which is not sulfated and has not been shown to exist covalently attached to protein, other glycosaminoglycans are linked covalently to protein, forming proteoglycans.

The kidney is rich in heparan sulphate proteoglycans (80-90%). It also consists of chondroitin sulphate B (4-8%) and chondroitin sulphate C (<2%) and traces of hyaluronic acid[58,59]. Heparan sulphate proteglycans have been recognized as important regulators of charge selectivity of

glomerular permselectivity [42, 60] . Decreased expression of heparan sulphate in glomerular basement membrane has been reported in both rats and humans [40, 61-64,] and also in *in vitro* studies. Glomerular epithelial cells in the environment of glucose synthesizes less heparan sulphate proteoglycans, and hence charge density of these sites gets reduced, leading to porous nature and increased passage of positively charged molecules, such as albumin. Significant negative correlations between charge, size, number and albuminuria have been reported in IDDM. During diabetes there is an increase in the activity of degradative enzymes of proteoglycans and glycoproteins such as β-glucuronidase, β- and α-N-acetyl hexosaminidase, α-glucosidase, sulphatase, hyaluronidase, β-galactosidase, etc. Most of these enzymes are localized primarily in lysosomes[65].

Management of diabetes

Management of diabetes is one of the important aspects in clinical practice. Globally, increasing urbanization, industrialization and changing life styles are suggested to be contributing to increasing prevalence of diabetes. India is now declared by WHO as the diabetes capital of the world. Any where in the world, diabetes is treated by one of these or combination of these treatments [66].

1. ***Diet*** and exercise
2. ***Diet*** and drugs
3. ***Diet*** and insulin

Hypocaloric diet with exercise improves health and outlook of life. Regular and controlled exercise helps to increase glucose utilization and improves long-term glucose control[67, 68].

Hypocaloric diet with insulin are always used by patients with Type-I diabetes and may be required in patients with Type-II diabetes or gestational diabetes[67]. Insulin can be broadly classified on the basis of the species (Table II).

Oral glycemic agents (Table III-IV) are primarily used in Type-II diabetes adjunct to nutrition therapy and exercise [68] . They are broadly categorized into, a. First generation sulfonylureas (Table III), b. Second generation sulfonylureas (Table IV), C. Agents enhancing the effect of insulin (Table V), d. Other oral agents (Table VI).

Table II. Source of insulin

Species	Structural difference	Immunogenecity
Human	Identical to physiological Insulin	Least
Porcine	Differs in one amino acid from human insulin	Negligible
Bovine	Differs in three amino acids from human insulin	More

Table III. First generation sulfonylureas [2]

Generic name	Dosage range / day	Duration of action	Side effects of class
Glipizide	2.5-40 mg	12-24 hrs	Prolonged hypoglycaemia
Glibenclamide	5-20 mg	12-24 hrs	Hypoglycaemia, Hypersensitiveity
Glimeperide	1.8 mg	24 hrs	Hypoglycaemia, Hypersensitiveity
Gliclzide	40-240 mg	12-24 hrs	Hypoglycaemia, Hypersensitiveity

Table IV. Second generation sulfonylureas

Generic name	Dosage range/ day	Duration of action	Side effects of class
Chlorpropamide	100-500 mg	>48 hrs	Prolonged hypoglycaemia
Tolbutamide	500-3000 mg	6-12 hrs	Hypoglycaemia, Hypersensitiveity
Tolazamide	100-1000 mg	12-24 hrs	Hypoglycaemia, Hypersensitiveity
Aceohexamide	500-1500 mg	12-24 hrs	Hypoglycaemia, Hypersensitiveity

Table V. Agents enhancing the effect of insulin

Generic name	Dosage range / day	Duration of action	Side effects of class
Metformin (Obimet)	500-2500 mg	6-8 hrs	GI upset; diarrhea; possible resumption of ovulation in premenopausal anovolvutary patients; acidosis (if renal, liver, heart impairment present)
Rosiglitazone	4-8 mg	Very long	Renal and liver function studies should be done to monitor liver dysfunction, salt and water retention, edema, congestive heart failure
Pioglitazone	15-45 mg	Very long	Renal and liver function studies should be done to monitor, liver dysfunction, salt and water retention, edema, congestive heart failure

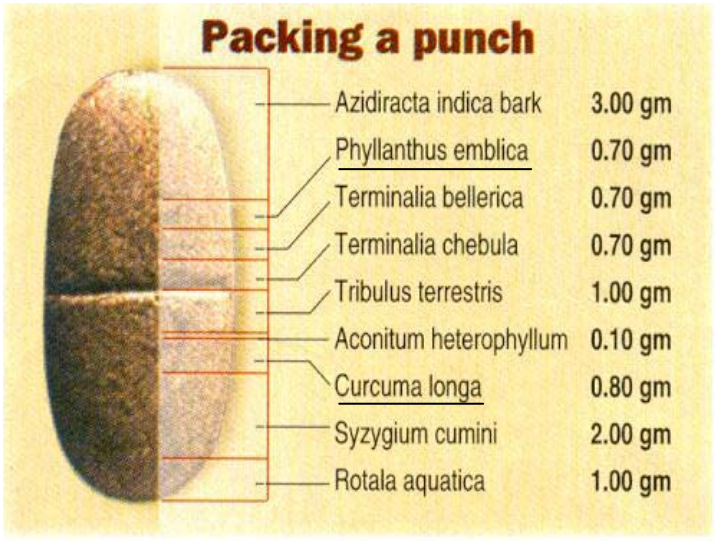
Table V. **Other oral agents**

Generic name	Dosage range / day	Duration of action	Side effects of class
Repaglinide	1.5-16 mg	2-6 hrs	Hypoglycaemia, Arthralgia, Leukopenia
Acarbose	4-8 mg	<4 hrs	Diarrhoea, Abdominal Discomfort, Flatulence
Miglitol	15-45mg	<4 hrs	Diarrhea, Abdominal discomfort, use not recommended when significant renal dysfunction is present.

The sulfonylurea drugs lower plasma glucose concentration in diabetic patients by stimulating insulin secretion and by potentiating the biological effect of the insulin on such tissues as skeletal muscle and liver. The mechanism of the latter may be by increasing the deficient number of insulin receptors on muscle and liver cells [69].

Diet

Dietary intervention is one of the means in the management of diabetes. Besides, emphasis is also to be laid on pharmacotherapy, Dietary treatments are known to maintain blood glucose levels, both short and long term diabetic complications are known to be prevented or minimized. Some of the traditional medicines based from plant source, posses antidiabetic potency[70,71]. Some of them are, fenugreek (*Trigonella foenumgraecum*), green beans (*Phaseolus vulgaris*), black plum (*Syzyzium cumini*), bitter gourd (*Momocardia charantia*), cabbage (*Brassica oleracia*), drum stick (*Moringa olifera*), mustard (*Brassica juncea*), turnip (*Brassica rapa*), greenbean (*Phaseolus aures*), etc. In recent days many Indian Ayurvedic medicines are becoming popular. One such is Cogent DB, which is in capsule form and contains *Azidiracta indica bark, Terminalia bellerica, Terminalia chebula, Syzygium cumini, Rotala aquatica, Emblica officinalis* as shown in Fig. VIII. These hypoglycemic plants may mediate their action through an insulin secretagogue effect or by influencing enzymes involved in glucose metabolism or may have extrapancreatic effect[70].



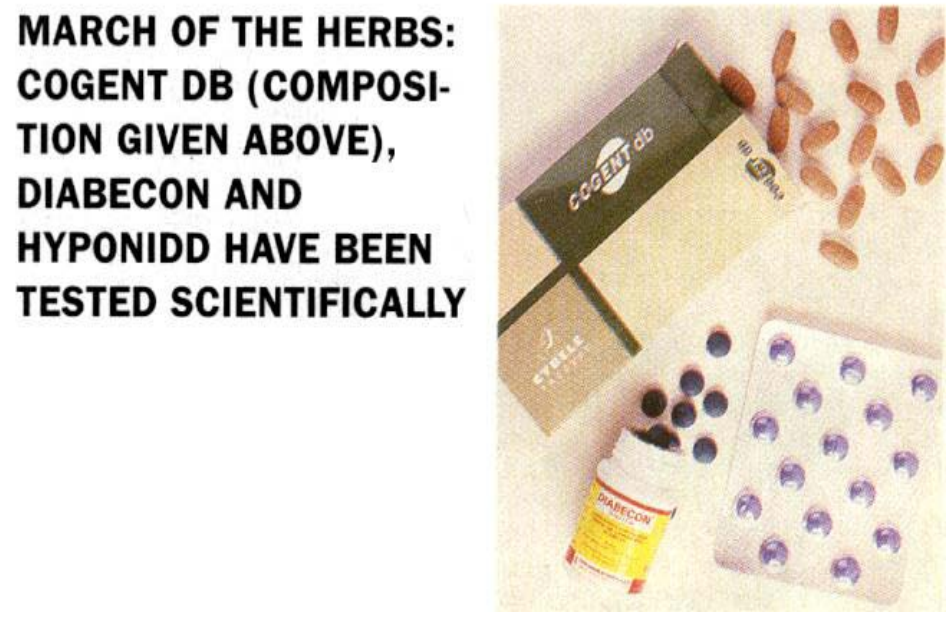


Fig. VIII. Ayurvedic capsule - Cogent DB, and its constituents.

Source: *The week* - A biweekly magazine (1998).

Some of the spices are also known to have hypoglycemic effect. Garlic (*Allium sativum*), onion (*Allium cepa*), turmeric (*Curcumal longa*), red pepper (*Capsicum annum*) are shown to ameliorate glycaemic status and renal lesions[74, 75].

Oxidants and antioxidants in humans are maintained in balance under normal physiological state and overproduction of oxidants in certain conditions such as smoking, hazardous environmental exposures, infectious diseases etc., cause oxidative stress leading to oxidative damage to biomolecules and cells[76-78]. Diet consisting of fruits, vegetables and spices has been shown to contain health beneficial components and are mainly attributed to antioxidants. Among them, phenolic compounds having antioxidant property are receiving much attention[79]. Phenolic components have been known to act as antioxidants not because of their ability to donate hydrogen or electron but because of their stable radical intermediates, which can effectively prevent the oxidation at cellular and physiological level [80].

Amla (*Emblica officinalis*) and turmeric (*Curcuma longa*) have long been known in India and many other countries for their use in traditional medicine. Several investigators have determined the efficacy of both *Emblica officinalis* and *Curcuma longa* as antiatherosclerotic [81], antidiabetic[82], antimutagenic[83, 84], anticancer[85, 86], etc. Oxidative stress is now well established to play important role in these disorders including diabetes. Notably, ascorbic acid and curcumin have been shown to be the major active components attributing health beneficial properties in *Emblica officinalis* and C*urcuma longa,* respectively[87, 88]. However,

precise phenolic acids and their antioxidant potency and their relation to health attributes are not well investigated. It should be noted here that several studies have indicated that the components other than ascorbic acid and curcumin also play potential health beneficial roles in *Emblica officinalis* and C*urcuma longa* [89, 90] .

American Diabetic Association (ADA) has recommended that diet should be rich in low caloric carbohydrates [83] . Dietary fibers are one such low caloric carbohydrates. Hipsely in 1953 coined the word dietary fibre and defined them as those polysaccharides which are not digested by the endogenous enzymes of the human gastrointestinal tract and also included lignin - non carbohydrate polymer. In recent years, role of resistant starch (RS) as a component of dietary fibre is receiving much attention[91]. The non-starch polysaccharides include mainly, gums, pectins, hemicelluloses and cellulose[92] .

***Cellulose*:** It is the main constituent of cell walls of the higher plants. Chemically speaking cellulose is a polysaccharide of sufficient chain length to be insoluble in water or dilute acids and alkalies, consisting of glucose units linked through β(1-4) glycosidic linkages[93-95]. The cellulose in cell wall gives high tensile strength and rigidity for plants survival.

***Hemicelluloses*:** The polysaccharides, which dissolve in the alkaline solution, are termed as hemicelluloses. Xyloglucans, glucomannans, galactomannans, β-D-glucans and xylans constitute the hemicellulose.

***Pectins*:** Pectins are a family of complex polysaccharides present in plant cell wall. Pectins contain 1,4-linked α-D-galactosyluronic acid

(GalpA) residues. Three pectic polysaccharides homogalacturonan, rhamnogalacturonan-I, and substituted galacturonan have been isolated from primary cell walls and their structures characterized.

Homogalacturonan is a linear chain of α-D-galactopyranosyluronic acid (GalpA) residues in which some of the carboxyl groups are methylesterfied. Homogalacturonans depending on the source, are partially O-acetylated at C-3 or C-2.

Rhamnogalacturonan – I is a family of pectic polysaccharides that contain a backbone of the repeating disaccharide [-4)-α-D-GalpA-(1-2)-α-L-Rhap-(1-]. The backbone GalpA residues may be *O*-methylated on C-2 and/or C-3 [96].

Substituted galacturonans are a diverse group of polysaccharides that contain backbone of linear 1,4-linked α-D-GalpA residues[94]. The substituted galacturonan referred to as rhamnogalcturonan-II (RG-II) are present in primary cell walls of higher plants [95].

***Gums*:** These are group of substances, which give viscous, mucilaginous, gelatinous solution in water. The gums occur as exudates on the fruit and bark. They have industrial importance as adhesives, thickeners of liquids and stabilizers of emulsions, etc. Medicinal uses include their application to soothe irritated tissues and their substitution for blood transfusion in the treatment of surgical shock. These generally represent continuous chains of galactose residues with 1,3 or 1,6 linkages and are with aldobiuronic acid side chains.

Bioactive polysaccharides: In recent years, pharmacological properties of polysaccharides are coming to light[96,95]. Polysaccharides from

different sources are shown to have antitumour, immunological, anticomplementary, antiinflammatory, anticoagulant, hypoglycemic and antiviral activities. Polysaccharides possessing such biological activities are found mostly in seaweeds, fungi and higher plants[96, 97]. Among the biological functions of polysaccharides, immuno-modulating functions of polysaccharides from herbal medicines are gaining importance. Some of their medicinal values have been attributed to the biological functions of polysaccharides [95,96,98,99]. The polysaccharides possessing complement activating and wound healing activity have been shown to be pectic polysaccharides and are identified from various types of medicinal herbs such as *Angelica acutiloba, Bupleurum falcatum, Glycyrrhiza uralensis* [96,100]. These polysaccharides consist of galacturonan region and a remified region. Pectic polysaccharides from *Bupleurum falcatum* is shown to have potent immune complex clearance activity. The activity was reduced when the polysaccharide was treated with periodate, but not with pronase or endopolygalacturonase digestions. These results clearly indicted that neutral carbohydrate chains are important for expression of activity[101,102]. The bioactive polysaccharides were extracted from *Bupleurum falcatum*. They showed potent antiulcer activity and this activity was higher than the presently employed clinical antiulcer drug-sucralfate [96, 103]. Endopolygalacturonase digestion decreased the activity, indicating presence of some of the activities with polygalacturonan backbone, while remified region containing other sugars exhibited different activities. β-Glucans are also receiving interest for the antitumorogenic acitivity. They are isolated from mushrooms and cereals and have been characterized [104]. Dietary fibers (DF) both

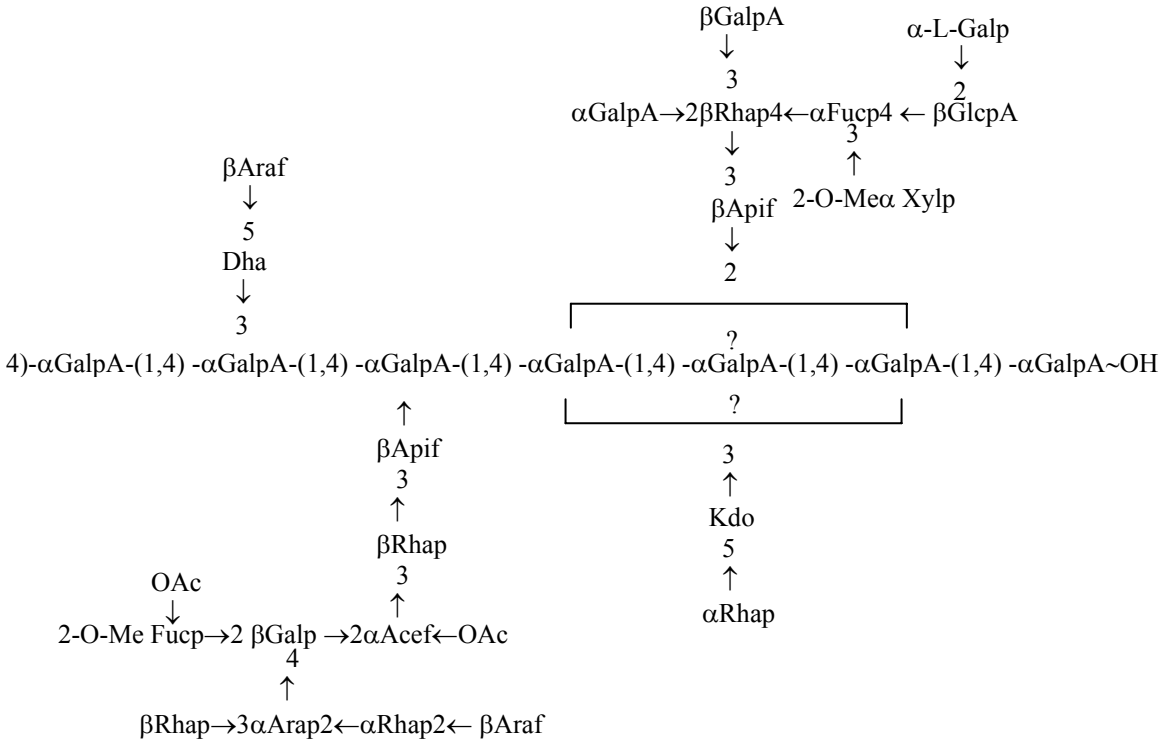


Fig. IX. Structure of feruloyl pectic polysaccharide.

Source: Ridley BL, O'Neill MA, Mohnen D. *Phytochemistry,* **57**(2001) 929.

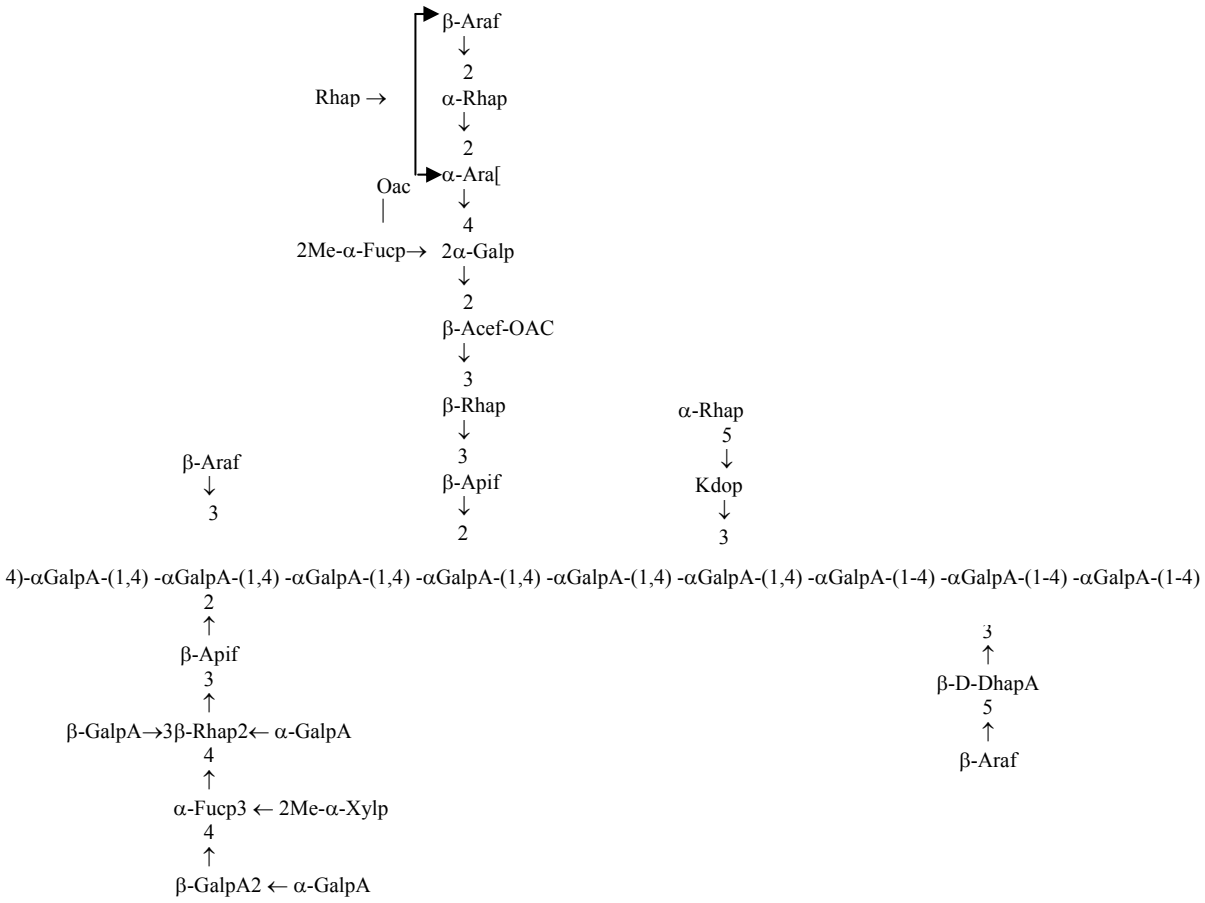


Fig. X. Structure of a rhamnoglacturonan.

Source: Vidal S, Doco T, Williams P, Pellerin P, York WS, O'Neill MA, Glushka J, Darvill AG, Albersheim P. *Carbohydr Res*, **326** (2000) 277.

soluble and insoluble, exert a wide range of physiological effects (Table VII) including hypoglycemic effect when consumed, and their complex nature of physical and chemical properties are responsible for these physiological effects. After they escape digestion in the small intestine, they pass into the large intestine, where they become substrate for the microflora of the large intestine, which can degrade many of the non-starch polysaccharides [105] into short chain fatty acids (SCFA). These SCFA have number of biological activities. Apart from diabetes, fibres have been found to be useful in other diseases like atherosclerosis, colon cancer, etc[106,107,111] .

A variety of environmental and physiological conditions are known to influence microbial composition and metabolic activities of the intestinal microflora[108,109]. Among the recognized factors within the bowel lumen are; diet, substrate, nutrient availability, redox potential, gas composition, acidity, pH, osmotic and ionic effects, surface tension and liquid flow. Endogenous and exogenous substances may inhibit bacterial growth (bile salt, volatile and nonvolatile fatty acids, bacteriocides, intestinal antibodies, and drugs), bacterial interaction and competition and intestinal motility. Individual microbial species vary in their sensitivity to these parameters and their physiological effects depend on specific properties. Some of these can influence the intestinal flora and they are partical size, binding properties, water-holding properties [109] and transit time in the bowel. The fermentation of dietary fibers (DF) and unhydrolysed starch (resistant starch) by colonic bacteria generates short chain fatty acids (SCFA) [110,111]. By decreasing

Table VII. Physiological effects of dietary fibre

Site	Actions
Food	Solidifier, hardener, water trapper
Mouth	Saliva stimulant, cleaner, work demander
Stomach	Diluter, distender, storage-prolonger
Small intestine	Diluter, distender, absorption-delayer
Large intestine	Diluter, distender, ion-binder, bacteria substrater/inhibitor, water trapper
Stool	Softener, enlarger, trauma/strain preventer

Source: Heaton KW, *Human Nutr Clin Nutr*, **37**(1980)151.

the pH in the colonic lumen, SCFA inhibit bacterial enzymes that are responsible for the formation of carcinogens and promote flow of bile acids, and increase oxygen supply to the colonic mucosa[109], stimulate the absorption of water and sodium from the large intestine hence does not induce diarrhea[112]. Administration of SCFA into large intestine increase flow of pancreatic juice, amylase secretion[113,114], blood levels of glucagons [115] , mucosal and submucosal mass and crypt cell number[116]. SCFA are involved in the formation of extracellular matrix and wound repair process[117]. Induction of differentiation and apoptosis by SCFA in colon cancer cells may be involved in the prevention of colon carcinogenesis[118].

Approximately 200 mM of short chain fatty acids are produced from the intake of about 20 g of fibre (acetate - 120 mM, propionate - 50 mM, and butyrate - 30 mM)[119,120]. Qualitative and quantitative profile of short chain fatty acids produced by different dietary fibres depend on their chemical nature. Variation in the nature of complex carbohydrates, for example, wheat bran produces, acetate 52%, propionate 11.4%, butyrate 19.2%, but pectins produce acetate 84.0%, propionate 14.0%, and butyrate 2%. The amount of short chain fatty acids observed in blood is presented in Table VIII.

The metabolism of SCFA varies for acetate, propionate and butyrate. Short chain fatty acids are produced by colonic bacteria. Acetate is metabolized by the colonocytes and only a part of it enters (Fig. IX) the portal systemic blood flow to be metabolized in kidney, mammary gland and adipose tissue and acts as a substrate for energy production

and synthesis of fat in liver [121] . Propionate is metabolized primarily in the liver and partly by coloncytes. Butyric acid provides approximately 70% of energy to coloncytes *in vivo* [122] . Butyrate, which is not utilized,

Table VIII. Concentrations of short chain fatty acids in blood

Short chain fatty acids	**Portal**	**Sub-hepatic**	**Peripheral**
Non fed status (μmol/ L) [123]			
Acetate	128.0±70.8	-	67.0±13.0
Propionate	34.4±2.3	-	3.7±1.2
Butyrate	17.6±8.4	-	Traces
Nutritional status unknown[124]			
Acetate	257.8±89.7	115.3±63.0	70±41.5
Propionate	88.3±22.2	21.2±01.9	5±00.4
Butyrate	28.7±07.6	11.7±09.8	3.8±00.8

Source: Bornet FRC, Alanowitch C, Slama G, **1994.** In: Philips. G.O. Williams. P.A. Wed.lock. D.J. (Ed.) Gums and stabilizers for the food industry-7. Oxford University Press. New York. pp 217.

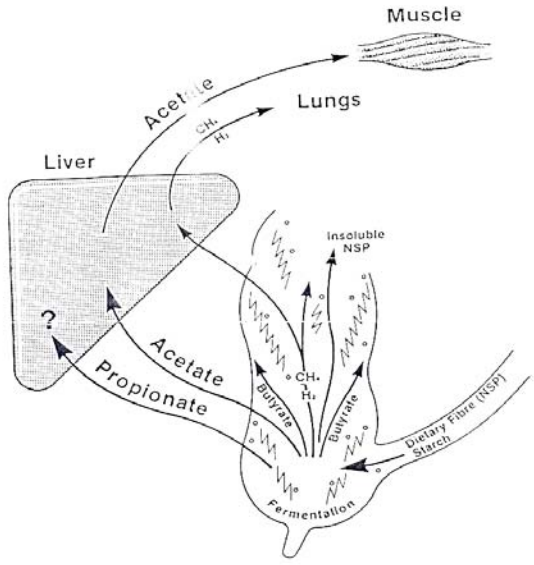


Fig. IX. Fermentation of dietary fibre in human large intestine.

Source: Alison MS. *Crit Rev Food Sci Nutr*, **34**(1994) 499.

enters the portal circulation. Among SCFA, butyrate has been shown to perform important biological functions (Fig. X) both *in vivo* and *in vitro.* Butyrate and propionate significantly offer beneficial effects against colon cancer, and have antiproliferative activity [123] . Sodium butyrate - induced erythroid differentiation was associated with upregulation of the epsilon PKC and acts by down regulating beta PKC isoforms and its signals may be mediated through PKC-dependent pathway. Butyric acid (BuA) has also been studied as a nuclear-acting perturbant and is shown to act at gene level. BuA is known to modulate chromatin structure [124, 125], phosphorylation of histone H_1 and H_{2A} [126], poly (ADP) ribosylation [127] and cell cycle arrest at G_1-S phase interface[128]. Various butyrate analogues and their effects are shown in the Table IX.

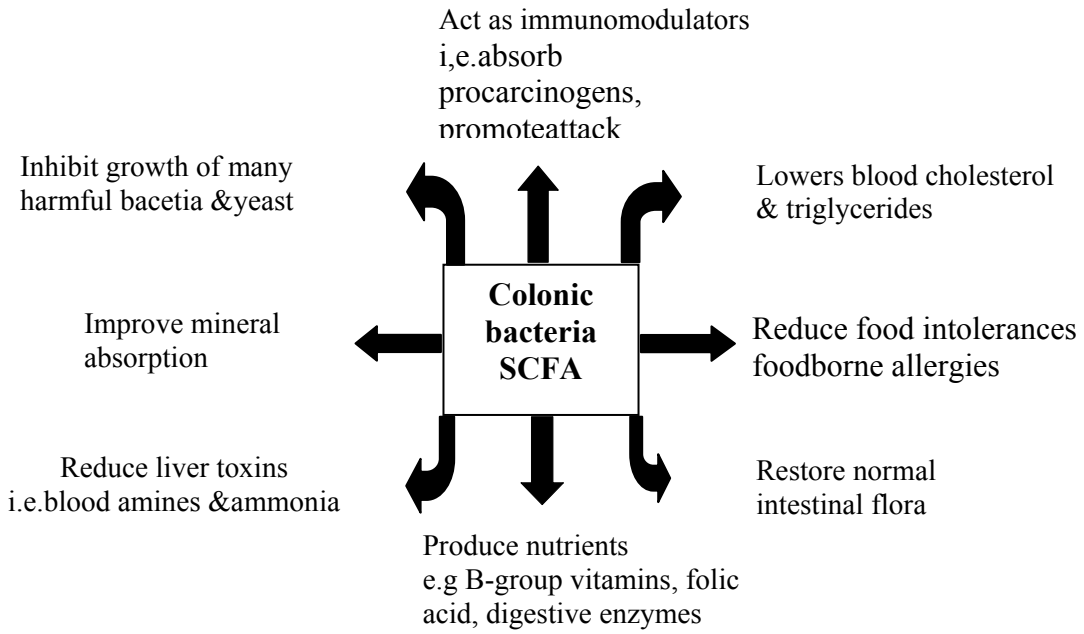


Fig. X. Schematic representation showing a relationship between colonic bacteria and short-chain fatty acid (SCFA) and their related physiological effects.

Source: Mecleary, Prosky **2001**. Advanced dietary fiber technology: Non-digestable oligosaccharides and polysaccharides: D. Meyer and B. Tungland. Their physiological effects and health implication, London. pp459.

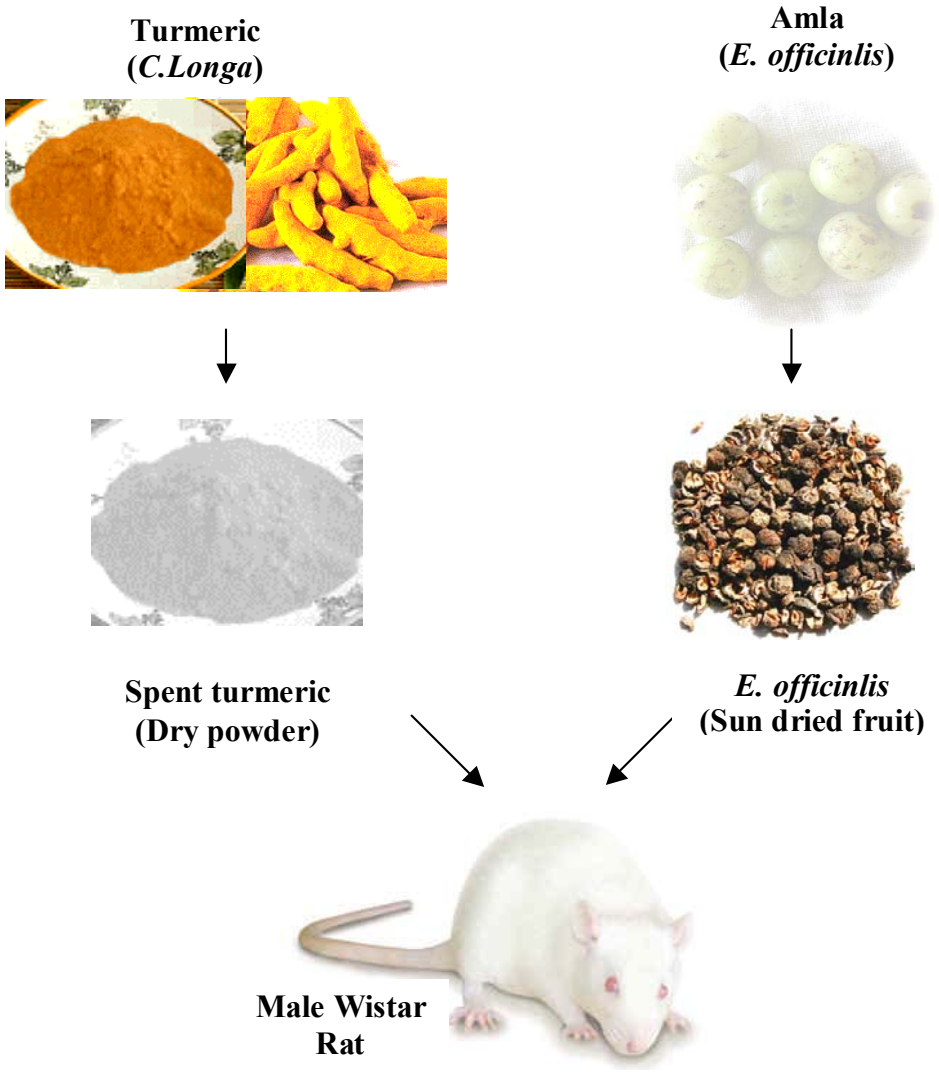
Table IX. Butyric acid analogs and their functions

Analogs	Functions
Arginine butyrate	Stimulates expression of human fetal globin gene *in vivo*
Butyramide	Inhibits cell proliferation and induces differentiation
Monoacetone glucose-3-butyrate	Induces apoptosis in human myeloid leukemia cell *in vitro*, induces differentiation and reduces cell proliferation *in vitro*, enhances *in vitro* antitumour activity of interferon
Isobutyramide	Stimulates expression of human fetal globin gene *in vitro*
Monobutyrin	Inhibits cell proliferation and induces differentiation, weaker activity than butyric acid.
Sodiumbutyrate	Alleviates leukemia *in vivo*

Source: Smith JG, Yokoyama WH, German JB. *Crit Rev Food Sci Nutr,* **38** (1998) 259.

Objective of present investigations

Thus dietary fibers and its constituents are well documented to perform important functions as antidiabetic, anticancer, antiulcer etc., Hence, our objective was to study antidiabetic property and beneficial effects on diabetic nephropathy state with particular emphasis on glomerular filtration matrix and to study structure/function relationship of some of the dietary fiber components. The study was performed using spent turmeric, a rich source of dietary fibre, which is a by product of curcumin industry and *Emblica officinalis,* which is known for its medicinal properties.



Chapter II

Materials and Methods

2.1 MATERIALS

2.1.1 General Chemicals and Instruments

Glucose oxidase, p-nitrophenyl-N-acetyl-β-glucosaminide, p-nitrophenyl-β-glucuronide, streptozotocin, toluidine blue, alcian blue, carbazole, p-dimethylaminobenzaldehyde, type IV collagen primary anti-body, chondroitinase ABC, peroxidase, calf thymus DNA, Dowex-2 (chloride form), blue dextran, Coomassie brilliant blue G-250, carbodiimide (1-cyclo-hexyl-2 (4-methylmorpholino)-ethyl p-toluene sulfonate), carbazole, catechin, dinitrosalicylic acid, deuterium oxide, iodomethane (methyl iodide), ruthenium red, sodium azide, sodium borohydride, sodium borodeuteride, Tris (hydroxy methyl) methyl amine [2- amino-2-(hydroxyl methyl) propane-1,3-diol], 1,1-diphenyl-2-picryl hydrazyl (DPPH), 2-thiobarbituric acid (TBA), were from Sigma Chemical Company, St. Louis, USA.

Dimethylmethylene blue was procured from Aldrich, Milwaukee, USA. Folin and Ciocalteu phenol reagent, agarose (low EEO), acetyl acetone, dimethyl sulphoxide (Spectroscopic grade), Folin phenol reagent (2N), bovine serum albumin and hydrogen peroxide were procured from Sisco Research laboratories, Mumbai, India.

Trifluoroacetic acid (Spectroscopy grade) was procured from Spectrochem, Mumbai, India. Vitamins and casein were obtained from Hi-Media, Mumbai, India. Corn starch, groundnut oil (refined, Postman Brand) and *Emblica officinalies* (Amla) were procured from a local

market in Mysore. Spent turmeric was from Flavor and Essence Industry, Mysore, India.

All other chemicals and reagents used were of analytical reagent grade.

Instruments

- Centrifugation of samples was done either using Hermle (Z 320K) or Remi (RC8) or Sigma (202C) centrifuge.
- Evaporation of samples was carried out using Buchi rotavapor with a water bath temperature of 35-40^{o}C.
- Samples from column chromatographic analysis were collected by using LKB Bromma 2211 fraction collector.
- Lyophilization was done using Virtis freeze mobile (12SL).
- Colorimetric and spectrophotometric determinations were carried out using Shimadzu double beam spectrophotometer (UV-160A).
- GC-15 A - Shimadzu.
- GLC-MS-17A QP-5000 Mass spectrometer - Shimadzu.
- FTIR was Perkin Elmer spectrum 2000 equipped with windows 2.1 version.
- HPLC-LC 6A was from Shimadzu equipped with UV and RI detectors and LC 10A equipped with RI, fluorescence and photodiode array detectors.

- NMR was Bruker 400MHz.
- Cellulose acetate electrophoresis unit was from Beckman, USA.
- Florescent Microscope, Diplan, Leica**,** Germany.
- Scanning Electron Microscope was from Pharmacia, Uppsala, Sweden.

2.1.2. Enzymes

Glucoamylase from *Aspergillus niger*, glucose oxdase from horse radish, and pancreatin were from Sigma Chemical Company, St. Louis, USA. Termamyl was obtained from Novo, Denmark, and sodium hydride (99%) from Aldrich Chemical Company, Milwaukee, USA. Papain was obtained from Fluka, Buchs, Switzerland.

2.1.3 Sugar standards

Sugar standards viz., rhamnose, fucose, xylose, arabinose, glucose, galactose, mannose, maltose, and inositol were from ICN Pharmaceuticals Inc., Cleveland, USA.

2.1.4 Dextran standards

T-Series viz., T-10, T-20, T-40, T-70, T-150, T-500, T-2000 were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden.

2.1.5 Phenolic acid standards

Phenolic acid standards such as gallic acid, caffeic, coumaric, ferulic, gentisic, protocatechuic, syringic and vanillic acids, butylated hydroxy

anisole (BHA), butylated hydroxy toluene (BHT) were from Sigma Chemical Company, St. Louis, USA.

2.1.6 Gel matrices and ion exchange resins

Sepharose CL-4B (4% cross-linked, fractionation range for dextrans 30,000-50,000 Da), DEAE-cellulose (0.99 meq/g), Amberlite IR-120-P (8% cross-linked, 16-50 mesh) were from Pharmacia Fine Chemicals, Uppsala, Sweden.

Sep-Pak C_{18} cartridges were from Waters Associates, Millford, USA. Nujol (UV spectroscopic grade) was obtained from E-Merck, Mumbai, India. Vials, crimper and decapitator for methylation were from Pierce Chemical Company, Rockford, USA.

Cellulose acetate membranes were from Beckman Instruments International, S.A., Geneva, Switzerland.

2.1.7 GC Columns

3% OV-225 (1/8"×6') on Chromosorb W (80-100 mesh) was from Pierce Chemical Company, Rockford, USA.

SP-2330 (0.32 mm × 30 M) was from Supelco, Tokyo, Japan.

2.1.8 HPLC

- μ-Bondapak-NH_2 carbohydrate column (4.1 mm × 30 cm); E-Linear (7.8 mm × 30 cm) and E-1000 (3.9 mm × 30 cm) gel permeation columns were from Waters Associates, Milford, USA.

- Shimpak-C_{18} column (4.6 mm × 250 cm) was from Shimadzu Corporation, Tokyo, Japan.

All chemicals and solvents used for HPLC and GLC were HPLC grade. All other analytical reagents were obtained from E-Merck, SRL or BDH Mumbai, India.

Triple distilled and degassed water was used for HPLC analysis.

2.1.9 Extractions

Extractions were carried out using double distilled water**.**

Dialysis against double distilled water was carried out using dialysis bags with cut off of 12,000 Da. Few drops of toluene were added to aqueous solutions to prevent microbial growth.

2.1.10 Purification of solvents

Phenol, acetic anhydride and pyridine were purified by distillation in an all glass apparatus.

Anhydrous ether was obtained by distilling over calcium chloride and the distillate was preserved in a brown colored bottle over metallic sodium wire.

Anhydrous dimethyl sulphoxide (DMSO) was obtained by distilling over fresh calcium hydride, under vacuum (7 mm) and the constant boiling fraction was collected and stored over molecular sieve ($4A^{o}$).

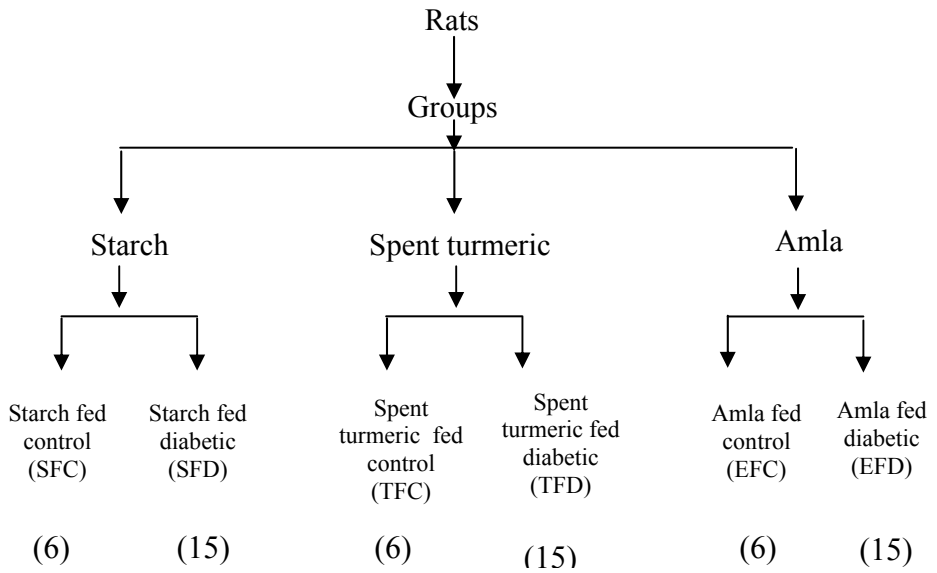
2.2 Nutritional studies

2.2.1 Animals

Male Wister rats [OUTB-Wistar, Ind-cft (2c)] weighing around120-140 g were obtained from Animal House facility of Central Food Technological Research Institute, Mysore. The study had the clearance of Institute Animal Ethical Committee. Animals were housed individually in stainless steel cages having raised bottom.

The Animals were grouped in to 3 main groups and each group was divided into two subgroups. Diabetes was induced in one of the subgroups and the other subgroup served as control. They were fed with AIN-76 (American Institute of Nutrition) basal diet.

Schematic representation of experimental design



**Number in the parenthesis indicate number of animals in that group.*

2.2.2 Composition of diet

Composition of different diets used in the present study is given in Table 1.

Table 1. Composition of basal AIN-76 diet

Composition	SFC/SFD	TFC/TFD	EFC/EFD
	g /kg Diet		
Casein	200	200	200
AIN-76 vitamin*mixture	10	10	10
AIN-76 mineral ** mixture	35	35	35
Choline chloride	2	2	2
Fat	100	100	100
Corn starch	653	553	648
Spent turmeric	-	100	-
Emblica officinalis	-	-	5

SFC: Starch Fed Control, SFD: Starch Fed Diabetic,
TFC: Spent turmeric Fed Control, TFD: Spent turmeric Fed Diabetic,
EFC: *Emblica officinalis* Fed Control, EFD: *Emblica officinalis* Fed Diabetic.

*AIN-76 vitamin mixture: (g/kg mixture) Thiamine hydrochloride 0.6; riboflavin 0.6; pyridoxine hydrochloride 0.7; nicotinic acid 3.0; D-calcium pantothenate 1.6; folic acid 0.2; D-biotin 0.02; cyanocobalmine (Vit B_{12}) 0.001; retinyl acetate 0.4 (4,00,000 I.U); DL-α-tocopherol acetate 7.7 (5000 I.U); cholecalciferol 0.0025 (1,00,000 I.U); menadione 0.005; starch (finely powdered was added to make to 1000 g.

**AIN-76 Mineral mixture (g/kg mixture)

Calcium phosphate (dibasic) 500; sodium chloride 74; potassium citrate monohydrate 220; potassium sulphate 52; magnesium oxide 24; manganous carbonate 3.5; ferric citrate (16-17%, Fe) 6; zinc carbonate (70%, ZnO) 1.6; cupric carbonate (53-55%, Cu) 0.3; potassium iodate 0.01; sodium selenite 0.01; chromium potassium sulphate 0.55; starch (finely powdered) was added to make to 1000 g.

Source: Bieri JG, Stoewsand GS, Briggs GM, Phillips RW, Woolard JC, Knapka J J. *J Nutr*, **107**(1977)1340.

2.2.3 Feeding period

Rats were acclimatized for 1 week with control diets before induction of diabetes. After induction of diabetes the rats were kept on experimental diet. The rats were sacrificed when mortality was about to set in, which was around 35-45 days. All the rats were fed diet *ad libitum* and water.

2.2.4 Induction of diabetes [129]

Diabetes was induced by a single intraperitoneal injection of streptozotocin at a dosage of 55 mg/kg body weight. Streptozotocin was dissolved in freshly prepared citrate buffer (0.1M, pH 4.5). Control rats were injected with citrate buffer only. After injection, the rats were given 5% glucose solution for two days and were then given drinking water.

2.2.5 Collection of blood and urine

Blood was collected in tubes containing heparin (20 U / mL of blood) either from retro-orbital plexus during the experiment or from the heart at the time of sacrificing the rats to measure fasting blood glucose.

Urine was collected after keeping rats in individual metabolic cages in the bottles under a layer of toluene.

2.2.6 Sacrificing of animals and collection of blood and tissues

Blood was collected in tubes containing heparin (20 U / mL of blood) either from retro-orbital plexus during the experiment or from the heart at the time of sacrificing the rats to measure fasting blood glucose.

Tissues (kidney, liver, spleen, lung, brain, stomach and intestine) were excised, washed with cold saline, blotted dry, weighed and stored at -20^{o}C till further analysis.

2.2.7 Urine and plasma analysis

2.2.7.1 Reducing sugar estimation[130]

Preparation of reagent

Dinitrosalicylic acid (DNS, 1.0 g) and sodium potassium tartarate (30.0 g) were dissolved in 100 ml of sodium hydroxide (0.4M).

To 1.0 mL of the urine sample containing approximately 100-1000 μg of reducing sugar, 1.0 mL of DNS reagent was added and placed in boiling water bath for 10 min. After cooling, the contents were diluted by adding 4 mL of distilled water. Absorbance was read at 500 nm in a spectrophotometer. Standard graph was generated using glucose (0-1000 μg).

2.2.7.2 Glucose estimation[131]

10 µL of plasma was mixed with 1 mL of glucose reagent (GOD/POD) and kept at room temperature for 15 min. Absorbance was read at 505 nm in a spectrophotometer.

$$\frac{\text{Absorbance of sample at 505 nm x 100}}{\text{Absorbance of standard at 505 nm}} = \text{mg /100 mL}$$

2.2.7.3 Creatinine estimation in plasma and urine[132]

To 250 µL of plasma or urine, 0.5 mL of distilled water, 3 mL of picric acid reagent and 2 mL of sodium hydroxide were added. The reaction mixture was mixed well and allowed to stand at room temperature for exactly 20 minutes, Absorbance of Blank (B), Standard (S) and Test (T) were measured against distilled water in spectrophotometer at 520 nm. Serum creatinine was calculated using the formula;

$$\text{Serum creatinine (mg \%)} = \frac{\text{A of (T)} - \text{A of (B)}}{\text{A of (S)} - \text{A of (B)}} = \text{mg /100 mL}$$

2.2.8 Glomerular filtration rate (GFR) [133]

GFR was calculated from urinary and plasma creatinine using the formula,

$$\frac{\text{Urinary creatinine (mg/dL) x urine volume (mL) x 1000 (g)}}{\text{Plasma creatinine (mg/dL) x body weight (g) x 1440 (min)}} = \text{mL/min}$$

2.2.9 Estimations in tissues

2.2.9.1 Processing of tissues

All the tissues were freed of fat, cut into small pieces and stored in acetone in cold room until use. Acetone was changed every week. After drying in acetone, the tissues were defatted by Soxhlet extraction with petroleum ether (40-60) for 6 h[134]. Dried and defatted tissues were ground to a fine powder using pestle and mortar and used for various estimations.

2.2.9.2 Hydrolysis of sample

To the sample (25 mg), 1 mL of trifluoroacetic acid (2 N) was added and hydrolysed at 100^{o}C for 8 h in sealed tubes in an oven[134]. Samples were cooled and aliquots were taken for estimation of total sugars, uronic acid and amino sugars.

2.2.9.3 Analytical methods/ Estimations

2.2.9.3.a Total carbohydrate estimation[135]

Total carbohydrate content was determined by taking 0.5 mL of the sample in a test tube into which were added 0.3 mL of phenol (5%) and 1.8 mL of concentrated sulphuric acid and the contents were mixed thoroughly. After cooling the tubes at room temperature (~20 min), the absorbance was read at 480 nm against a reagent blank. Sugar content was determined by referring to the standard graph, prepared by using D-glucose (0-25 μg).

2.2.9.3.b Uronic acid estimation[136]

Uronic acid was determined by taking 0.5 mL of the sample solution in a test tube and kept in ice cold water bath for 10 min, to which was added concentrated sulphuric acid (3 mL) slowly and the contents were mixed thoroughly and kept in boiling water bath for 20 min. The contents were cooled to which carbazole solution (0.1 mL, 0.1% prepared by dissolving re-crystallized carbazole in alcohol) was added. The tubes were kept in dark for 2 h and the absorbance was recorded at 530 nm. Uronic acid content was determined by referring to the standard graph prepared by using D-galacturonic acid (0-50μg).

2.2.9.3.c Amino sugar estimation[137]

Preparation of reagents

Solution A: Acetyl acetone (1.5 mL) was mixed with sodium carbonate (0.7 M, 50 mL).

Solution B: p-Dimethyl aminobezaldehyde (1.6 g) was dissolved in a mixture containing 30 mL of concentrated hydrochloric acid and 30 mL of ethanol.

To 0.25 mL of the sample solution containing approximately 10-50 μg of amino sugar, 0.5 mL of solution A was added and heated to 80^{o}C for 1 h in a water bath. After cooling the tubes to room temperature, 5 mL of ethanol and 0.5 mL of solution B were added. The contents of the tubes were thoroughly mixed and left at room temperature for 1h. The absorbance was read at 535 nm in a spectrophotometer. Glucosamine hydrochloride (0-50 μg) was used to prepare the calibration curve.

2.2.9.3.d Sulphate estimation [138]

Tissues were hydrolysed with 60% formic acid prior to estimation. To the sample (20-30 mg), 3 mL of 60% formic acid was added and hydrolysed at 100^{o}C in sealed tubes in an oven for 8 h. The hydrolysates were evaporated to dryness in a flash evaporator at 30^{o}C and reconstituted with 2.0 mL of water.

Preparation of reagents

Solution A: Trichloroacetic acid (4.0 g) in 100 mL distilled water.

Solution B: Bactograde gelatin (2.0 g) was dissolved by gentle shaking in 400 mL of hot (60-70^{o}C) distilled water. It was cooled and allowed to stand for 6 h at 4^{o}C. Barium chloride (20 g) was dissolved in this gelatin solution and was allowed to stand for 2-3 h before use.

To 0.5 mL of the hydrolysate containing 5-50 μg of sulphates, 3.8 mL of solution A and 1.0 mL of solution B were added and allowed to stand at RT for 10 min. Turbidity was read at 500 nm in a spectrophotometer. Standard graph was prepared using potassium sulphate having 0-50 μg of sulphate content.

2.2.9.3.e Protein estimation[139]

Tissues (5.0 mg) were suspended in 5 mL of sodium hydroxide (0.5 M). After allowing it to swell for 1 or 2 h, the contents were repeatedly sonicated and vortexed to dissolve it completely. If required, it was allowed to stand overnight.

Preparation of reagents

Solution A : Sodium carbonate (2 g) in 100 mL of sodium hydroxide (0.1M).

Solution B_1: Copper sulphate ($CuSO_4.5H_2O$, 1.0 g) in 100 mL of distilled water.

Solution B_2: Sodium potassium tartarate (2.0 g) in 100 mL distilled water.

Solution C : Mix 100 mL of A + 1 mL of B_1 + 1 mL of B_2 before use.

Solution D : Folin and Ciocalteu phenol reagent stock (1.0 mL, ~2.0 N) diluted to 2.5 mL with distilled water before use.

To the tissue sample solution (1.0 mL), containing 0-200 μg of protein, 5.0 mL of solution C was added and allowed to stand at room temperature for 10 min. To it, 0.5 mL of solution D was added while vortexing the contents. It was again allowed to stand for 30 min. Absorbance was read at 750 nm in a spectrophotometer. Standard graph was prepared using bovine serum albumin (0-200 μg).

2.2.9.3.f Estimation of glycosaminoglycans [140]

Preparation of reagent

Dimethylmethylene blue (8.0 mg) was added to 500 mL of distilled water containing glycine (1.52 g), sodium chloride (1.135 g) and 47.5 mL hydrochloric acid (0.1N).

To 0.1mL of the sample solution containing 1-5 μg of sulphated polysaccharide, 2.5 mL of dimethylmethylene blue reagent was added and absorbance was read at 525 nm in a spectrophotometer with in a minute. Standard graph was prepared using chondroitin sulphate (0.5 μg).

2.2.9.3. g Collagen estimation[141]

Collagen content was estimated in terms of hydroxyproline. The dry kidney powder samples were hydrolyzed by keeping at 100°C in sealed tubes in an oven for 8 h in 6 N HCl. To the hydrolyzed sample, 1 mL of copper sulfate (0.01M) and 1.0 mL of NaOH (2.5N), followed by 1.0 mL of hydrogen peroxide (6% v/v), were added to each tube. Tubes were capped tightly and heated for 5 minutes at 80°C and were cooled to room temperature. To all tubes, 4 mL of 3 N sulfuric acid was added by agitation in cold condition. 2 mL of p-dimethylaminobenzaldehyde (5% solution in n-propanol) solution was added to each tube and vortex-mixed for 2 minutes. Once again, all the tubes were heated to 70°C for 15 minutes. Tubes were cooled, and optical density was read against a reagent blank at 540 nm.

2.2.10 Isolation of glycosaminoglycans (GAGs) from kidney and other tissues[142]

Tissue (1.0 g) was suspended in 20 mL of phosphate buffer (0.1M, pH 6.5). Papain solution [10 mg papain in 1.0 mL phosphate buffer containing EDTA (0.005 M)] was first activated by keeping at 65°C for

30 min in a water bath. The activated papain solution (1.0 mL) was added to the tissue suspension and digested for 2 days at 65^{o}C in an oven. Enzyme solution (1.0 mL) was again added at the end of 24 h. After digestion, the reaction mixture was centrifuged (3000 g, 15 min). To the supernatant, one-third volume of trichloroacetic acid (40%) was added to precipitate the proteins. The precipitate was discarded after centrifugation. To the supernatant, four volumes of ethanol containing potassium acetate (1.2%) was added and left at 4^{o}C overnight. The precipitate was separated by centrifugation. The precipitate was reconstituted with water. Aliquots were taken for analysis.

2.2.11 Fractionation of glycosaminoglycans[143]

To 0.05 mL of glycosaminoglycan solution containing 10-15 µg of sulphated polysaccharide, 0.05 mL Tris - HCl buffer (0.25 M) and 0.01 mL chondroitinase ABC (5 units) were added and digested at 37^{o}C for 17 h under a layer of toluene. Simultaneously, a control without chondroitinase ABC was subjected to digestion as described above. After the digestion, aliquots were used for estimation of sulphated polysaccharide by dimethylmethylene blue reagent in control and digested samples. The difference between the two gives the amount of heparan sulphate. The hydrolysed sample gives the amount of chondroitin sulphate.

2.2.12 Agarose gel electrophoresis [144]

Preparation of reagents

Solution A: Agarose (1g, low EEO) was dissolved in 100 mL distilled water by warming.

Solution B: Barium acetate (0.05M) was adjusted to pH 5.0 with glacial acetic acid.

Solution C: Barium acetate (0.05M, pH 5.0) containing glycerol (20%).

Solution D: Toluidine blue (0.1g) in 100 mL solution containing sodium acetate (0.05M) and magnesium chloride (0.01M) adjusted to pH 3.5 with hydrochloric acid (0.1M).

Solution E: Sodium acetate buffer (0.01M, pH 5.5).

Agarose gel was casted on a boat to 0.5 cm thickness using solution A. Samples were freeze dried and reconstituted in 15 μL of solution C. Samples were applied after placing the boat with gel in the electrophoresis chamber (Broviga, Chennai) containing solution B. The samples were run for 5 h at 80V using Brovega - power pack. After the run, the gel was stained in solution D overnight. It was then destained with solution E till the gel was cleared of background colour.

2.2.13 Measurement of Enzyme activity

2.2.13.1 Intestinal maltase, sucrase and lactase activities[145]

Enzyme preparation

Intestine was freed from food particles by flushing cold saline (0.9%). The intestine was cut along its length and the mucosa was scrapped using a glass slide into stoppered, graduated tubes. The mucosal scrapings were made to 10 or 20 mL using saline (0.9%).

The mucosa was homogenized in Potter-Elvehjem homogenizer for 10 min at 4^{o}C. Homogenate was taken for measuring the enzyme activity.

Assay

Maltase, sucrase, and lactase activities in intestinal mucosa were measured by incubating maltose (56 mM), sucrose (56 mM) or lactose (56 mM), prepared in maleate buffer (0.1M, pH 6.0) with the enzyme preparation after suitable dilutions. Control was done in the same way, but the enzyme preparation was added after stopping the reaction. The contents were incubated at 37^{o}C in a water bath for 1 h and the activity was stopped by adding Tris glucose oxidase (1.2 mL). The amount of glucose released was measured as described in section 2.2.7.2.

Protein in the aliquot was estimated by Folin Ciocalteu reagent as described in section 2.2.9.2.e.

2.2.13.2 Renal maltase, sucrase and lactase activities

Enzyme preparation

Kidney tissue (100 mg) was taken in 1.0 mL cold saline (0.9%) and homogenized in Potter-Elvehjem homogenizer at 4^{o}C for 10 min. It was then centrifuged at 800 g at 4^{o}C for 10 min. The supernatant was used as an enzyme source.

Assay

This was done as described in Section 2.2.13.1.

2.2.13.3 L-Glutamine-fructose-6-phosphate aminotransferase[146]

Enzyme preparation

Kidney tissue (200 mg) was homogenized in Potter-Elvehjem in phosphate buffer (0.1 M, pH 6.5) containing potassium chloride (0.154 M), EDTA (0.001M) and glucose-6-phosphate (0.012 M). It was then centrifuged at 800 g for 10 min at 4^{o}C. Supernatant was used as an enzyme source.

Assay

To the glucose-6-phosphate solution (0.1 M in phosphate buffer, pH 7.0) containing glutamine (0.01 M) and reduced glutathione (0.1 M), 0.3 mL of the enzyme preparation was added and incubated at 37^{o}C for 1 h. At the end of the incubation period, the reaction was stopped by adding 1.0 mL of trichloroacetic acid (0.4 M). The mixture was centrifuged and the supernatant (1.5 mL) was evaporated to dryness in a rotary

evaporator maintained at 35°C. It was reconstituted with 0.25 mL water. Amino sugar was estimated as described in section 2.2.9.2.c and protein as described in section 2.2.9.2.e.

Activity

It is expressed as μmoles of glucosamine formed / h /g protein.

2.2.13.4 β-N-Acetyl glucosaminidase[147]

Enzyme preparation

Kidney tissue (100 mg) was homogenized in 1 mL of acetate buffer (0.1M, pH 4.5) and centrifuged at 2000 rpm for 10 min at 4°C. The supernatant was used as a source of enzyme.

Assay

p-Nitrophenyl-β-N-acetyl glucosamine was used as the substrate. To the substrate (50 μg/0.2 mL acetate buffer, pH 4.5, 0.1M), 0.3 mL of acetate buffer (0.1M, pH 4.5) and 10 μL of enzyme solution were added and incubated at 37°C for 10 min. Reaction was stopped by the addition of 2.5 mL of sodium carbonate (0.2 M) and absorbance was read at 400 nm in a spectrophotometer. Paranitrophenol (0-20 μg) was used to prepare the standard curve. Protein was estimated as described in 2.2.9.2.e.

Activity

It is expressed as μmoles of paranitrophenol formed /h/ mg protein.

2.2.13.5 β –Glucuronidase[147]

Enzyme preparation

It was prepared in a similar way as in section 2.2.13.4.

Assay

p-Nitrophenyl-β-glucuronide was used as substrate. To the substrate (50 μg/0.2 mL acetate buffer (pH 4.5, 0.1 M), 0.1 mL of the enzyme solution and 0.2 mL acetate buffer (0.1M, pH 4.5) were added and incubated at 37^{o}C for 1h. Reaction was stopped by the addition of 2.5 mL sodium carbonate (0.2 M) and absorbance was read at 400 nm in a spectrophotometer. Paranitrophenol (0-20 μg) was used to prepare the standard curve. Protein was estimated as described in section 2.2.9.2.e.

Activity

Activity of the enzyme is expressed as μmoles of paranitrophenol formed/h/mg protein.

2.2.14 Statistical analysis[148]

In experiments, where the estimations or assays were not pooled, the statistical significance of values obtained was evaluated by using 't' test.

Standard error of mean (SEM) was obtained by the formula:

$$SEM = \sqrt{\frac{\sum d^2}{n\,(n-1)}}$$

Where,'d' is the deviation of the individual value from arithmetic mean and 'n' is the number of observations.

$$t = \frac{AM_1 - AM_2}{\sqrt{SEM_1^2 - SEM_2^2}}$$

Where, AM_1 and AM_2 are arithmetic mean.

Results are expressed as mean values ± SEM. Differences in mean values were analysed using Student 't' test and ANOVA. Significance is defined as $p < 0.05$.

2.2.15 Histopathology

2.2.15.1 Processing of tissues[149]

The tissues immediately after removal from the animals were kept in the fixative (10% formalin) and were passed through series of alcohol (70% to 100%) for 1 h in each step. The dehydrated tissues were kept in xlyene for 1h.

After xylene treatment, the tissues were kept in melted paraffin (60^oC) for 1h. The tissues were finally embedded in paraffin blocks and allowed to cool. Tissue sections of 5 μ thickness were prepared using a microtome and were fixed to slides using Mayers egg albumin with mild heat treatment. The slides so obtained were used for different stainings.

2.2.15.2 Reagents

a) Formalin 10%

Formaldehyde (37-40%, 100 mL) was made up to 1000 ml with distilled water.

b) Mayer's egg albumin

Egg white and glycerol were taken in the proportion of 1:1, mixed and filtered through coarse filter paper and to the filtrate a few crystals of thymol were added as a preservative.

2.2.16 Staining

2.2.16.1 Combined alcian blue and periodic acid stain[150]

Reagent preparation

1 g of Alcian blue was dissolved in 100 ml of 3% acetic acid solution.

1 g of periodic acid was dissolved in 100 ml of distilled water.

Schiffs reagent: Dissolve 1.0 g of basic fuchsin in 200 mL of hot distilled water, allow to cool and add 2.0 g potassium metabisulfite, and 10.0 ml hydrochloric acid. Leave for 24 h, and then add 0.5 g activated carbon. Shake for a minute and filter through coarse filter paper. The solution was filtered until solution became colorless, and then stored in a refrigerator.

Staining procedure;

Tissues were deparaffinised in xylene (30 min, x2) and then hydrated by passing through 100%, 90%, 70% and 30% ethyl alcohol for 10 min in each. After rinsing the sections in water, they were placed in alcian blue stain for 5 min and washed with distilled water. Then they were in periodic acid for 10 min followed by rinsing with distilled water and were placed in Schiff's reagent for 20 min and washed under running tap water for 10 min. Then they were lightly stained with Gills hematoxylin and washed and glued in 1% lithium carbonate, dehydrated and sections were mounted in D.P.X.

2.2.16.2 Periodic acid-Schiff's stain (PAS)[151]

The method as in sections 2.2.16.1 was used except that alcian blue was not used.

2.2.16.3 Immunohistochemistry of type IV collagen[152]

Sections were dewaxed, rehydrated and immunostained as per the protocol supplied by the manufacturer (Santa Cruz Biotechnology, USA). In brief, paraffin sections were washed thrice with xylene for 5 min. The sections were hydrated with 100% ethanol for 5 min followed by 95% for 2 min and 80% for 2 min. Then, the sections were rinsed with distilled water and incubated with 3% skimmed milk powder to block non-specific sites. After 2 min, the sections were rinsed with PBS and baked at 450 Hz microwave oven for 10 min to retrieve the antigen and again hydrated with PBS for 2 min. Sections were incubated with primary antibodies (goat polyclonal IgG) for Type IV collagen

overnight at 4°C. After tapping off the antibody, the slides were dipped for 5 min in PBS. The sections were then incubated with secondary antibodies (rabbit anti goat IgG) tagged with FITC of type IV collagen for 30 min at RT. The slides were washed in PBS for 5 min and observed under florescent microscope.

2.2.17 Scanning electron microscopy of glomeruli

Preparation of sections was done as explained in section 2.2.15.1 and deparaffinised as in section 2.2.16.1. The section was kept on a double-sided conducting adhesive tape and pasted on a metallic stub. It was coated with 100μ gold in a sputter coating unit for 5 min and observed in the SEM-LEO-435-VP, Scanning Electron Microscopy at 20 kv.

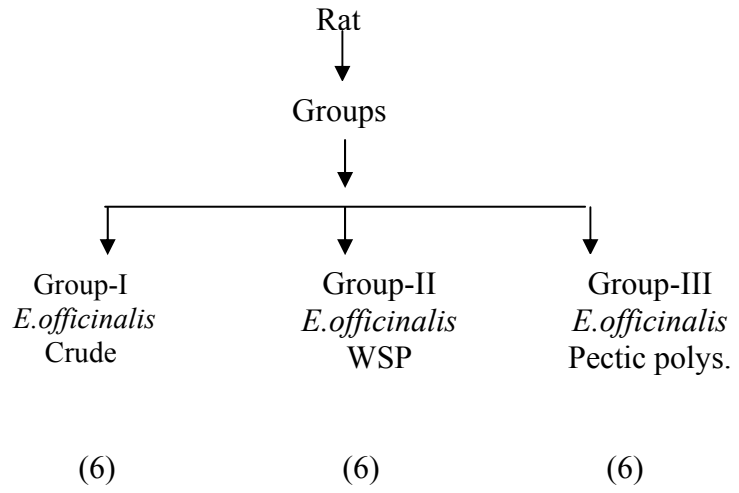
2.3 Wound healing studies

2.3.1 Animals

Wistar rats of either sex weighing 180-200g (4-5 months old) were selected for the study and were obtained from Animal House facility of Central Food Technological Research Institute, Mysore, India. The study had clearance of Institute Animal Ethical Committee.

Animals were grouped into three groups, and were treated with crude *Emblica officinalis*, water-soluble polysaccharides (WSP) and pectic polysaccharides.

Schematic representation of experimental design



2.3.2 Formulation of gel [153]

The gel composed of 5% cabapol-934 (carboxy polymethylene of molecular weight 3×10^6), propylene glycol (25.75%), propylparaben (0.25%), triethanolamine (0.8 mL), and distilled water in a quantity sufficient to prepare 100 g of gel in the case of blank gel, whereas in the case of sample extract (polysaccharide fractions, 500 mg) was incorporated for 100 g of total gel. The gel prepared in this method was kept under vacuum for 2 h to remove entrapped air. The pH of the gel prepared in this manner was neutral. This gel was placed in collapsible tubes and stored in a cool, dry place during the studies.

2.3.3 Excision wound model[154]

Rats were clipped free of hair on the lateral surface on either side, thus making out wound of known size (wounds were made on cleanly shaved area by removing the outer layer of skin in the marked area equidistant from midventra line). Wounds of left side served as control and that of right side was administered with formulation containing extracts.

Animals were accessed for normal diet. Animals were kept under cleaned bedding, which was changed daily by cleaning the cage with disinfectant and material used for bedding was dried for 2 h at 80^{o}C daily. This was done mainly to avoid chances of secondary infection. During the study, animals were kept in individual cages. Equal amount of gel was applied to all the wounds till healing was complete.

2.3.4 Parameters used for assessing the wound healing activity

- Photos of wound area was taken after 3 days.
- Estimation of collagen content in healed skin, was done on 6th day (2.2.9.2.g).

2.4 Studies on carbohydrates

2.4.1 Determination of starch[155]

Sample (0.5-1.0 g) was taken in conical flask and dispersed in 50 mL water. Termamyl (0.1 mL) was added and then kept in boiling water bath for 10 min. After cooling, acetate buffer (pH 4.6, 0.05 M) was added and equilibrated at 60^{o}C for 2 h. The solution was filtered and made upto 100 ml and the liberated glucose was determined by the Tris glucose oxidase (TGO) method (2.2.7.2). The glucose value multiplied by a factor 0.9 gave the starch content.

2.4.2 Isolation of total dietary fiber [156]

The flour was first extracted with petroleum ether for 30 min to remove fat. The defatted sample (1.0 g), was suspended in phosphate buffer, (25 mL, pH 6.0, 0.1 M) followed by the addition of Termamyl (0.1 mL) and kept in a boiling water bath for 15 min to digest starch. The contents were cooled and water (20 mL) was added and the pH was adjusted to 1.5 with 4 N HCl. Proteins were removed by digesting with pepsin (100 mg) at 40^{o}C for 1 h. Once again water (20 mL) was added and the pH adjusted to 6.0 with 4 M NaOH. To this, pancreatin (100 mg) was added, and incubated at 40^{o}C for 1 h. Finally the contents were cooled, and the pH was adjusted to 4.5 with 4 N HCl and filtered through a dried and weighed crucible containing celite (0.5 g).

2.4.2.1 Insoluble dietary fiber

The residue retained in the crucible was washed with ethanol (95%, 20 mL) followed by acetone (20 mL). The crucible was kept in an oven (105^{o}C) till the weight became constant and the final weight was taken accurately (D_1). The crucible was then incinerated at 550^{o}C for 5 h, and once again its weight (I_1) was recorded.

2.4.2.2 Soluble dietary fiber

The volume of the filtrate was adjusted to 100 ml and the soluble fibers were precipitated by adding 4 volume of warm ethanol (60^{o}C). The precipitate was filtered through celite, dried and weighed after drying at 105^{o}C (D_2) followed by incineration at 550^{o}C (I_2).

C. Blank was prepared as above without sample.

D. Soluble and insoluble dietary fiber contents (%) were calculated by using following formula:

$$\% \text{ Insoluble fiber} = \frac{D_1 - (I_1 - B_1)}{W} \times 100$$

$$\% \text{ Soluble fiber} = \frac{D_2 - (I_2 - B_2)}{W} \times 100$$

2.4.3 Isolation of free sugars [157]

The sample (10 g) was defatted with petroleum ether and chloroform (1:1) by using Soxhlet extraction. Then sample was extracted with 70%

alcohol to extract the sugars completely. The extracts were pooled and concentrated by flash evaporation. They were further centrifuged, and the resulting supernatant was subjected to ion exhange chromatography, initially on Dowex-50 H^+ (to remove amino acids/organic acids) followed by Dowex-1 OH^- (to remove cations/organic acids). The sugar fraction was collected by eluting with water. The fractions were pooled, concentrated, clarified by centrifugation (1600 g, 10 min) and quantitated by HPLC.

2.4.4 Extracton of polysaccharides

2.4.4.1 Isolation of non-starch polysaccharides (NSP) [158]

The alcohol insoluble residue of the flour was suspended in water (1:100, w/v). The suspension was kept in boiling water bath for 1h to facilitate gelatinisation. Termamyl (1.0 mL) was added at intervals to digest the starch. Starch was digested till it shows negative test to iodine solution. The contents were cooled to 60^{o}C and then subjected to glucoamylase digestion. The digestion was carried out for 1-2 h. The digest was centrifuged. The supernatant (water-soluble polysaccharides) was dialyzed, and lyophilized (water-soluble polysaccharides). Destarched residue was further extracted with 0.5% ammonium oxalate in a boiling water bath at 85^{o}C for 2 h in order to extract pectins. The residue was further extracted twice with 10% alkali[10] (1:10, w/v) under nitrogen atmosphere for 4 h each. The extract was centrifuged and the residue (alkali-insoluble residue - AIR) was washed with water till the pH became neutral. The pH of the extract was adjusted to 4.5 by adding 50% acetic acid. The polysaccharides

obtained at this pH were separated by centrifugation at 3500 g for 15 min, and was designated as hemicellulose A. To the supernatant, 3 volumes of ethanol was added to precipitate hemicellulose B. Scheme of extraction is depicted in Fig.1.

2.4.5 Hydrolysis of polysaccharides

2.4.5.1 Trifluoracetic acid hydrolysis

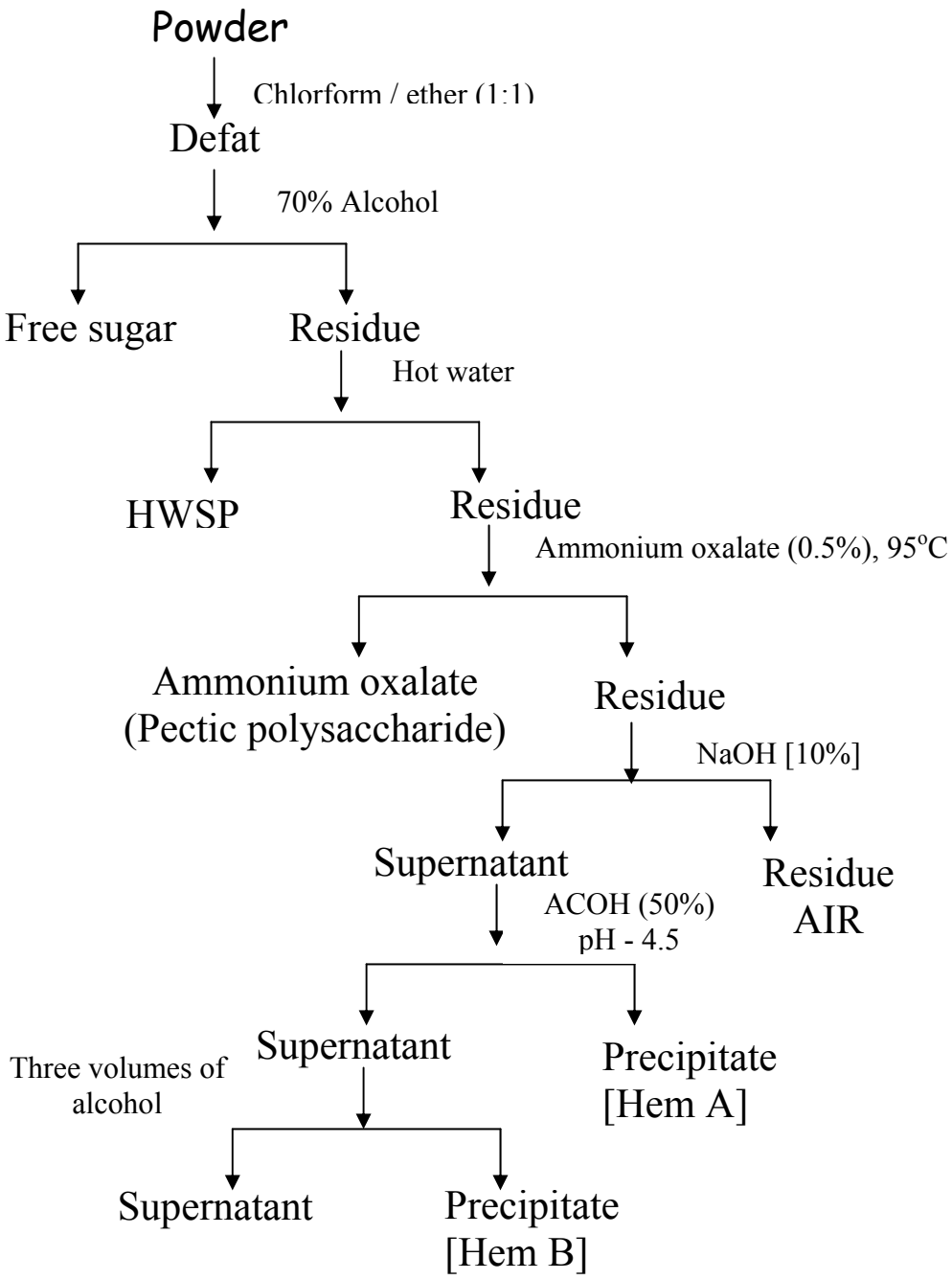
Sample (10 – 15 mg) was taken in 1 mL of trifluoroacetic acid (2 N) and the tube was sealed. Hydrolysis was carried out at 100^{o}C for 6-8 h in an oven. After the hydrolysis, the acid was removed by flash evaporation at water bath temperature of 40^{o}C and co-distilled with water (1 mL, x 3).

2.4.5.2 Sulphuric acid hydrolysis[159]

The polysaccharide (10 mg) was suspended in water and was hydrolyzed by prior solubilization with 72% sulphuric acid at ice cold temperature followed by dilution to 8% acid and heating in a boiling water bath at 100^{o}C for 10-12 h. The above mixture was neutralized with barium carbonate (solid), filtered, deionized with Amberlite IR 120 H^{+} resin and concentrated.

2.4.6 Hydrolysis of permethylated polysaccharides

Permethylated sample was hydrolysed in 2 mL of formic acid (90%) at 100^{0}C for 2 h in a boiling water bath. Excess acid was evaporated by codistillation with methanol (1.0 mL, ×4). The samples were then hydrolysed with TFA (2 N, 1 mL) in sealed tubes at 100^{o}C in an oven



HWSP - Hot water soluble polysaccharide, Hem A - Hemicellulose A,
Hem B - Hemicellulose B, AIR - Alkali insoluble residue.

Fig. 1. Schematic representation of extraction of non-starch polysaccharides.

for 6-8 h. Acid was removed as in section 2.4.5.1 but the water bath temperature was maintained at bellow 35^{o}C.

2.4.7 Regeneration of Amberlite IR-120 H^{+}resin

The Amberlite resin was washed with water to remove the fines, colour and other impurities. The water was drained by filtering it through a nylon cloth. The resin was then regenerated by suspending in HCl (2 N) for 1 h at room temperature with intermittent shaking. The resin was then filtered through nylon cloth and washed thoroughly with water till the filtrate gave neutral pH.

2.4.8 Chromatographic methods

2.4.8.1 Paper chromatography

Whatman No.1 paper was used to run descending paper chromatography. For the separation of neutral sugars, solvent system consisting of n-butanol, pyridine and water in the ratio of 6:4:3 was used. The paper was run for 24 h, air-dried and then sprayed with aniline - phthalate reagent or silver nitrate reagent.

2.4.8.1.a Preparation of aniline- phthalate reagent[160]

Phthalic acid (1.66 g) was dissolved in water - saturated butanol (99 mL butanol + 1 mL water). To this 1.0 mL of aniline was added and mixed well. The air-dried chromatogram was dipped in the reagent, air dried and then placed in an oven for 5 min at 110^{o}C.

2.4.8.1.b Preparation of silver nitrate reagent[161]

The air-dried chromatogram was dipped in silver nitrate solution (1.0 mL saturated $AgNO_3$ in water, diluted to 6.0 mL with water + 200 mL acetone). After drying, it was dipped in methanolic potassium hydroxide (1 volume of 10% aqueous potassium hydroxide + 5 volumes of methanol). When black spots appeared in the region of sugars, the chromatogram was washed with sodium thiosulphate (0.05 M) solution to clear the background colour. Finally the chromatogram was washed with water and dried.

2.4.9 Determination of total nitrogen by Kjeldhal method[162]

The sample (0.5-1.0 g) was digested with concentrated sulphuric acid (20 mL) in the presence of catalyst (98 parts K_2SO_4 + 2 parts $CuSO_4$) till the solution became clear. The contents of the flask were cooled and the volume was made up to 100 ml with water in a volumetric flask. A known aliquot from the digested material (5 ml) was made alkaline by adding sodium hydroxide (10 ml, 40%) and methyl red indicator was added. The solution was titrated with 0.01 N HCl till the solution became bluish green. Simultaneously a running blank was processed as above with water, in place of sample. Titre value of the blank was deducted from test values. Ammonium sulphate solution (1 M) was used as the standard.

% Protein was calculated by using the formula:

$$\frac{\text{Titre value of sample (mL)}}{\text{Titre value of standard (mL)}} \times \frac{\text{Dilution factor}}{\text{Volume of solution (0.5 mL)}} \times \frac{\text{Factor (6.25)}}{\text{Weight of the sample (mg/g)} \times 1000}$$

2.4.10 Gas liquid chromatography

2.4.10.1 Preparation of alditol acetates [163]

The neutralized and deionised sample was concentrated to about 0.5 mL. Sodium carbonate was added to a concentration of about 0.07 M to decompose uronic acids. Sodium borohydride (20 - 30 mg) was added and the test tubes were stoppered and taped with adhesive plaster around to hold the stoppers. They were left overnight. Next day, excess borohydride was destroyed with acetic acid (2N). The excess borate and other salts were removed by co-distilling with methanol (1mL, x4) and then evaporated to dryness. Dry and distilled acetic anhydride and pyridine (0.5 mL each) were added and kept in an oven at 100^0C for 2 h after tightly stoppering the tubes. Excess reagents were removed by co-distilling with water (1mL, x3) and toluene (1mL, x3). After thorough drying, the contents were taken in chloroform and filtered through glass wool and dried by passing nitrogen gas. They were taken in chloroform for analysis.

2.4.10.2 Operating conditions of GLC

Shimadzu GLC (Model-CR4A) fitted with flame ionization detector was used for analysis. OV-225 (1/8" x 6') was the column used with column, injector and detector block temperatures maintained at 200, 250 and 250^{o}C, respectively. Nitrogen with the flow rate of 40 mL/min was used as the carrier gas.

2.4.11 High performance liquid chromatography (HPLC)[164]

E-linear (7.8 mm x 30 cm) connected in series with E-1000 (3.9 mm x 30 cm) column was used. Degassed triple distilled water was the mobile phase used. Flow rate was maintained at 0.6 mL/min. Oven temperature was 50^{o}C and RI setting was at 8 x 10^{-6} RIU. Sample (5 to 10 μL) solutions having concentration of about 10 mg/mL (w/v) were injected. Calibration curve was prepared using dextran standards of different molecular weights (T-40, T-70, T-150, T-500, T-2000).

2.4.12 DEAE-cellulose column chromatography

2.4.12.1 Regeneration of DEAE-cellulose[165]

DEAE-cellulose was washed with water to remove fine particles. It was then treated successively with HCl (0.5 N) and NaOH (0.5 M). After each treatment, the pH was adjusted to neutrality by washing thoroughly with water. The regenerated exchanger was suspended in ammonium carbonate (0.5 M, pH 9.3), packed in a column (3.5 cm x 26 cm) and excess carbonate was washed off with water.

2.4.12.2 Fractionation[165]

The fraction (1g) was dissolved in water and loaded on to DEAE-cellulose column and the elution was carried out with water, followed by ammonium carbonate (0.1 to 0.3 M) and sodium hydroxide (0.1 and 0.2 M) solutions. The flow rate was maintained at 60 mL/h and fractions (10 mL) were collected, assayed for total sugar by phenol-sulfuric acid method (2.2.9.2.a). Carbohydrate positive fractions were pooled, dialyzed and lyophilized.

2.4.13 Homogeneity criteria

2.4.13.1 Molecular sieving[166]

Gel permeation chromatography was performed on Sepharose CL-4B (1.6 cm × 92 cm). The polysaccharide fractions (10 mg) were dissolved in distilled water, centrifuged and loaded (1 mL) on to the column. The elution was carried out by using NaCl (0.1 M) containing sodium azide (0.05%) at a constant flow rate of 16 mL/h. Fractions (3 mL) were collected and analyzed for the presence of total sugar and appropriate fractions were pooled. Dextran series standards (T-40, T-70, T-150, T-500, T-2000) were used to determine molecular weight. Blue dextran was used to determine void volume. A calibration curve was prepared by plotting V_e /V_0 versus log molecular weight. Where, V_e = void volume, V_0 = elution volume. Molecular weight of the unknown polysaccharide was determined, from this graph.

2.4.13.2 Electrophoresis [167]

Electrophoresis was performed on cellulose acetate membranes using Beckman microzone electrophoretic cell (Model R 101). Ammonium carbonate - NaCl buffer (pH 9.3, 0.05 M) was used at a constant voltage of 180 V. Prior to electrophoresis the membranes were wetted with running buffer and the excess buffer was removed by using blotters. The polysaccharides (1 mg each) were dissolved in water (0.1 mL) and loaded (10 μL each) by using an applicator. The rate of migration was followed by using procion red marker dye. The polysaccharides were stained with ruthenium red (0.5%) in water and excess dye was removed by washing with water.

2.4.14 Carboxyl group reduction of polysaccharide[168]

To the clear solution of polysaccharide (100 mg/20 mL) was added 1-cyclohexyl-2 (4-methylmorpholino)-ehylcarbodiamide p-toluene sulfonate (1 g) in small portions over a period of 2 h. The pH of the solution during the reaction was maintained between 4-5 by the addition of dilute HCl (0.1 N). The reduction was carried out by the addition of sodium borohydride (2 M, 10 mL) over a period of 4 h during which the pH was maintained around 7.0 by the addition of HCl (4 N). Octanol in small aliquots was added to prevent foaming during the additions. The above mixture was dialyzed and lyophilized. This reaction was repeated twice for quantitative conversion of carboxyl groups into primary alcohols.

2.4.15 Structural analysis

2.4.15.1 Methylation analysis[169]

2.4.15.1.a Preparation of methyl sulphinyl carbonion (MSC)[169]

Sodium hydride (99%, 500 mg) was taken in a reaction vial and washed with 5 mL petroleum ether (dried with sodium wire). This was repeated 4-5 times and finally dried by passing dry nitrogen gas. Dry dimethyl sulphoxide (5.0 mL) was added in small portions over a period of time. It was kept at 37^{o}C for 12-14 h with occasional venting of hydrogen, which was formed during the reaction. The prepared MSC gave red blood color with triphenyl methane.

2.4.15.1.b Methylation

Polysaccharide (5-10 mg) was dissolved in distilled dry DMSO (0.5 mL) with stirring and/or occasional ultrasonication. MSC (1.0 mL) was added to the above solution and the mixture was stirred at room temperature for 3-4 h. After the reaction, the mixture was tested for excess reagent and was made sure that the test was positive for triphenyl methane test. Iodomethane (1.0 mL) was added to the reaction mixture at ice-cold temperature with the help of a syringe. The reaction mixture was stirred overnight.

2.4.15.1.c Purification of methylated polysaccharides [170]

This was done using Sep-Pak C18 cartridge. The cartridge was activated by flushing ethanol (40 mL) followed by acetonitrile (2 mL) and water (4 mL). The methylated reaction mixture was diluted with

equal volume of water and was passed through the cartridge. Then the vial was rinsed with DMSO-water (0.5 mL, 1:1) and passed through the cartridge**.** More polar contaminants of the reaction such as DMSO and sodium iodide were eluted with water (2.0 mL, x4). Less polar contaminants held on the cartridge were eluted with 2 mL of the following: acetonitrile-water 3:17 (v/v, x4), 100% methanol (x4) and 95% ethanol (x4). The cartridge was flushed with solvent mixtures at a rate of about 1-2 drops/sec. The fractions eluted were tested on silica gel TLC strip by charring with 100 mL ethanol containing 1 mL sulphuric acid. The tubes giving positive test for carbohydrates were pooled and concentrated by flash evaporation.The methylated samples eluted with 100% acetonitrile and 100% methanol.

2.4.15.1.d Derivatization

The methylated polysaccharides were hydrolysed (2.4.6), reduced using $NaBD_4$ in D_20 and acetylated (2.4.10.1).

2.4.15.1.e Gas liquid chromatography-Mass spectrometry (GLC-MS)[171]

GLC-MS analysis was carried out on Shimadzu (Model-QP5000) using SP 2330 capillary column (0.32 mm x 30 M). A temperature gradient of 180-200^{o}C with an increase of 4^{o}C/min was maintained for the analysis. Ionization potential was 70 eV and mass range (m/z) was 40-400. Helium was the carrier gas used.

2.4.15.2 Periodate oxidation [172]

To 5.0 mL of aqueous solution of the polysaccharide, sodium meta periodate, (5.0 mL, 20 mM) was added. The reaction mixture was mixed well and kept at 4°C in the dark. At regular intervals, 0.5 mL aliquots were withdrawn and mixed with saturated sodium bicarbonate (1.0 mL), sodium arsenate (2.0 mL, 0.005 M) and potassium iodide (0.2 mL, 20%). The reaction mixture was kept aside for 15 min in the dark and titrated against iodine solution (standardized, 0.01 N) using 1-2 drops of starch solution (2.0%) as indicator. Simultaneously, a blank was run by taking 0.5 mL water in place of the sample. The consumption of periodate was calculated using the formula:

$$\text{Periodate oxidation} = \left(E - \frac{(V_1 - V_2) \times C}{1000} \right) \times \frac{M}{G}$$

Where, E = Actual moles of periodate taken,

V_1 = Titre value of the blank,

V_2 = Titre value of the sample,

C = Concentration of standardized iodine solution,

M = Molecular weight of sugar (180),

G = Weight of sample taken in grams.

2.4.15.3 Formic acid liberation [173]

Aliquot (0.5 mL) was withdrawn from the above reaction mixture after periodate consumption had leveled off. To this, 2.0 mL of ethylene glycol in water (50%), water (5 mL) and 2-3 drops of phenolphthalein (0.02% in ethanol) were added and titrated against sodium hydroxide (0.01 M). Appearance of the pink colour signified the end point. The liberated formic acid was calculated according to the formula:

$$\text{Formic acid liberated} = \frac{(V_1 - V_2) \times N_{NaOH} \times MW}{0.5}$$

Where, V_1 = Sample titre value,

V_2 = Blank titre value,

N_{NaOH} = Normality of sodium hydroxide,

MW = Molecular weight of sugar (180),

0.5 = Volume of sample taken for titration.

2.4.15.4 Smith degradation [174]

To the polysaccharide solution (10 mg / 5 mL) was added sodium meta periodate (20 mM, 5 mL) and kept at 4^{o}C for 48 h. The reaction was stopped by adding ethylene glycol (0.1 mL) and the oxidized polysaccharide was kept for reduction with sodium borohydride (100 mg) for 16 h at room temperature. The excess borohydride was destroyed by using acetic acid (2 N) and the solution was dialyzed and

the polyalcohol was hydrolyzed with 0.5 N H_2SO_4 at room temperature for 48 h and analyzed.

2.4.15.5 Optical rotation

Aqueous solution of polysaccharide (0.5 – 1.0 %) was used to measure the optical rotation in a Perkin - Elmer (Model 243) polarimeter. Optical rotation was calculated using the formula:

$$\text{Optical rotation } [\alpha]_D = \frac{100\theta}{lc}$$

Where, θ = Angle of rotation of plane polarized light,

l = The path length (1cm),

c = Concentration (%) of the polysaccharide solution.

2.4.15.6 Infrared spectroscopy[175]

Polysaccharide (~1.0 mg) was blended thoroughly with Nujol (liquid paraffin) so that a homogeneous smear is obtained on the window. Its spectra was obtained with Perkin-Elmer (2000 system GC-IR) operating at 4 cm^{-1} resolution.

2.4.15.7 ^{13}C Nuclear magnetic resonance spectroscopy [176]

^{13}C NMR spectra were recorded on a Bruker 400 MHz spectrometer at 70^oC for 5 h. The samples was exchanged twice with D_2O (99%). The samples (50 mg) was then dissolved in D_2O (1mL) for recording the spectra.

2.5 Phenolic acids and their biological activity

2.5.1 Free phenolic acids [177]

Samples (1 g each) were extracted with aqueous ethanol (70%, 50 mL, 1h, x 4) and the supernatant was obtained by centrifugation. The combined supernatant was concentrated, and the pH was adjusted to 2-3 with 4 N HCl. Phenolic acids were extracted with ethyl acetate (50 mL, x 5) and phase separation was followed and dried with anhydrous sodium sulphate. Sodium sulphate was removed by filtration and evaporated to dryness. The dried material was taken in methanol (1 mL) and analyzed on C_{18} column (4.6 x 25 cm) by HPLC using photo - diode array detector (operating at 280 nm) with a solvent system of water : acetic acid : methanol (isocratic, 80:5:15, v/v/v). Caffeic, coumaric, ferulic, gentisic, gallic, protcatecheuic, syringic and vanillic acids were used as standards and the quantitation of phenolic acids in the samples was done by measuring the area under each peak.

2.5.2 Bound phenolic acids[178]

Samples (1 g each) were first extracted with ethanol (70%, 50 mL, 1 h, x 4) and then with hexane (50 mL, 1 h, x 4) to remove free phenolics, sugars and fat. The residues were extracted with 1 M NaOH (100 mL, 2 h, x 2) containing sodium borohydirde (0.5%) under nitrogen. The clear supernatants obtained after centrifugation were pooled, acidified with 4 N HCl till the pH became 1.5. Released phenolic acids were extracted, quantified and characterized, as above (Section 2.5.1).

2.5.3 Determination of total phenolic content[179]

The total phenolic content of the samples was determined colorimetrically using the Folin-Ciocalteu method. To the 100 μL sample, 900 μL of water, 5 mL of 0.2 N Folin - Ciocalteu reagent and 4 mL of saturated sodium carbonate solution (100 g/L) were added and mixed. The absorbance was measured at 765 nm using a Shimadzu UV-Visible spectrophotometer after incubation for 2 h at room temperature. The total phenolic content was expressed as gallic acid equivalent (GAE) in milligrams per gram sample.

2.5.4 Reverse phase chromatography

C_{18} (Shimpack) column was used to analyze the phenolic acids. Mobile phase consisted of water, acetic acid and methanol in the ratio of 80:5:15. The flow rate was maintained at 1 mL/min. UV detection at 280 nm was employed. Samples were taken in methanol (1 mg/1 mL) of which 5-10 μL was injected.

2.5.5 Antioxidant activity

2.5.5.1 Scavenging effect of extracts on DPPH radical[180]

The effect of free and bound phenolics on DPPH radical was estimated according to the method of Lih-Shiuh Lai *et.al*[180]. An aliquot of 200 μL of different phenolic fractions (2-10 μg, GAE) of the sample and standard antioxidants (2-10 μg) were mixed with 100 mM Tris-HCl buffer (800 μL, pH 7.4) and then added to 1 mL of 500 μM DPPH in ethanol (final concentration of 250 μM). The mixture was shaken

vigorously and left for 20 min at room temperature in the dark. The absorbance of the resulting solution was measured spectrophotometrically at 517 nm. The capability to scavenge the DPPH radical was calculated using the following equation.

Scavenging effect (%) = (1 - absorbance of sample at 517 nm/absorbance of control at 517nm) x 100.

2.5.5.2 Measurement of reducing power[181]

The free and bound phenolic extract (2-10 μg) of the sample and standards BHA and BHT (2-10 μg) were mixed with an equal volume of 0.2 M phosphate buffer, pH 6.6 and 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min. Then an equal volume of 10% trichloroacetic acid was added to the mixture and then centrifuged at 1600 g for 10 min. The upper layer of solution, distilled water and 0.1 % $FeCl_3$ at a ratio of 1:1:2 was taken and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

2.5.5.3 DNA protection assay[182]

DNA protection activity was performed using calf thymus DNA. Briefly, calf thymus DNA (1 μg) was added to Fenton's reagent (30 mM H_2O_2, 50 μM ascorbic acid and 80 μM $FeCl_3$) containing DNA, with sample, with out sample and only DNA. The final volume of the mixture was brought up to 20 μL and then incubated for 30 min at 37°C and the DNA was analyzed on 1% agarose gel and stained with ethidium bromide.

Chapter III

Results and Discussion

3.1 Effect of spent turmeric on diabetic status

Male Wistar rats weighing around 120 g were used for the study. The rats were maintained on AIN-76 diet (Table 1, Section 3.2.2). The diabetic status of the rats was assessed by measuring the level of fasting blood glucose in the blood drawn from retro-orbital plexus and they were grouped in to uniform diabetic status and were given either starch based (SFC/SFD) or spent turmeric based (TFC/TFD) diet. To all the rats water and fresh diet were given *ad libitum*. The rats were monitored regularly for diet intake, water intake, gain in body weight, urine volume, urine sugar and fasting blood glucose. The rats were maintained with the experimental diet for 45 days and were sacrificed when the mortality was about to set in, which was initially in starch fed diabetic group (SFD).

Effect of spent turmeric on water, diet intake and gain in body weight in control and diabetic rats

High amount of water intake is a characteristic symptom of diabetes. The control rats (SFC/TFC) consumed 20-30 mL of water/day (Table 2). The consumption of water was 101 mL, which was high in starch fed diabetic group and was due to the development of polydipsia condition, and was ameliorated in the spent turmeric fed diabetic group (TFD), and was statistically significant when compared to SFD.

Diet consumption was followed in both control and diabetic rats. The starch fed control rats (SFC) consumed around 14 g/day (Table 2). Consumption of diet was more in starch fed diabetic group (SFD) and

Table 2. Effect of spent turmeric on water intake, diet intake and body weight in control and diabetic rats

Group	Water intake (mL/day)	Diet intake (g/day)	Body weight (g)		
			Initial	Final	Gain
SFC	22.5 ± 1.1	13.8 ± 0.8	116.3 ± 3.8	212.0 ± 15.7	95.7 ± 11.8
SFD	101.3 ± 6.3 [a]	15.6 ± 1.3[a]	124.1 ± 2.3	123.4 ± 7.1[a]	0.76 ±0. 007[a]
TFC	32.3 ± 1.1	18.6 ± 0.4	119.3 ± 6.5	234.0 ± 6.9	114.7± 10.4
TFD	89.3 ± 4.1	20.3 ±1.3[b]	120.2 ± 2.1	161.0 ± 5.6[b]	40.8 ± 03.5[b]

SFC: Starch fed control, SFD: Starch fed diabetic,

TFC: Spent turmeric fed control, TFD: Spent turmeric fed diabetic.

Values are Mean ± SEM of 6 rats in control and 8 rats in diabetic groups,

[a] Statistically significant when compared to SFC at $p<0.05$,

[b] Statistically significant when compared to SFD at $p<0.05$.

may be due to the development of hyperphagic condition and was statistically significant when compared to SFC. Consumption of diet was more in spent turmeric fed control group (TFC) when compared to SFC. The treated group (TFD) consumed more diet when compared to SFD. Higher consumption of diet by the rats fed with spent turmeric may be due to the presence of high amount of dietary fibre present in the diet. Foods rich in dietary fibers are known to be consumed in higher amounts, which may be due to better palatability and caloric dilution. [183,184]

Body weight was examined in all the groups. Average body weight of all the rats taken for the study was around 120 g at the beginning of the experiment (Table 2). The control rats (SFC/TFC) kept on increasing their weight uniformly and at the end of the experiment weight of the control rats ranged between 210-235 g. The body weight was more in spent turmeric fed control groups (TFC). The diabetic rats did not gain weight and the starch fed diabetic rats (SFD) apparently had less body weight. Improvement in body weight was observed in spent turmeric fed diabetic group (TFD) and was 30.4% during the experimental period.

Effect of spent turmeric on urine sugar and urine output in control and diabetic rats

Excretion of sugar in urine was followed on weekly basis (Fig. 2). The control rats (SFC/TFC) excreted sugar in mg level. The diabetic rats (SFD) excreted sugar in g level (glucosurea) throughout the experimental period. The excretion of sugar in the urine was

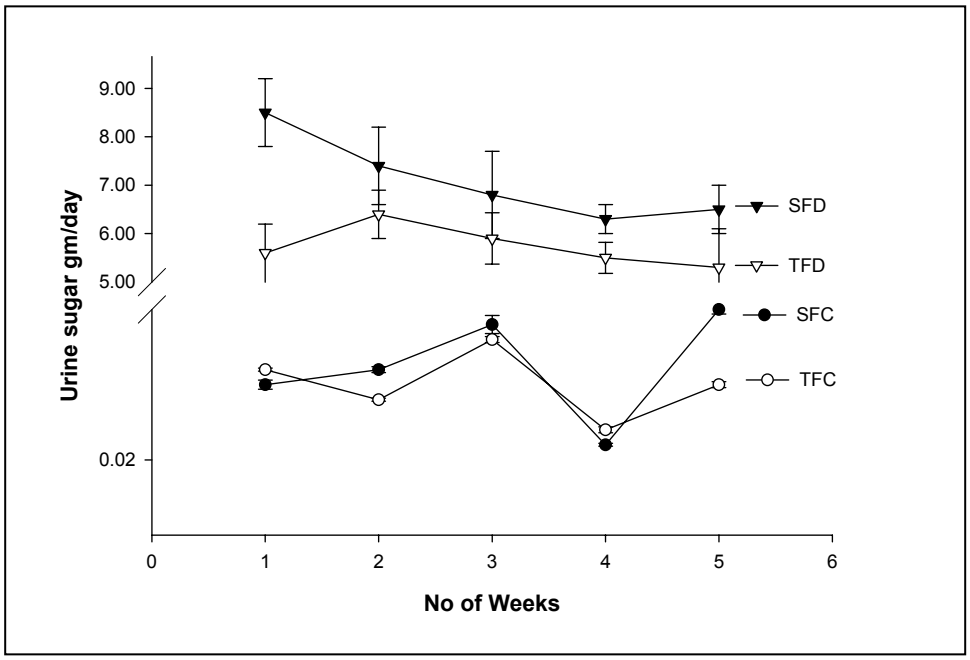


Fig. 2. Effect of spent turmeric on urine sugar in control and diabetic rats.

Abbreviations as in Table 2.

ameliorated by 31.0% by the feeding of spent turmeric in the diet. These results clearly indicate an alleviation in the diabetic status by spent turmeric.

Excretion of urine was monitored on weekly basis (Fig. 3). The control rats (SFC/TFC) excreted around 20 mL/day. More excretion of urine (polyurea) prevailed in starch fed diabetic rats (SFD). Spent turmeric feeding to diabetic rats showed 30% amelioration when compared to SFD. This indicated augmentation in diabetic status.

Effect of spent turmeric on fasting blood glucose in control and diabetic rats

Fasting blood glucose was measured at the end of the experiment after drawing blood from heart puncture at the time of sacrificing the rats. The fasting blood glucose level was around 120 mg/dL in all control groups (Fig. 4). The starch fed diabetic group (SFD) had fasting blood sugar level of 302 mg/dL. The spent turmeric fed diabetic group (TFD) showed an improvement in fasting blood glucose level and was 247.7 mg/dL.

Dietary fibers, have protective influence against diseases like diabetes, colon cancer, etc [108,184]. Dietary fiber (DF) rich foods are known to have hypoglycemic properties[185,186]. Spent turmeric being rich in dietary fibers (46.3%; 5.9% soluble and 40.4% insoluble) ameliorated the diabetic status in streptozotocin induced diabetic rats in terms of urine sugar, urine volume, fasting blood glucose and body weight. The observed beneficial effects may be due to structural and functional

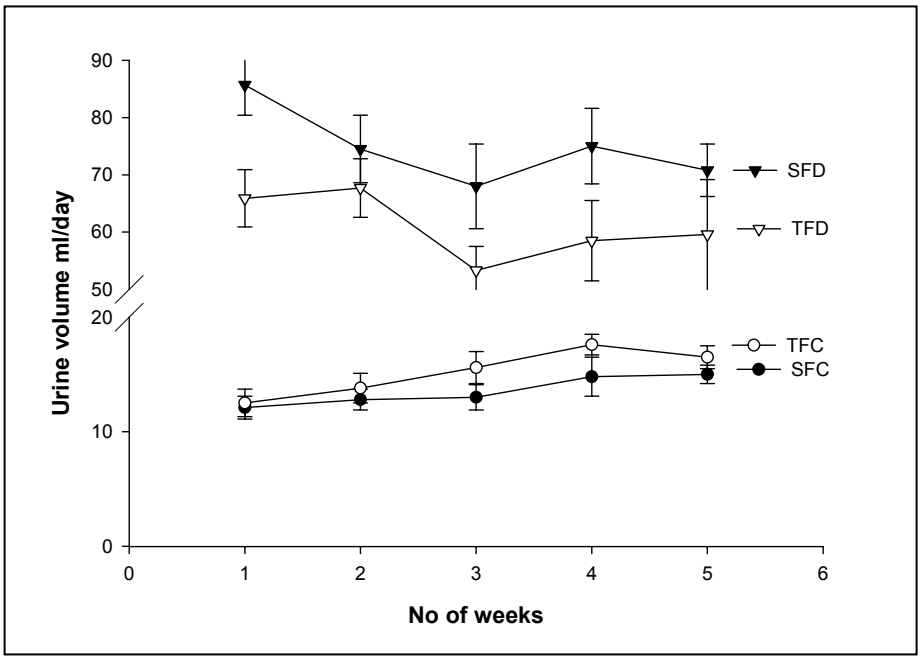


Fig. 3. Effect of spent turmeric on urine volume in control and diabetic rats.

Abbreviations as in Table 2.

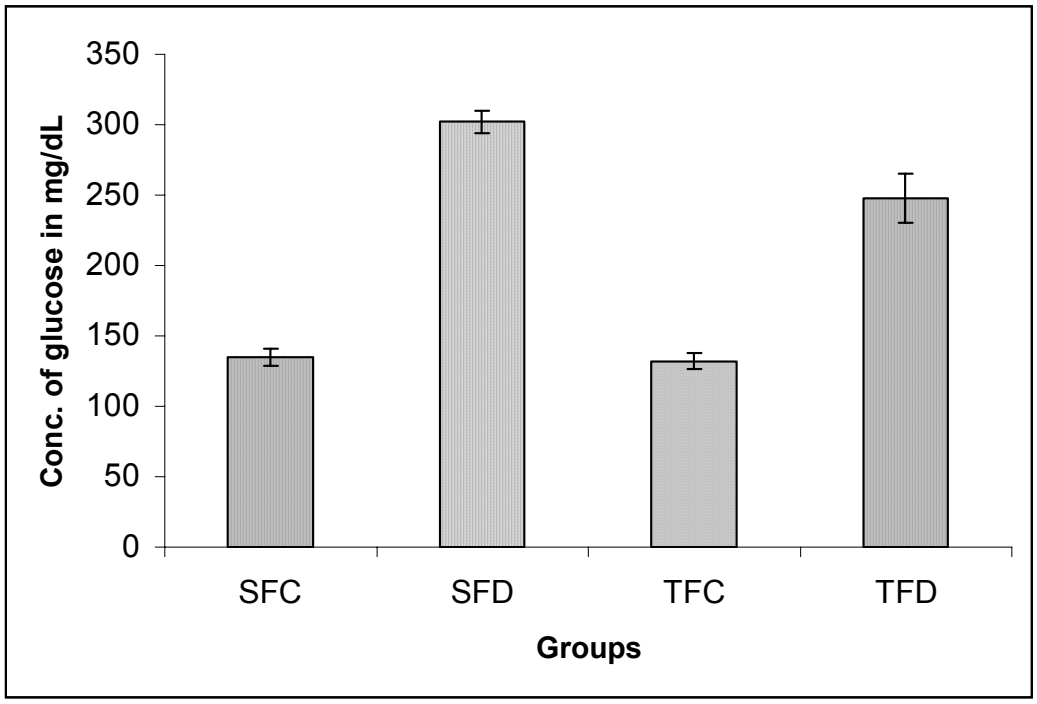


Fig. 4. Effect of spent turmeric on fasting blood glucose in control, diabetic and treated rats.

Abbreviations as in Table 2.

diversities of DF. The DF act as insoluble matrix and facilitate slow release of glucose and the DF that are not digested by gastro-intestinal enzymes are fermented in the large intestine by colonic microflora producing short chain fatty acids (SCFA) [187,188], such as acetate, propionate and butyrate. In recent years role of SCFA and butyric acid in particular are well recognized to facilitate normal health and well being and under disease conditions like cancer and diabetes. Our laboratory has shown that butyric acid modulates diabetic status [189].

3.2 Effect of spent turmeric on intestinal and renal disaccharidases

Diabetes frequently results in severe metabolic imbalance and pathological changes in many tissues. In the small intestine this causes significant changes in the morphology and function of mucosa[190,191]. Effect of spent turmeric on the activities of intestinal enzymes is given in Table 3. During diabetes (SFD), there was a significant increase in maltase activity (2.2 fold). Spent turmeric in the diet alleviated the maltase activity by 21%. Activity of sucrase showed an increase in SFD by two fold when compared to starch fed control (SFC) and the activity was ameliorated in spent turmeric fed diabetic group (TFD) by 35%, when compared to starch fed diabetic rats (SFD). Lactase activity was increased during diabetes (SFD) by nearly two folds and was augmented by spent turmeric feeding (25.6 %).

In kidney, presence of disaccharidases activities in sub-cellular fractions of cortex reflects an involvement in the digestion and transport of the

Table 3. Effect of spent turmeric on activities of intestinal maltase, sucrase and lactase in control and diabetic rats

Groups	Maltase	Sucrase	Lactase
	μmoles of product formed /g of protein /min		
SFC	310.16±23.52	37.50±9.76	2.02±0.28
SFD	699.08±62.43[a]	88.23±6.95[a]	3.99±0.41[a]
TFC	267.30±8.31	29.71±6.09	2.15±0.28
TFD	552.44±44.76[b]	57.21±4.82[b]	2.97±0.21

Abbreviations as in Table 2.

sugars across the membrane[192]. Renal disaccharidases activities also showed variation in diabetic groups (Table 4). Maltase activity was highest among three disaccharidases. The activity of maltase decreased significantly in SFD when compared to SFC. Spent turmeric in the diet ameliorated the maltase activity (18%). Decrease in the activity of sucrase was observed during diabetes (SFD) compared to the control and alleviation in the sucrase activity was observed by the feeding of spent turmeric. Activity of lactase in the kidney was decreased during diabetes and was alleviated by spent turmeric feeding.

Disaccharidases are present in small intestinal brush border, and play major role in the hydrolysis of disaccharides to monosacchardes, there by facilitating their absorption. Maltase is one of the major enzymes present in the intestinal brush border. Maltase from kidney and intestine have the same molecular weight and K_m values [193] but there is a difference in the gene expression of disaccharidases in kidney and intestine, although they share many structural and enzymatic similarities. Repletion of insulin in this situation brings back the enzyme activity to normal level[194]. Thus it indicates that insulin has an inhibitory effect on disaccharidases in intestine.

Increase in intestinal disaccharidases activities and decrease in renal disaccharidases activities and alleviation by fiber feeding has been reported[195,196]. It was reported that pectin delays the uptake of glucose and maltose in rats[197]. This may be due to physical adsorption which may alter nutrient availability at the level of intestinal brush border resulting in less amount of sugar absorption and making the diabetic

Table 4. Effect of spent turmeric on activities of renal maltase, sucrase and lactase in control and diabetic rats

Groups	Maltase	Sucrase	Lactase
	μmoles of product formed /g protein /min		
SFC	120.16±6.3	2.13±0.10	3.10 ± 0.30
SFD	81.22±2.8[a]	1.66±0.09[a]	2.07 ± 0.90[a]
TFC	129.00±2.3	2.13 ±0.20	3.01 ± 0.38
TFD	96.00±4.6[b]	1.76 ± 0.08[NS]	2.18 ± 0.05[NS]

Abbreviations as in Table 2.

animals more tolerant to hyperglycemia. The short chain fatty acids (SCFA) released by the fermentation of dietary fibers may alter activities of disaccharidases both in the intestine and kidney. These beneficial effects on disaccharidases may also be due to SCFA including butyric acid. It was shown that the intraluminal colonic infusion or the intraperitoneal injection of SCFA stimulates jejunal and ileal mucosal cell proliferation[198]. Butyrate showed amelioratory effect on intestinal and renal disaccharidases[199] .

3.3 Effect of spent turmeric on carbohydrate profile in different tissues

Effect of spent turmeric on organ weights

In this study main emphasis was to study changes of glomerular filtration matrix in relation to diabetic nephropathy status with particular emphasis on heparan sulfate in kidney. Hence detailed studies are carried on kidney and are presented in sections 3.5 and 3.6. Preliminary data on changes in carbohydrate and glycosaminoglycan profile in different tissues is presented below (Sections 3.3 and 3.4).

Weights of various organs of control and streptozotocin induced diabetic rats and the influence of spent turmeric on their weights is presented in Table 5. An increase in organ weights of liver, lungs, brain and testis was observed when compared to starch fed control groups (SFC/TFC). Relative weights of the organs were decreased during the course of the experiment (Table 5). These changes in organ weights are alleviated to some extent by the feeding of spent turmeric (TFD).

Increase in the liver weight is reported in diabetes[200] and is in agreement with our results.

Effect of spent turmeric on carbohydrate constituents in different tissues

Variations in the constituents of carbohydrates was observed in tissues like, liver, spleen, lungs, heart, and brain during diabetes. Various tissues from the rats were harvested, dried and defatted. The tissues of particular groups were pooled and then taken for analysis. All are average of triplicates of pooled samples.

Effect of spent turmeric on total sugar content in different tissues

The content of total sugars in the organs of control, diabetic and the spent turmeric fed groups is presented in Table 6. The content of total sugars decreased in liver, spleen and brain in experimentally induced diabetic rats (SFD) and there was not much change in lungs but heart showed an increase when compared to controls (SFC/TFC). The feeding of spent turmeric (TFD) ameliorated the decreased content of total carbohydrates in liver and spleen. Increase in the content of total sugars in the heart was marginally alleviated by spent turmeric. Lungs did not show much change both in the diabetic or experimental group.

Effect of spent turmeric on uronic acid content in different tissues

The content of uronic acid decreased during diabetes (SFD) in liver, spleen and brain (Table 7) when compared to SFC/TFC. Spent turmeric (TFD) marginally prevented the decrease in uronic acid content. There was not much change in uronic acid content in lungs and heart.

Table 6. Effect of spent turmeric on total sugar content in control and diabetic rats

Groups	Liver	Spleen	Lungs	Brain	Heart	Testis
	g/100g dry tissue					
SFC	5.98	2.34	3.25	3.55	1.85	2.62
SFD	3.30	1.47	3.35	2.55	2.20	3.26
TFC	6.20	1.90	3.36	3.44	1.70	2.47
TFD	3.81	1.72	3.50	3.01	1.82	2.85

Abbreviations as in Table 2.

Table 7. Effect of spent turmeric on uronic acid content in control and diabetic rats

Groups	Liver	Spleen	Lungs	Brain	Heart	Testis
	g/100g dry tissue					
SFC	0.45	0.22	0.06	0.29	0.25	0.21
SFD	0.26	0.13	0.05	0.15	0.23	0.25
TFC	0.43	0.20	0.13	0.24	0.22	0.26
TFD	0.34	0.16	0.14	0.20	0.24	0.29

Abbreviations as in Table 2.

Excretion of uronic acid was also shown to be increased during diabetes[201], which may be due to degradation of glycoconjugates of different tissues.

Effect of spent turmeric on amino sugar content in different tissues

The content of amino sugars decreased during diabetes (SFD) in liver, spleen and lungs (Table 8). The decrease was significant (25%) in liver compared to other tissues. The decreased amount of amino sugar was ameliorated by feeding spent turmeric (TFD). Lungs showed decreased content of amino sugars in all the diabetic groups and not much change was observed by dietary treatment. Heart did not show much change between control (SFC/TFC), diabetic (SFD) and treated (TFD) groups.

Effect of spent turmeric on sulfate content in different tissues

Sulfate groups present on glycosaminoglycans and glycoproteins are now well established to perform variety of important physiological functions and have aroused considerable amount of interest[202-204]. During diabetes (SFD) content of sulfate decreased in liver, spleen, lungs and heart (Table 9). The decrease was significant in liver 21.5% and heart 15.3% when compared to control (SFC/TFC) and considerable amount of amelioration was observed by feeding spent turmeric (TFD). Dietary treatment did not bring much alteration to the increase in sulfation in testis and there was not much change between control, diabetic and treated groups. Incorporation of $^{35}SO_4$ in diabetic glomerulus and aorta has been studied in glycosaminoglycans and glycopeptides both *in vivo* and *in vitro* s tudies. Uptake of $^{35}SO_4$ was

Table 8. Effect of spent turmeric on amino sugar content in control and diabetic rats

Groups	Liver	Spleen	Lungs	Brain	Heart	Testis
	g/100g dry tissue					
SFC	0.79	1.81	0.57	0.64	0.27	0.65
SFD	0.59	1.35	0.40	0.61	0.24	0.47
TFC	0.77	1.70	0.48	0.63	0.27	0.63
TFD	0.68	1.34	0.41	0.59	0.26	0.55

Abbreviations as in Table 2.

Table 9. Effect of spent turmeric on sulfate content in control and diabetic rats

Groups	Liver	Spleen	Lungs	Brain	Heart	Testis
	g/100g dry tissue					
SFC	2.13	1.85	1.88	1.33	0.78	0.55
SFD	1.67	1.54	1.35	1.35	0.66	0.70
TFC	2.01	1.78	1.68	1.42	0.89	0.57
TFD	1.97	1.61	1.30	1.42	0.86	0.69

Abbreviations as in Table 2.

shown to be decreased in rat tissues both in streptozotocin and alloxan induced diabetic rats[204].

Effect of spent turmeric on protein content in different tissues

The content of proteins in control, diabetic and treated groups are presented in Table 10. In brain the content of proteins increased in diabetes with respect to control (SFC/TFC) and alleviation was observed by feeding spent turmeric (TFD). Testis showed an increase in protein content in control and diabetic groups and the groups fed with spent turmeric did not show much change. Spleen, lungs and heart did not show significant variations between control, diabetic and treated groups.

During diabetes abnormalities in glycoconjugates in vasculature of liver, muscle and brain is reported[202]. Incorporation of $^{35}SO_4$ in diabetic glomerulus and aorta has been studied in glycosaminoglycans and glycopeptides both in *in vivo* and *in vitro* studies. Our experiment showed modulatory effect of spent turmeric in experimentally induced diabetic rats on constituents of complex carbohydrates, such as, total sugar, uronic acid, amino sugar and sulfate and the content of protein. During diabetes, decrease in the content of glycosaminoglycans has been observed in liver[203] and aorta[204]. The total carbohydrate content decreased in liver during diabetes. This is reported by other workers also [206]. The decrease in sugar content in liver may be due to depletion of glycogen during diabetes, and our results agree with decrease in the carbohydrate content in liver. Modulatory effect on the carbohydrate content is observed in liver by the feeding of spent turmeric. Uronic

Table 10. Effect of spent turmeric on protein content in control and diabetic rats

Groups	Liver	Spleen	Lungs	Brain	Heart	Testis
	g/100g dry tissue					
SFC	87.14	79.82	93.36	71.12	54.30	65.04
SFD	82.92	81.11	90.25	76.20	62.70	72.23
TFC	82.19	77.91	86.50	72.20	63.30	61.12
TFD	81.19	75.31	90.34	77.31	69.22	68.81

Abbreviations as in Table 2.

acid content represents glycosaminoglycan profile in different tissues and was found to decrease in tissues like liver, spleen and brain. The role of glycosaminoglycans in brain is well established and is a study of great academic interest[205, 206] and it is interesting to note a decrease in the content of uronic acid in brain and spent turmeric alleviated the decreased content of uronic acid in brain. Changes are also observed with respect to amino sugars content and it is interesting to note that the content of amino sugars did not alter much in brain between control, diabetic and treated groups. However a decrease in the content of amino sugars is observed in liver, spleen and lungs. Sulfate groups present on glycosaminoglycans, glycoproteins and glycopeptides are now well established to play many vital functions and are receiving much scientific attention in recent years[203]. The content of sulfate decreased in liver, spleen, lungs and heart. Variation in sulfation brings about alteration in charge in the glycoconjugates and hence their function. Reduced content of sulfation on heparan sulfate is reported in the liver of streptozotocin induced diabetic rats[204]. The content of proteins decreased in liver, while an increase was observed in brain. There was no change in spleen, lungs and heart with spent turmeric feeding.

Role of spent turmeric in the management of diabetes could be mainly due to the presence of dietary fibers (46.3%; soluble - 5.9% and insoluble - 40.4%). The dietary fibres serve not only in the slow absorption of glucose in the gastrointestinal tract but are also fermented by the microflora present in the colon, which release short chain fatty acids. In recent years beneficial effect of short chain fatty acids and that of butyric acid are receiving much attention in the improvement of

disease condition like cancer [106,107] and diabetes[185,186]**.** Our own results have provided experimental evidence to show that decreased synthesis of heparan sulfate in diabetes is modulated by dietary fiber[207]. Changes in glycoconjugate metabolism observed during diabetes in different tissues were alleviated by dietary fibers such as wheat bran and guar gum. Further studies on the glycosaminoglycan profile that are affected during diabetes in these tissues during diabetes and to what extent spent turmeric modulates such changes in the glycosaminoglycans is presented in the following section [3.4].

3.4 Effect of spent turmeric on glycosaminoglycans (GAGs) in different tissues

Glycoconjugates perform important biological functions in various tissues[208, 209]. Hyperglycemia, which is pronounced in diabetes leads to abnormalities in the metabolism of proteoglycans and glycosaminoglycans in different organs of the body[210, 211].

Defatted dry powdered tissues were subjected for papain digestion (twice). The extract was then subjected to TCA precipitation and centrifuged. To the supernatant, 2 volumes of alcohol containing 2% potassium acetate was added to precipitate GAGs. This process was repeated again. The precipitate was solubilized in water and the aliquots were used for various estimations. The values are average of triplicate determinations.

Effect of spent turmeric on the content of GAGs in different tissues

The content of isolated GAGs was estimated by DMMB method and is presented in Fig. 4. Decrease in the content of GAGs was maximum in heart (40%) and testis (37%) and was moderate in liver, spleen and lungs (SFD). Spent turmeric (TFD) was effective in ameliorating the decreased content of GAGs during experimental diabetic condition and not much change was observed in the control (SFC/TFC) groups. Decrease in the content of glycosaminoglycans in liver[203] and aorta[204] during diabetes is reported.

Effect of spent turmeric on total sugar content in isolated GAGs of different tissues

The content of total sugars in the isolated GAGs in control and diabetic rats and the effect of spent turmeric on the total sugar content is presented in Table 11. Decrease in the content of total sugars in diabetes (SFD) in the isolated GAGs was maximum (43%) in testis and it was moderate in liver (27%) and spleen (30%) with respect to controls (SFC/TFC). The decreased content of total sugars in GAGs was alleviated nearly by 58% by feeding spent turmeric in heart. Spleen also showed amelioration by feeding spent turmeric (TFD).

Effect of spent turmeric on amino sugar content in isolated GAGs of different tissues

The content of amino sugars in the isolated GAGs during diabetes and modulation by feeding spent turmeric is presented in Table 12. During diabetes (SFD) decrease in the content of amino sugars in the isolated GAGs was maximum in testis and moderate in liver (25%) and heart

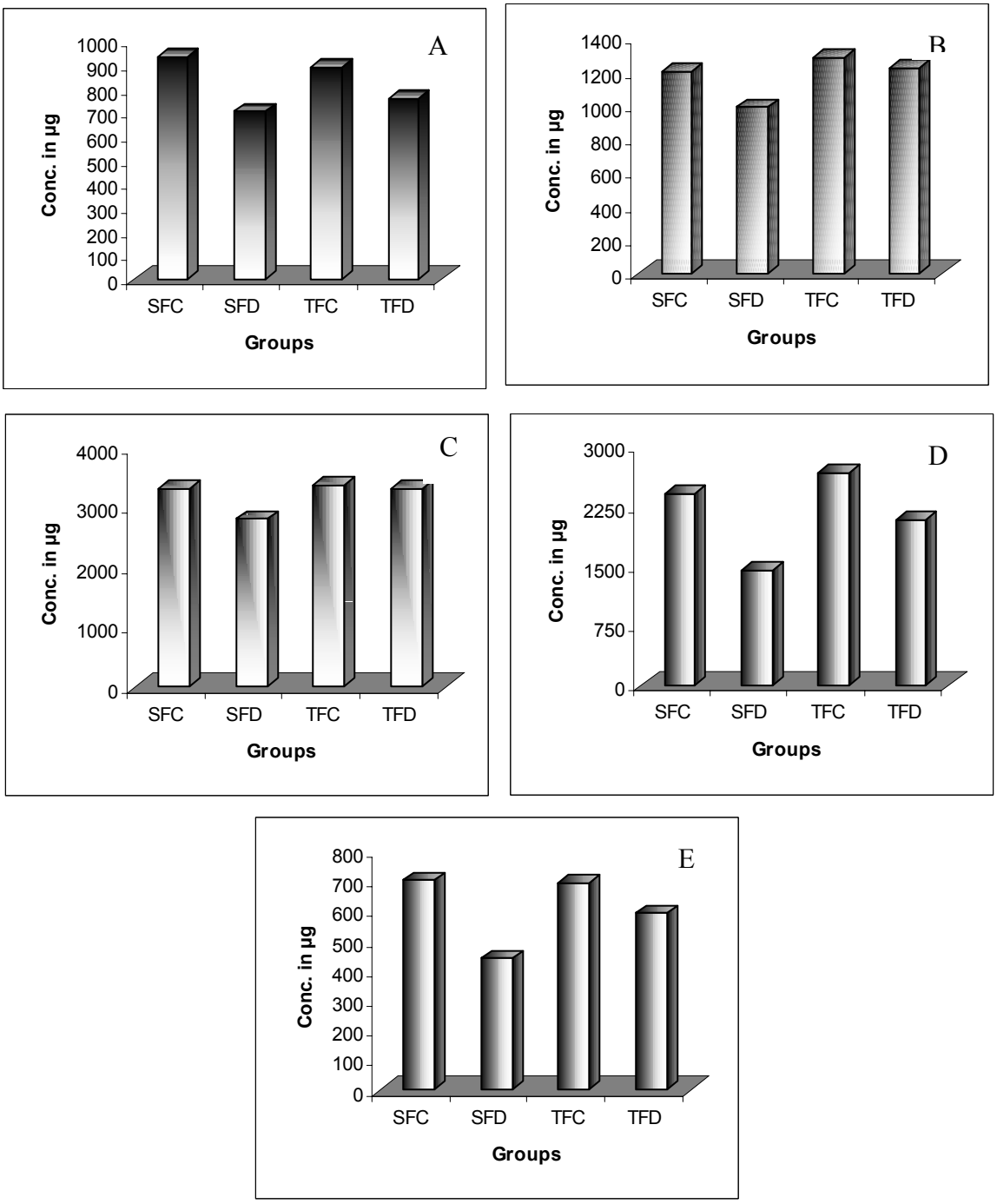


Fig. 4. Effect of spent turmeric on total GAGs in control and diabetic rats [A - Liver, B - spleen, C - lungs, D - Heart, E - Testis]

Abbreviations as in Table 2.

Table 11. Effect of spent turmeric on total sugar content in GAGs of control and diabetic rat tissues

Group	Liver	Spleen	Lungs	Heart	Testis
	μg / g dry tissue weight				
SFC	5951	4981	9961	6301	3054
SFD	4292	3485	8434	5352	1752
TFC	6100	4882	10102	6309	2902
TFD	5211	3953	9392	5950	1954

Abbreviations as in Table 2.

Table 12. Effect of spent turmeric on amino sugar content in GAGs of control and diabetic rat tissues

Group	Liver	Spleen	Lungs	Heart	Testis
	μg / g dry tissue weight				
SFC	351	432	792	523	181
SFD	264	356	670	415	113
TFC	312	461	792	582	178
TFD	283	427	701	517	128

Abbreviations as in Table 2.

(21%) followed by spleen and lungs. The decreased content of amino sugars in the isolated GAGs of different tissues was alleviated to different extents by feeding spent turmeric (TFD). About 20% amelioration was observed in spleen.

Effect of spent turmeric on uronic acid content in isolated GAGs of different tissues

Decrease in the content of uronic acid in the isolated GAGs was observed in all the tissues studied (Table 13). Decrease in uronic acid content in diabetes (SFD) was maximum in testis followed by heart (29%) and liver (25%) when compared to control (SFC/TFC). Alleviation of the decreased content of uronic acid in the isolated GAGs was considerable (about 50%) in liver, spleen and lungs by the feeding of spent turmeric (TFD).

Effect of spent turmeric on sulfate content in isolated GAGs of different tissues

The content of sulfate in the isolated GAGs in different tissues in the control and diabetic rats was modulated by spent turmeric (Table 14). During diabetes (SFD) decrease in sulfate content in the isolated GAGs was maximum (40%) in heart, testis and liver and moderate in spleen (19%) and lungs (17%). About 50% alleviation to the decreased content of sulfate in the isolated GAGs was observed in liver, spleen and lungs in diet containing spent turmeric (TFD). Sulfate groups are well established to play an important role in various physiological functions[212, 213]. Variation in sulfation brings about alteration in charge in the GAGs and hence their function. Reduced content of sulfation in

Table 13. Effect of spent turmeric on uronic acid content in GAGs of control and diabetic rat tissues

Group	Liver	Spleen	Lungs	Heart	Testis
	μg /g dry tissue weight				
SFC	602	791	1251	951	451
SFD	453	652	1053	673	303
TFC	569	847	1272	1062	417
TFD	480	781	1206	842	327

Abbreviations as in Table 2.

Table 14. Effect of spent turmeric on sulfate content in GAGs of control and diabetic rat tissues

Group	Liver	Spleen	Lungs	Heart	Testis
	μg / g dry tissue weight				
SFC	888	1140	3180	2280	665
SFD	559	941	2664	1375	417
TFC	851	1217	3206	2541	693
TFD	636	1123	2969	1971	514

Abbreviations as in Table 2.

heparan sulfate is reported in the liver of streptozotocin induced diabetic rats[204].

Chronic diabetes leads to abnormalities in glycoprotein and proteoglycan metabolism. Our results showed that during diabetes uronic acid and sulfate contents decreased in all the organs and was upregulated by feeding of spent turmeric during diabetes. Sulfation pattern in glycosaminoglycans (GAGs) has received much attention and the content of sulfate in the isolated GAGs decreased during diabetes and it was more prominent in heart, testis and liver [203]. Decrease in the content of sulfate groups in GAGs reduces their charge and interaction with other molecules. Sulfate content of heparan sulfate isolated from liver of streptozotocin induced diabetic rats was shown to be decreased compared to control rats. It is suggested that activity of one of the regulatory enzymes involved in the synthesis of GAGs, N-acetylase is altered during diabetes thus resulting in the decreased content of sulfate in the isolated GAGs in liver of diabetic rats[204].

In heart, heparan sulfate is proposed to play an important role as anti-atherogenic. The content of heparan sulfate is shown to be decreased during diabetes in arteries exhibiting atherosclerotic lesions[214]. Uptake of $^{35}SO_4$ was shown to be decreased in rat tissues both in streptozotocin and alloxan induced diabetic rats. Excretion of uronic acid is shown to increase during diabetes[201]. Changes in amino sugars were also observed indicating altered synthesis and catabolism of GAGs.

Modulatory effect of dietary fibers is reported by the feeding of cooked tapioca which is shown to be better compared to rice and the beneficial

effects are attributed to the presence of dietary fiber present in tapioca[215]. Dietary fiber rich foods are known to have antidiabetic properties[216]by providing insoluble matrix during the passage of food through the intestine thus resulting slow release of glucose and also by fermentation of dietary fiber to produce short chain fatty acids. Lot of interst is generated on the biological functions of SCFA and butyrate in particular has aroused lot of attention and may be mediating some of the long term effect of dietary fiber[217].

3.5 Effect of spent turmeric on glycosaminoglycan metabolism with particularly emphasis on heparan sulfate in kidney

Effect of spent turmeric on renal enlargement and glomerular filtration rate (GFR)

Among the complications of diabetes, diabetic nephropathy is one of the serious concerns and has aroused lot of interest all over the world[218]. Glomerular filtration matrix plays a crucial role in kidney function and is damaged during diabetes[219]. Hence studies on the effect of dietary fibre rich foods such as spent turmeric is of interest and hence is studied in detail.

During diabetes (SFD) size and weight of the kidney increased (Table 15) and was augmented by 26% by the feeding of spent turmeric in the diet (TFD). There was not much change in control groups (SFC/TFC). Kidney size was increased in early stages of diabetes[220]. Renal enlargement starts on the day of induction of diabetes and continues for at least 6 weeks [221-223].

Table 15. Effect of spent turmeric on kidney weight, glomerular filtration rate and size of glomeruli in control and diabetic rats

Groups	Kidney weight (g/100g)	Glomerular filtration rate (mL/min)	Size of the gloumurli (μ)
SFC	0.66±0.02	0.34 ± 0.03	57.8±3.3
SFD	1.46±0.14[a]	7.33 ± 1.47[a]	75.3±0.8[a]
TFC	0.67±0.04	0.46±0.04	58.5±0.5
TFD	1.08±0.03	3.68±0.12[b]	70.1±0.5

Abbreviations as in Table 2.

Glomerular filtration rate (GFR) is an important marker of diabetic nephropathy and was measured in terms of creatinine clearance. The excretion of creatinine is constant and is unaffected by wide variety of circumstances[224]. Creatinine excretion is used as an index of glomerular filtration rate[224]. GFR gives an indication of the amount of blood passing through the kidney per minute during the test period. Lower values of GFR indicate reduced flow of blood through renal vessels and are significant in diabetic rats. Increase in kidney size and GFR nearly by 20-40% was observed in the earlier stages of diabetes due to hypertrophy and hyperfunction[221]. The GFR increased considerably in starch fed diabetic group (SFD) compared to the control group (SFC). Both the control groups had similar GFR rate. The increased GFR in the starch fed diabetic (SFD) group was ameliorated considerably in spent turmeric fed diabetic (TFD) group (Table 15). Reversal of increase in GFR and renal hypertrophy by blood glucose normalization has been observed by Stackhouse *et al* [221,225].

The kidney contains 1% glomerulus [33]. In the kidney sections, size of glomeruli (Table 15) was measured and was around 58μ in control (SFC/TFC) as shown in Fig. 6. During diabetes (SFD) size of the glomeruli increased (75.3μ) and was ameliorated (70.1μ) to some extent in spent turmeric fed diabetic group (TFD) as observed under scanning electron microscope. The size of the glomeruli presented in Table 15 are average of six determinations.

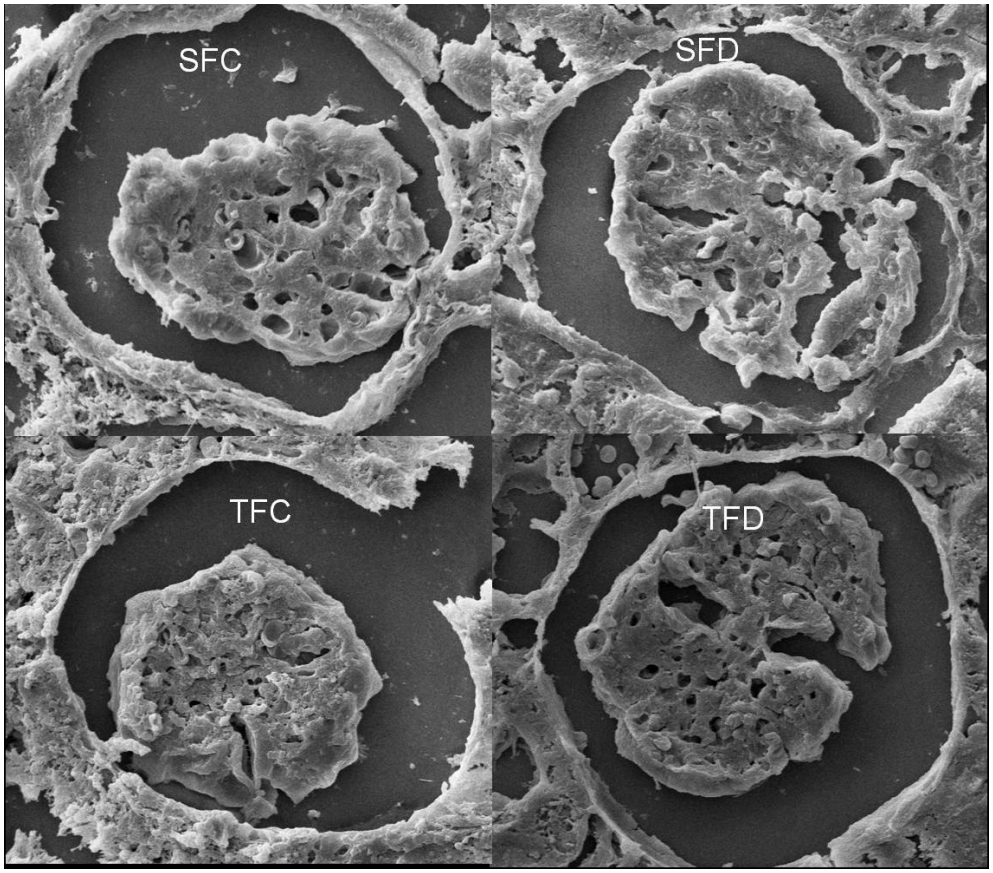


Fig. 6. Scanning electron microscopy of kidney sections on the effect of spent turmeric in control and diabetic rats.

Abbreviations as in Table 2.

Effect of spent turmeric on activities of renal enzymes involved in glycoconjugate metabolism

Activities of renal enzymes, such as L-glutamine fructose-6-phosphate amino transferase (GFAT) involved in the synthesis of amino sugars and degrading enzymes such as N-acetyl-β-glucosaminidase (NAG) and β-glucuronidase were examined in kidney tissue of different groups (Table 16). Activity of GFAT was increased by 40% in the starch fed diabetic (SFD) group compared to starch fed control (SFC) group. Spent turmeric (TFD) fed diabetic groups showed an improvement by 13.6% in GFAT activity, which was statistically significant. Activity of N-acetyl-β-glucosaminidase (NAG) increased by about 60% in the starch fed diabetic group when compared to control groups (SFC/TFC) and there was an improvement in the spent turmeric fed diabetic (TFD) group by 16%. Activity of β-glucuronidase increased in the starch fed diabetic (SFD) group compared to its control (SFC). Activity of the β-glucuronidase was ameliorated in spent turmeric fed (TFD) diabetic group as shown in Table 16. In diabetic nephropathy significant increase in GFAT expression in glomerular and epithelial cells is reported[226, 227]. GFAT involved in the hexosamine pathway converts glucose to UDP-sugars, which is further converted to glucosamine and becomes an important component of glycoconjugates. Increased synthesis of glucosamine indicates accumulation of glycoconjugates. This could be one of the reasons for the thickening of glomerular basement membrane, which occurs during diabetic nephropathy.

NAG activity has been shown to be elevated in diabetic mice [228] but few studies have shown a decrease in diabetic rats[229,230]. β-Glucuronidase

Table 16. Effect of spent turmeric on activities of some of the renal enzymes in control and diabetic rats

Groups	L-Glutamine fructose-6-phosphate aminotransferase (GFAT)	N-Acetyl β-glucosaminidase (NAG)	β-Glucuronidase
	μmoles of product formed /g of protein /min		
SFC	54.96±4.21	2393.00±367	2.35±0.260
SFD	77.09±4.79[a]	3869.00±192[a]	3.92±0.319[a]
TFC	58.78±5.37	2258.00±290	2.20±0.08
TFD	66.58±3.22[b]	3250.00±173[b]	2.68±0.27[b]

Abbreviations as in Table 2.

activity was shown to be unchanged or significantly elevated during diabetes depending on the diabeteogenic agent used to induce diabetes[231], and duration of diabetes and strains of animals employed in the experiments. NAG and β-glucuronidase activities were shown to be elevated in liver and aorta of atheromatous rats[232] and diabetic hypertensive rabbits[233].

Effect of spent turmeric on glycoconjugate metabolism in kidney

The contents of total sugar, amino sugars, uronic acid, sulfate and protein were estimated with out pooling the tissues and the results are presented in Table 17. During diabetes (SFD) content of total sugar, amino sugar and uronic acid was increased compared to its control (SFC) and was alleviated by feeding spent turmeric (TFD). Content of proteins was decreased during diabetes (SFD) and it was ameliorated by feeding spent turmeric (TFD). Sulphate is one of the improrttant constituents of proteoglycans and glycoproteins and there was not much change in the control (SFC/TFC), diabetic (SFD) and treated (TFD) groups (Table 17). Hydroxyproline is one of the characteristic amino acids present in collagen and the content was estimated interms of hydroxyproline. During diabetes (SFC) content of hydroxyproline increased when compared to its control (SFC) and it was augmented by 15.6% by the feeding of spent turmeric (TFD). The non-enzymatic glycation of tissue proteins increases the total sugar content during diabetes[208,209]. The increase in uronic acid and amino sugars could be due to increased synthesis of small GAGs [234]. Increased amino sugar may also be due to UDP-sugar synthesis in the hexosamine pathway which are the building blocks of glycoproteins and

glycosminoglycans[228]. During diabetes decrease in protein content may be due to increase in gluconeogensis and decrease in soluble proteins in kidney[235]. Increased hydroxyproline in kidney was reported during diabetes[42].

3.5 Effect of spent turmeric on glycosaminoglycans in kidney

For the isolation of glycosaminoglycans (GAGs), acetone dried kidneys from each group were pooled and GAGs were isolated[192]. The chemical composition of GAGs was examined by estimating the contents of total sugars, amino sugars, uronic acid and sulfates (Table 18). The content of total sugars in the GAGs decreased during diabetes in the starch fed diabetic group (SFD) compared to its control (SFC). Amelioration in the content of total sugars was observed by the feeding of spent turmeric in the diabetic group (TFD). The content of amino sugars was also decreased in the starch fed diabetic group (SFD) compared to its control (SFC) and an alleviation was observed by spent turmeric (TFD) feeding in the diabetic group. The uronic acid content decreased in the starch fed diabetic (SFD) group compared to its control (SFC) and amelioration was observed by feeding spent turmeric (TFD) to the diabetic groups. The content of sulfates in glycosaminoglycans decreased in uncontrolled diabetic status (SFD) compared to its control (SFC) and alleviation was observed by feeding spent turmeric (TFD) to the diabetic group. Decrease in sulphate content in kidney during diabetes has been reported[236]. Dietary fibers have been shown to affect metabolism of GAG[237]. It was observed that the polysaccharides from

Table 18. Effect of spent turmeric on the composition of kidney GAGs in control and diabetic rats

Groups	Total sugar	Amino sugar	Uronic acid	Sulfates
	µg/g dry kidney tissue			
SFC	9682	928	1354	2521
SFD	3431	604	715	1363
TFC	9583	895	1495	2385
TFD	4959	683	831	1670

Abbreviations as in Table 2.

black gram affected the metabolism of glycosminoglycans and glycoproteins in rats[238]. The GAG metabolism was shown to vary depending on the nature of the dietary carbohydrates like complex polysaccharides, glucose or sucrose[239].

Fractionation of glycosaminoglycans (GAGs)

GAGs were fractionated based on the specificity to the enzyme chondroitinase ABC. Chondroitinase ABC specifically cleaves chondroitin-4-sulphate, chondroitin-6-sulphate, non-sulfated chondroitin and dermatan sulphate. The undigested fraction represents heparan sulfate. The estimation of sulphated GAGs was done using dimethyl methylene blue dye. Heparan sulfate was isolated from the glycosaminoglycans by digesting with chondroitinase ABC. The content of heparan sulfate decreased in the starch fed diabetic group (SFD) compared to its control (SFC) and amelioration in the heparan sulfate content was observed by the feeding of spent turmeric (TFD) in the diabetic groups (Fig. 7B). The content of chondroitin sulfate (Fig 7C) decreased in the starch fed diabetic group (SFD) compared to its control (SFC) and amelioration in the content of chondroitin sulfate was observed in spent turmeric (TFD) fed diabetic groups. Studies have shown that the GBM behaves like a sieve. Studies using dextran fractions with similar molecular radii carrying either neutral, anionic (dextran sulfate), or cationic (diethylaminoethyl dextran) charges showed an enhanced permeability to cationic but a restricted permeability to anionic dextran fractions[240,241]. The GBM is organized as a network of fibrils or chords forming a small-meshed sieve. The glomerular basement membrane is believed to function as the major

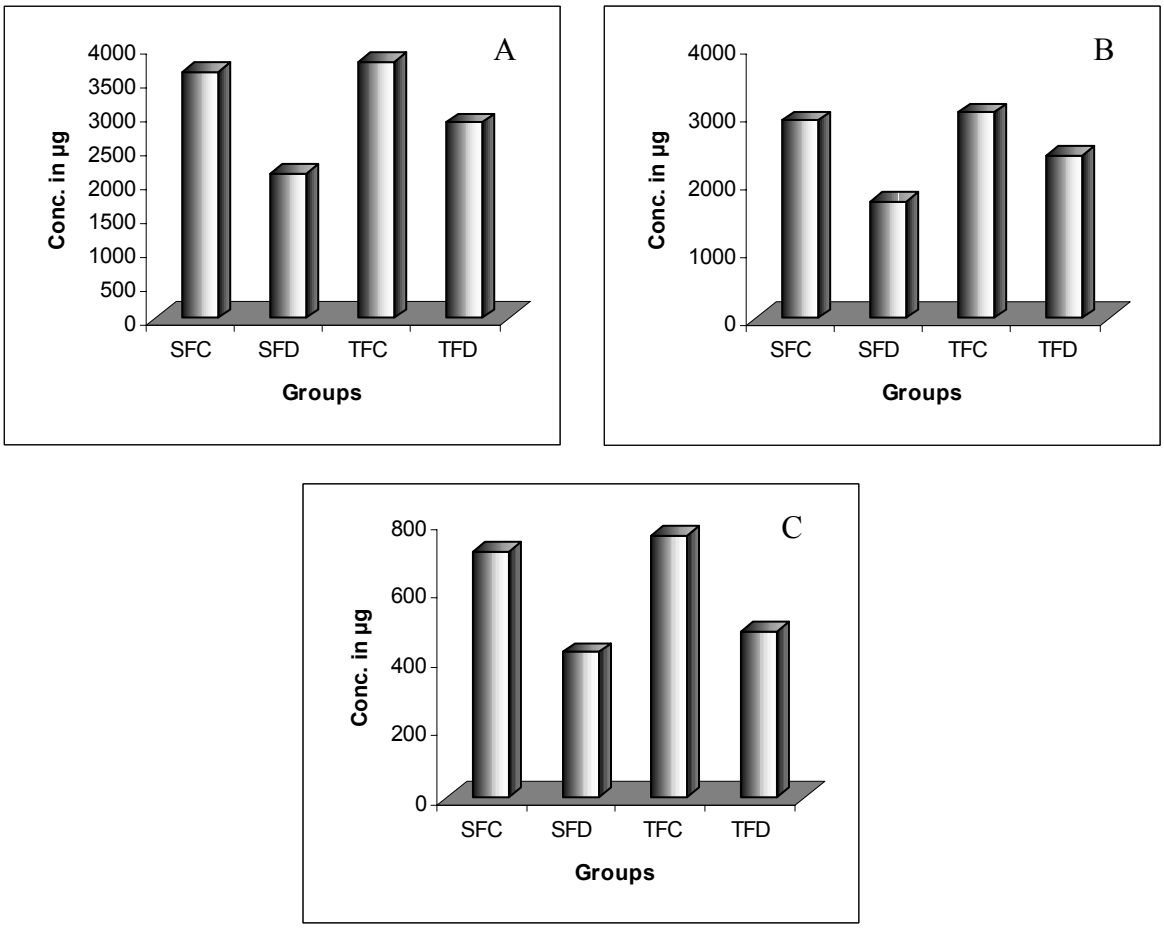


Fig. 7. Effect of spent turmeric on sulphated glycosaminoglycans (GAGs) of renal tissue in control and diabetic rats; A - Total GAG, B - Heparan Sulphate, C - Chondroitin Sulpahte.

Abbreviations as in Table 2.

filtration barrier between the blood and the urine[242]. In diabetes, where it undergoes marked thickening, it becomes defective in its function, and

filtration barrier between the blood and the urine[242]. In diabetes, where it undergoes marked thickening, it becomes defective in its function, and patients with diabetic nephropathy often loose large quantities of protein in the urine. This suggests that diabetes leads not only to altered functions of basement membrane but also alteration in its structure[39,40].

The composition of the GBM is different in most of the basement membranes. GBM contains highly organized architecture. The main GAG constituent in the glomerular basement membrane is heparan sulfate (HS). HS is an unbranched chain of repeating disaccharide units, which contain amino sugar and an uronic acid (glucuronic or iduronic acid). The presence of sulfate groups and carboxyl groups are responsible for the charge density in the lamina rara of GBM. Heparan sulfate forms 90% of ECM, and remaining 10% includes other ECM constituents such as condroitin sulfate, dermatan sulfate and hyaluronic acid. Recognition of proteoglycans, especially heparan sulfate proteoglycan (HSPG) as important regulator of both charge and size selective aspects of glomerular permselectivity is well recognised[243,244]. These anionic sites prevent clogging of the basement membranes by circulating plasma macromolecues[245]. It was observed that heparan sulphate was the major GAG present in all the groups, which amounted to 90% of the total GAGs. This is in agreement with the values reported in the literature[54,58]. Total sulfated GAGs decreased by about 41% during diabetes (SFD) compared to control (SFC/TFC). Since this estimation was basically based on the sulfate content, the decrease in GAGs could also be due to under sulfation (also observed in our study,

Table 18), and is known to occur during diabetes. The uronic acid content was also decreased and the decrease in sulfated GAGs may be probably due to decrease in GAGs as well as under sulphation. The decrease in total GAGs was due to decrease in both heparan sulphate and chondroitin sulphate. Reduction in heparan sulphate during diabetes has been observed in both glomerular basement membrane[211,246] and renal cortex[247,203]. Heparan sulphate and chondroitin sulphates are known to be located on two different core proteins[248]. Modulation of GAGs during diabetes in kidney tissue has been reported by earlier workers[249,250]. Stanlee observed that addition of non-absorbable fibre to the diet of genetically diabetic mice improved glycaemic control and retards the development of diabetic nephropathy[250]. Barley has been found to have modulating influence on the altered metabolism of glucose and of basement membranes in diabetic rats[249] and we have also provided experimental evidence to show that decreased synthesis of heparan sulfate in diabetes is modulated by dietary fibers such as wheat bran and guar gum [207].

Agarose gel electrophoresis

Separation of glycosaminoglycans (GAGs) was achieved by agarose gel electrophoresis. Equal amount of kidney samples were loaded along with chondroitin sulpahte B as reference. Heparan sulfate was the major glycosaminoglycan and both the control samples (SFC and TFC) showed approximately equal amounts of heparan sulfate and decrease in heparan sulphate in starch fed diabetic group (SFD) is clearly visible as seen in the electrophoretic profile (Fig. 8). The bands were compared with chondroitin sulphate B standard. Hence, the isolated GAGs from

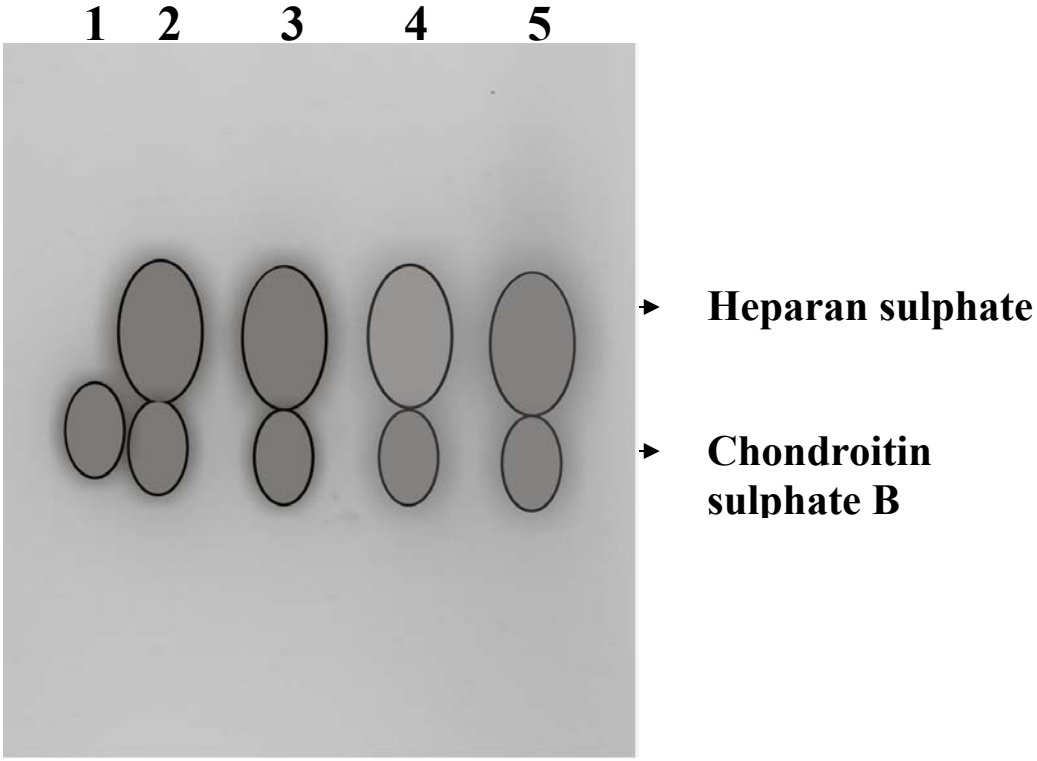


Fig. 8. Agarose gel electrophoresis of glycosaminoglycans(GAGs) isolated from control, diabetic, and spent turmeric treated rat kidneys.

Lane 1. Chondroitin sulphate B, Lane 2. Starch fed control, Lane 3. Spent turmeric fed control, Lane 4. Starch fed diabetic, Lane 5. Spent turmeric fed diabetic.

kidney mainly consisted of heparan sulfate. The decreased content of heparan sulfate in the kidney was ameliorated by the feeding of spent turmeric (TFD) in the diabetic group. Decreased heparan sulfate in the agarose electrophoresis was also observed previously[207,250].

3.6 Effect of spent turmeric on collagen content in kidney

Histopathological studies

Alcian blue and PAS stain (AB-PAS)

During diabetes along with the thickening of glomerular basement membrane (GBM), glycoproteins are also known to accumulate on the GBM and mesangial cells[206]. Kidney sections of control, diabetic and treated rats were stained with a combination of alcian blue (AB) and Periodic Acid - Schiff (PAS) stains (AB-PAS). Alcian blue is one of the widely used cationic dyes for the demonstration of GAGs in glomeruli. Alcian blue stains GAGs[150] and periodic acid stains glycoproteins[151]. In the kidney blue color stained with alcian blue indicates the presence of GAGs and staining with PAS gives pink color. Combination of blue and pink color indicates the presence of GAGs and glycoproteins. Staining intensity was graded from '–' to '+'. The control rat kidney sections (SFC/TFC) showed bluer in AB-PAS (+++). But in the diabetic rat kidneys were pinkish AB-PAS (+++) along with blue as shown in Fig. 9 and Table 19. The intensity of color due to alcian blue by GAGs was considerably reduced during diabetes and the blue color intensity was alleviated by the feeding of spent turmeric in diabetic rats and the

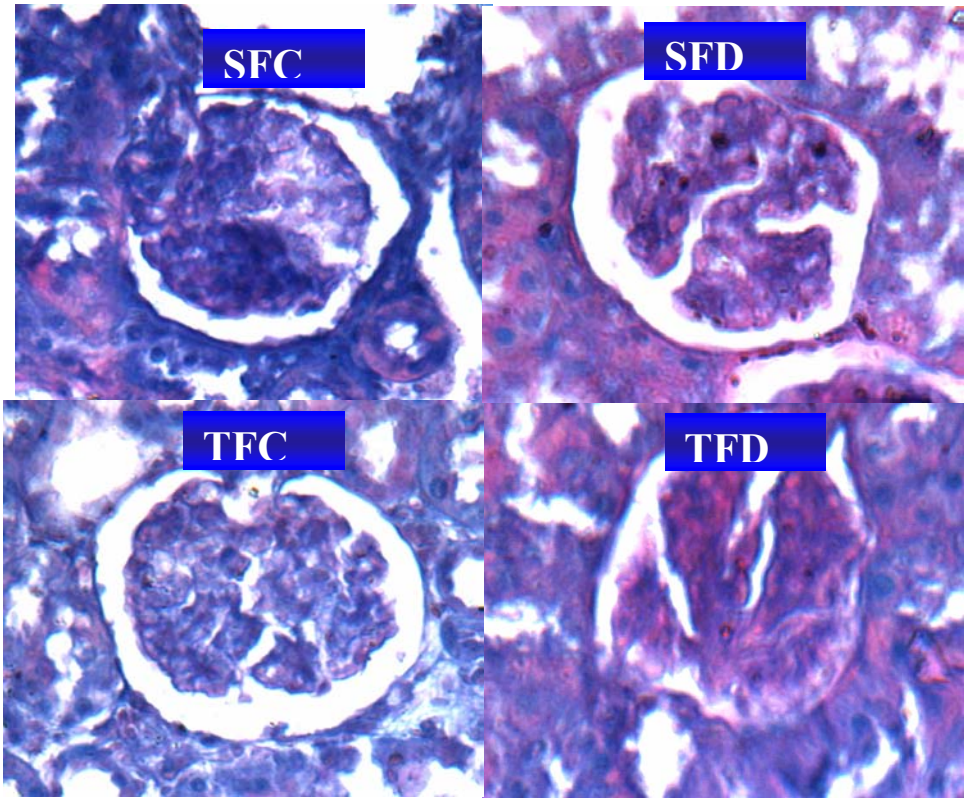


Fig. 9. Kidney sections of control, diabetic and spent turmeric treated rats.

Abbreviations as in Table 2.

Table 19. Intensities of kidney glomeruli by alcian blue with periodic Schiff's stain (AB-PAS) and immunoflorescent intensity of type IV collagen in control, diabetic and spent turmeric fed rats

Groups	AB-PAS (Blue)	AB-PAS (Pink)	PAS	Type IV collagen
SFC	+++	+	++	+
TFC	+++	+	++	+
SFD	+	+++	++++	++++
TFD	++	++	+++	+++

Abbreviations as in Table 2.

pinkish color observed in kidney sections, due to accumulation of glycoproteins in kidney during diabetes (TFD) was more (+++) compared to the controls (SFC/TFC). The increase in pink color intensity during diabetes (SFD) was ameliorated in the spent turmeric fed diabetic group (TFD). Alcian blue stain reveals structural elements bearing negatively charged sites within the glomerular capillary wall of the rat kidney, These charges represent one of the most important functional components of the glomerular filtration barrier[250,251,252], and is also used to measure glomerular plasma flow[253]. AB-PAS stain thus was also used in the detection of goblet cells[254]. Similarly PAS stain alone showed high PAS positive staining in diabetic group (SFD) as in Fig. 10. Spent turmeric feeding during diabetes (TFD) showed considerable amelioration (Table 19). The nuclei of cells in SFD glomeruli were swollen during diabetes compared to controls (SFC/TFC) as can be seen in Fig. 10 with black arrow was in agreement with other reports[255]. Light microscopy indicates increase in matrix and increased intensity can be seen with the periodic acid-Schiff (PAS) stain. In the renal glomerulus, alterations are particularly prominent in addition to thickening of the basement membranes of the capillary loops. Accumulation of glycoconjugates in the mesangial region was similar to that observed in basement membranes. The thickening eventually results in the characteristic Kimmelstiel-Wilson nodular lesions[256].

Immunohistochemical study was done for type IV collagen using primary antibodies (goat polyclonal IgG) and secondary antibodies (rabbit anti-goat IgG) tagged with FITC of type IV collagen and the

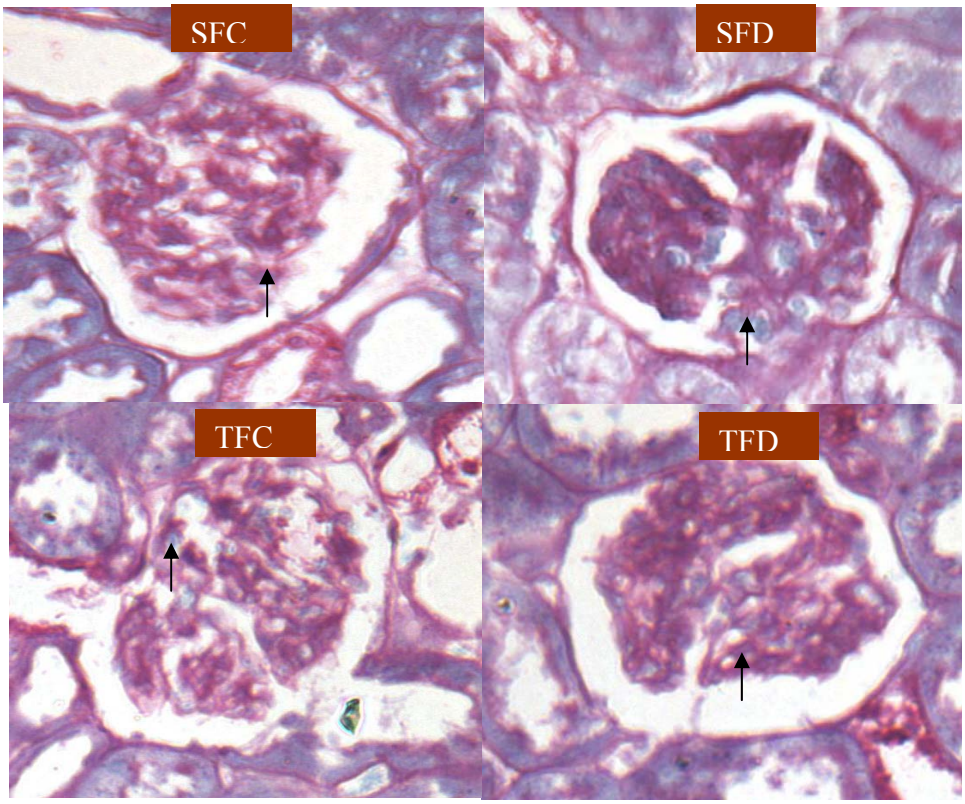


Fig. 10. Periodic acid stains of kidney sections of control, diabetic and spent turmeric treated rats.

Abbreviations as in Table 2.

sections were observed under fluorescent microscope. Results clearly showed (Table 19) that during diabetes content of type IV collagen (florescent positive) was increased as can be seen in Fig. 11 (with arrow mark), when compared to control (SFC/TFC). The increased content of type IV collagen was ameliorated by the feeding of spent turmeric in the diet (TFD).

Paul and Robert *et al.,* showed that total carbohydrate content was 7.5% of the dry weight of the membrane and the sugars identified were glucose, galactose, mannose, fucose, glucosamine, galactosamine, and N-acetyl neuraminic acid[257]. The alterations that occur in the diabetic membrane involves lysine or its derivatives hydroxylysine and glycosylated hydroxylysine. Both lysine and hydroxylysine participate in the peptide chains of collagens and elastin[258]. Such cross-links also exist in the glomerular basement membrane. The increase in glycosylation of hydroxylysine would reduce the availability of this amino acid to participate in their formation. Such a defect in cross-linking and the effect of the extra and bulky carbohydrate substituents on the packing of the peptide chains could contribute to the increased permeability of the basement membrane seen in diabetic nephropathy. The compositional studies on the basement membrane of normal human kidney indicated that it belongs to the collagen family of proteins, the most abundant are type-IV collagen and various laminin isoforms. Studies have shown that diabetic glomeruli had greater hydroxyproline content than non-diabetic[259]. Feeding of spent turmeric ameliorated type IV collagen content (Table 18). During hyperglycemia increase in the activity of β form of PKC inturn increases the activity of TGF-β and

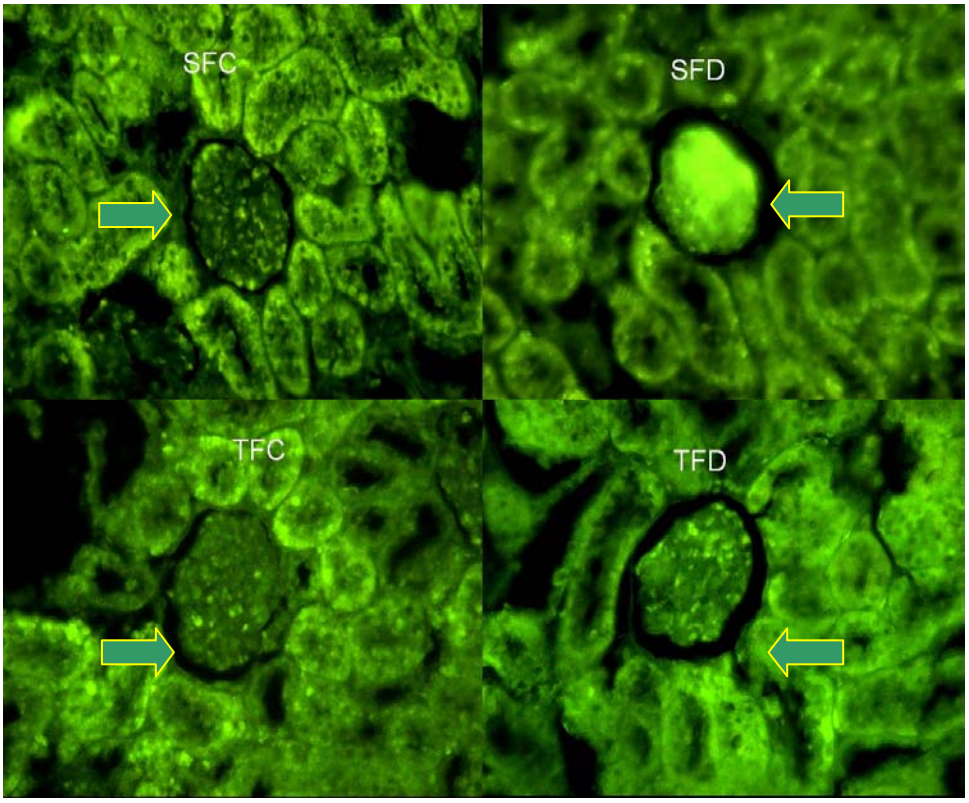


Fig. 11. Immunohistochemistry of Type 1V Collagen in the kidney sections of control, diabetic and spent turmeric treated rats.

Abbreviations as in Table 2.

hence increases type IV collagen[17,18] in the mesangial cells and glomerular basement membrane.

The amount of dietary fiber and their chemical nature brings about variation in the functionality and their fermentation characteristics leading to short chain fatty acids[260]. Fermentation of dietary fibers by colonic microflora leads to release of short chain fatty acids (SCFA) such as acetate, propionate and butyrate in an approximate ratio of 60:20:20[187,261-263]. Propionate is shown to exhibit hypocholesterolemic activity. Butyrate is known to modulate activities of many key regulatory enzymes involved in glycoconjugate metabolism like sialyl transferases[264] and sulfotransferase[265]. N-butyrate is shown to down regulate the β form of PKC[266], which may combat the increased synthesis of type IV collagen. Butyrate in *in vitro* studies using cell culture system is shown to induce insulin gene expression[267]. Mesangial matrix accumulation can be prevented and reversed in experimental animals after treatment with insulin. GBM thickening is also shown to be prevented but resolution is much slower[266]. Dietary fibers also delay the uptake of glucose by forming insoluble matrix in the intestine thereby interfering in the free absorption of glucose. Thus there is a decrease in blood sugar level in fibre fed groups. We have shown earlier that butyric acid modulates diabetic status[189]. Few reports have shown increased insulin binding to monocytes and adipocytes in subjects fed with high fiber diets[189,268].

3.7 Carbohydrate composition of spent turmeric

Turmeric is well known spice used in Ayurvedic medicine and is also extensively used as a flavor in cooking. Spent turmeric is nothing but turmeric devoid of curcumin (active principle of turmeric), and is a by-product of turmeric industry. Spent turmeric is rich in dietary fibers. Analysis of dietary fibers of spent turmeric is given in Table 20. Spent turmeric contained around 46.3% total dietary fibre of which 40.4% was insoluble dietary fiber (IDF) and 5.9% was contributed by soluble dietary fiber (SDF). Insoluble dietary fibre (IDF) contains mainly water insoluble non-starch polysaccharides and lignin. Soluble dietary fibers (SDF) contained mainly water-soluble non-starch polysaccharides. The fibers were rich in carbohydrates (Table 20). The main sugars present in IDF of spent turmeric were arabinose, galactose and glucose with minor amounts of fucose/rhamnose, xylose and mannose. Soluble dietary fiber (SDF) also had glucose as major sugar, along with arabinose (15%) and xylose (13.4%) and could come from arabinoxylan type of polysaccharides.

The carbohydrates of spent turmeric were extracted sequentially (Section 2.4.4) to water-soluble polysaccharides, pectic polysaccharides, hemicellulose A (Hem A), hemicellulose B (Hem B) and alkali-insoluble residue (AIR). All the fractions were rich in carbohydrates and uronic acid content ranged between 4.8 to 12.6%. The water-soluble polysaccharides contained glucose mainly and small amount of arabinose and galactose were also observed. This may be due to the presence of β-glucan type of polysaccharides[269] and resistant starch[270]

formed during gelatinisation or may be formed during processing in turmeric industry. Uronic acid was found to be 10.9%. Ammonium oxalate (0.5%) extracted polysaccharide contained arabinose and galactose and may be due to the presence of arabinogalactan type of polysaccharides[271] and glucose may be coming from resistant starch[270] formed during gelatinisation or may be formed during processing in turmeric industry. The ammonium oxalate extracted residue was extracted with 10% sodium hydroxide. The hemicellulose A and B had high amount of xylose and glucose and may be coming from xyloglucan type of polysaccharides[272] and alkali-insoluble residue (AIR) showed only glucose as shown in Table 21. Earlier, polysaccharides of *Curcuma longa* were shown to possess number of biological activities. Three polysaccharides namely ukonan A, ukonan B and ukonan C present in the rhizome, contained L-arabinose, D-xylose, D-galactose, D-glucose, L-rhamnose, D-galacturonic acid in the molar ratio of 12:4:12:1:4:10 (ukonan A). 12:4:12:1:2:4 (ukonan B) and 8:3:6:14:2:3 (ukonan C). Polysaccharide ukonan C had immunological activity[273], ukonan A phagocytosis activity[274], and the neutral polysaccharide (ukonan A, ukonan B, ukonan C, and ukonan D) had reticuloendothelial - potentiating activity[275].

3.1.1 Summary and Conclusions

The present investigation was carried out on the effect of dietary fibers on glycoconjugate metabolism in streptozotocin induced diabetic rats. Spent turmeric (turmeric devoid of curcumin), by-product of turmeric in flavour and essence industry is rich in dietary fiber and hence was used as a source of dietary fiber for the experiment. The experimental animals were maintained on diet containing 10% spent turmeric.

1. Diet consumption in spent turmeric fed rats both in normal as well as diabetic groups was higher and may be due to caloric dilution. Amelioration of the body weight was observed in diabetic rats by the feeding of spent turmeric. There was an increase in fasting blood glucose, urine sugar and urine out put during diabetes and they were alleviated to different extents by the feeding of spent turmeric.
2. During diabetes intestinal disaccharidases activity was increased and renal disaccharidases activity decreased when compared to controls. Feeding spent turmeric (10%) effectively ameliorated intestinal and renal disaccharidases activity during diabetes.
3. During diabetic state weights of liver, lungs, brain, testis and kidney increased, and spent turmeric was effective in countering these weights to different extents.
4. During diabetes changes in the metabolism of glycosminoglycans (GAGs) occurred in the tissues studied. Decrease in the content of glycosminoglycans (GAGs) was observed during diabetes

compared to control and was ameliorated by spent turmeric feeding.

5. Glomerular filtration rate (GFR) was tested as a marker of diabetic nephropathy and was found to increase during diabetes and spent turmeric in the diet ameliorated the increased GFR.
6. Activity of renal enzymes like L-glutamine fructose-6-phosphate amino transferases (GFAT), N-acetyl-β-glucosamindase (NAG) and β-glucuronidase increased during diabetes and were augmented to different extents in spent turmeric fed diabetic group.
7. The contents of total sugar, uronic acid and amino sugars in kidney decreased during diabetes and these were ameliorated by the feeding of spent turmeric.
8. In the isolated kidney GAGs, total sugar, aminosugar and uronic acid contents were decreased during diabetes. The content of sulfates in isolated GAGs was decreased during diabetes and spent turmeric feeding to diabetic rats augmented decreased content of sulfate in GAGs.
9. Fractionation of glycosaminoglycans (GAGs) into heparan sulfate and chondroitin sulfate from control, diabetic and treated kidney showed decrease in heparan sulfate content during diabetes as reported earlier and it was ameliorated (39.7%) by feeding spent turmeric and was confirmed by the electrophoretic profile.

10. Histopathlogical studies done with alcian blue, PAS stain and their combination showed decrease in GAG and an increase in glycoprotein content during diabetes and this was ameliorated by feeding spent turmeric in the diet.
11. Immunohistochemistry of type IV collagen showed that there was a significant increase in the type IV collagen content during diabetes when compared to control rats and it was alleviated in spent turmeric fed group.
12. Kidney sections examined under scanning electron microscope (SEM) showed that there was an increase in the size of glomeruli during diabetes and it was ameliorated in treated group receiving spent turmeric.

Thus it can be concluded that spent turmeric [which contained 46.3% total dietary fiber (IDF- 40.4%, SDF-5.9%)] when added at 10% level alleviates diabetic and diabetic nephropathy state. In diabetic nephropathy, metabolism of glomerular filtration matrix studied as heparan sulfate and type IV collagen was altered and was augmented by spent turmeric feeding.

3.2.a Studies on the role of *Emblica officinalis* on diabetic status in streptozotocin induced diabetic rats

Use of traditional medicines, mainly derived from plant sources, has been one of the major means in the management of diabetes anywhere in the world[276-278]. A large number of plants/spices are now well recognized to possess hypoglycemic potential[73,276-278]. Most of the studies have focused only on blood glucose levels, mainly as an alternative to exogenous insulin. *Emblica officinalis* commonly known as *amla* is extensively used in many preparations of Ayurveda and also against many chronic ailments including diabetes[77,279,280]. Other studies showed that 75% methanolic extract of *Terminalia chebula, Terminalia beleria*, *Emblica officinalis* and their combination named "Triphala" (equal proportion of above three plant extracts) showed significant beneficial effect on diabetic status[77].

Fresh fruits of *Emblica officinalis* were obtained from the local market. The pericarps were collected and sundried. Dried material was powdered and used for the experiment at different concentrations such as 1.0, 0.5, 0.1 and 0.025% in the AIN-76 diet and were fed to streptozotocin induced diabetic rats. Initially diabetic rats were grouped in such a way that all groups had same fasting blood sugar. Diabetic status was assessed by urine sugar, urine volume, fasting blood sugar, diet intake and water intake, weekly, and renal damage was studied by glomerular filtration rate at the end of the experiment.

Effect of Emblica officinalis at 1.0 and 0.5% level on diet intake and body weight in control and diabetic rats

The diabetic rats fed with 1.0% *Emblica officinalis* (EFD) developed diarrhea with in three days and hence experiment could not be continued and was terminated. But in the 0.5% *Emblica officinalis* fed diabetic (EFD) group, diet intake was more when compared to starch fed diabetic (SFD) group (Table 22). Body weight decreased by 30% in EFD group at 0.5% level when compared to SFD as presented in Table 22.

Effect of Emblica officinalis at 0.5% level on water intake, urine sugar, urine output and fasting blood glucose in control and diabetic rats

Effect of *Emblica officinalis* was studied at 0.5% level on water intake, urine sugar, urine volume and fasting blood glucose. The studies clearly showed that there was no beneficial effect on diabetic status in streptozotocin induced diabetic rats by feeding *Emblica officinalis* at 0.5% level. Glomerular filtration rate was measured as a measure of diabetic nephropathy status in streptozotocin induced diabetic rats. No beneficial effect was seen even on glomerular filtration rate by the feeding of *Emblica officinalis* at 0.5% level (Table 23).

Table 22. Effect of *Emblica officinalis* at the concentration of 0.5% on diet intake and body weight

Group	Diet intake (g / day)	Body weight (g)		
		Initial	Final	Gain
SFC	11.9±0.9	126.0±5.18	237.3±5.5	111.0 ±0.3
SFD	15.5±0.9[a]	123.7±2.60	135.1±7.6	12.0 ±5.0[a]
EFC	11.0±0.2	126.2±3.30	230.0±6.0	104.0 ±2.7
EFD	13.3±0.8[NS]	124.1±6.20	128.0±1.9	4.0 ± 4.3[NS]

SFC: Starch fed control, SFD: Starch fed diabetic,
EFC: *Emblica officinalis* fed control, EFD: *Emblica officinalis* fed diabetic.

Values are Mean ± SEM of 6 rats in control and 8 rats in diabetic groups.

[a] Statistically significant when compared to SFC at $p<0.05$,
[b] Statistically significant when compared to SFD at $p<0.05$,
[NS] Statistically not significant when compared to SFD at $p<0.05$.

Table 23. Effect of *Emblica officinalis* at the concentration of 0.5% on water intake, urine sugar, urine volume, fasting blood sugar, and glomerular filtration rate (GFR)

Groups	Water in take (mL/day)	Urine sugar (g /day)	Urine output (mL / day)	Fasting blood sugar (mg/12 h)	GFR (ml/min)
SFC	29.5±1.5	0.031±0.002	15.0±0.8	108.3±04.5	1.17±0.17
SFD	82.3±2.0[a]	6.3±0.54[a]	70.8±4.6[a]	340.3±45.2[a]	5.90±0.55[a]
EFC	32.0±1.5	0.022±0.0028	16.1±1.2	94.1±05.7	1.11±0.13
EFD	80.1±2.2[NS]	6.4±0.30[NS]	68.2±5.5	433.5±06.8[NS]	8.13±0.09[NS]

Abbreviation as in Table 22.

Effect of Emblica officinalis at 0.1 and 0.025% level on diet intake and body weight in control and diabetic rats

Since there was no beneficial effect on diabetic status in streptozotocin induced diabetic rats by feeding *Emblica officinalis* at 1.0% and 0.5% level, *Emblica officinalis* was fed at lower concentrations of 0.1% and 0.025% and its effect on diabetic status was studied. At low concentrations of *Emblica officinalis* (EFD), fed at 0.1 and 0.025%, there was no improvement both in diet intake and body weight when compared to starch fed diabetic group (SFD) as shown in Table 24.

Effect of Emblica officinalis at 0.1 and 0.025% level on water intake, urine sugar, urine output and fasting blood glucose in control and diabetic rats

There was no significant effect on water intake, urine sugar, urine out put, fasting blood sugar and glomerular filtration rate in *Emblica officinalis* fed diabetic group (EFD) at the concentration of 0.1 and 0.025% in the diet, when compared to starch fed diabetic group (SFD) as shown in Table 25.

Our results clearly showed that fruits of *Emblica officinalis* alone at 1.0, 0.5, 0.1 and 0.025% have no beneficial effect on streptozotocin induced diabetic rats.

Table 24. Effect of *Emblica officinalis* at the concentration of 0.1 % and 0.025% on diet intake and body weight

Group	Diet intake (g / day)	Body weight (g)		
		Initial	Final	Gain
SFC	13.5±0.37	130±7.2	227±11.5	97.0±4.3
SFD	22.3±1.5[a]	119±3.68	128±5.5	9.0±1.8[a]
EFC (0.1%)	13.2±0.5	135±5.9	229±13.6	94.0±7.7
EFD (0.1%)	21.9±1.1[NS]	123±17.2	124±9.5	1.0±7.7[NS]
EFC (0.025%)	16.1±1.1	134±6.2	221±12.7	87.0±6.5
EFD (0.025%)	21.9±2.6[NS]	123±6.1	126±9.6	3.0±3.5[NS]

Abbreviation as in Table 22.

Table 25. Effect of *Emblica officinalis* at the concentration of 0.1% and 0.025% on water intake, urine sugar, urine volume, fasting blood sugar and glomerular filtration rate (GFR)

Groups	Water intake (mL /day)	Urine sugar (g /day)	Urine output (mL / day)	Fasting blood sugar (mg /12 h)	GFR (mL /min)
SFC	30±1.2	0.021±0.001	14.8±1.7	133.4±4.1	1.14±0.12
SFD	112±13.0[a]	8.0±0.540[a]	106.73±8.8[a]	343.0±27.1[a]	6.1±0.55[a]
EFC (0.1%)	27±1.2	0.025±0.002	15.1±1.1	130.0±2.8	1.11±0.12
EFD (0.1%)	110±20.2[NS]	7.5±0.760	99.4±15.0[NS]	342.0±23.5	5.9±0.10[NS]
EFC (0.025%)	28.5±2.2	0.022±0.001	13.8±1.1	127.0±4.4	1.20±0.11
EFD (0.025%)	111±14.0[NS]	7.7±0.690[NS]	108.2±10.5[NS]	341.7±29.3[NS]	6.32±0.65[NS]

Abbreviation as in Table 22.

3.2.b Chemical and biological studies on the polysaccharides of *Emblica officinalis*

The fruits of *Emblica officinalis* are widely consumed either as raw, cooked or in the form of pickles and are used as one of the constituents of many of the potent Ayurvedic preparations[187]. Since no beneficial effects on diabetic status were observed with the fruits of *Emblica officinalis* at 1.0, 0.5, 0.1 and 0.025%, we made an attempt to study, if, any of the polysaccharides have any biological functions. In recent years biological functions of polysaccharides are receiving much attention and hence an attempt was made to isolate various polysaccharide fractions of *Emblica officinalis* and study their biological activities. The flour was rich in carbohydrates and contained 6.2% uronic acid and was identified as galacturonic acid. Various carbohydrate rich fractions were isolated from dry fruits of *Emblica officinalis* as shown in Fig. 1 (Section 2.4.4.1). Free sugars were estimated at 9.0%. Content of starch was estimated by hydrolyzing the flour with amylases and glucose released was estimated by glucose oxidase method. *Emblica officinalis* did not contain any starch. The flour was digested with Termamyl and glucoamylase to digest starch and the water-soluble polysaccharide was thus extracted is 9.9% yield. The hot water soluble polysaccharide was rich in carbohydrates and was rich in galactose (49.6%) as shown in Table 26. Glucose was present in minor amounts and also contained arabinose, xylose, mannose and rhamnose/fucose. Thus the hot water-soluble polysaccharide was complex mixture of arabinogalactan and galactan-type polysaccharides. Glucose may be coming from unhydrolyzed starch and resistant

starch[281]. The pectic polysaccharides were isolated with ammonium oxalate (0.5%) at boiling water bath temperature. Since pectic polysaccharides are associated with Ca^{2+} ions, they were chelated with ammonium oxalate and was used for the extraction of pectic polysaccharides[282,283]. The pectic polysaccharide was rich in carbohydrates (86.6%) and uronic acid content was (7.9%). Galacturonic acid and rhamnose are reported in ammonium oxalate extract[282,283]. The pectic fraction was rich in glucose and contained high amounts of galactose, xylose, arabinose, mannose, rhamnose/fucose, indicating it to be a complex mixture of arabino-galactan type of polysaccharides[284]. The ammonium oxalate insoluble residue was extracted with NaOH (10%) to extract alkali soluble-hemicellulose A and B. Both were isolated in approximately equal concentrations. They were rich in carbohydrates and contained uronic acid. The Hemicellulose A was rich in xylose and glucose and hence may contain xyloglucan type of polysaccharide or may be a mixture of xylan and glucan type polysaccharides. Hemicellulose B was also rich in xylose and glucose and contained sugars like galactose and mannose. Though there was not much difference in sugar composition, solubility differences were observed between hemicellulose A and B. Reasons for the difference in their solubility need further investigation. The alkali-insoluble residue was isolated is 31% yield and was rich in carbohydrates and contained glucose alone as the constituent sugar indicating it to be cellulosic in nature. Presence of glucose exclusively in alkali-insoluble residue indicates complete extraction of non-starch polysaccharides.

Biological activity of polysaccharides

Wound healing

Wound healing property was checked with water soluble and pectic polysaccharides, isolated from *Emblica officinalis*. Area of the wound observed after 3 days is shown in Fig. 12. Wound healing property was further confirmed by estimating hydroxyproline as a measure of collagen formed. Collagen content in normal skin was 21%. The content of collagen in basal ointment treated skin was 6.1%. Polysaccharides of *Emblica officinalis* showed better wound healing property than basal ointment and was 11.8% with water-soluble polysaccharides and 14.8% with pectic polysaccharides (Table 27). Increased collagen content indicates healing up of wound[285]. Pectic polysaccharide fraction showed good wound healing property. The pectinized gauze seems to absorb the decomposed tissue materials and debris and the wound becomes nonpurulent, looks clean, and healed rapidly. The pectin treatment seemed to stimulate the formation of healthy granulation. This beneficial effect of pectin on wound healing may also be due to its alleged bactericidal action[286].

Antioxidant activity

Isolated polysaccharide fractions of *Emblica officinalis* such as water-soluble polysaccharide (WSP), pectic polysaccharide, hemicellulose A, and hemicellulose B were subjected to free radical scavenging activity and was found to be 99, 210, 35 and 30 unit/g respectively (Fig. 13). Native fraction showed 100% activity. Pectic polysaccharide showed good antioxidant activity compared to other fractions.

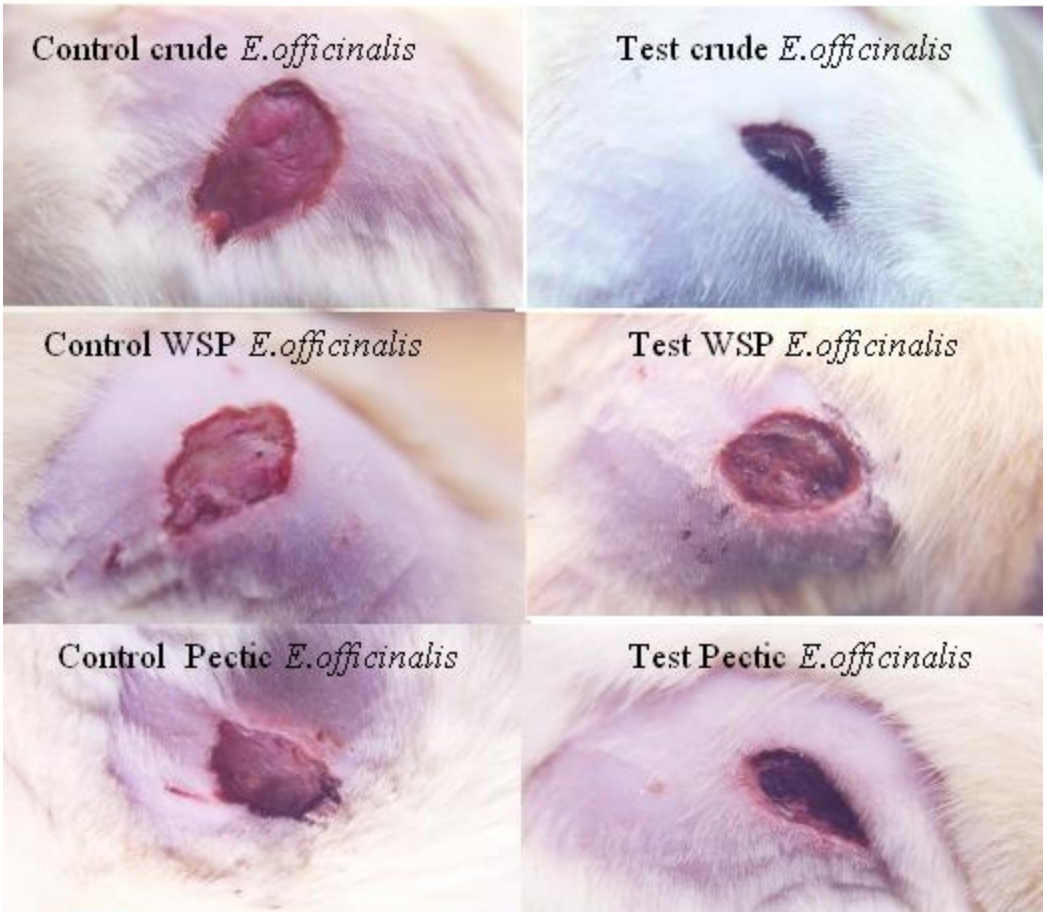


Fig. 12. Wound healing property (after 3 day) of *Emblica officinalis* and its polysaccharide fractions.

WSP - Water-soluble polysaccharide,

Pectic - Pectic polysaccharide.

Table 27. Content of collagen (%) in skin during wound healing

Treatment	Collagen content in skin
Normal skin	21.0 ± 0.11
Wound treated with basal ointment	6.1 ± 0.06[a]
Wound treated with water-soluble polysaccharides	11.8 ± 0.07
Wound treated with pectic polysaccharides	14.8 ± 0.08[b]

[a.] Statistically significant when compared to normal skin at $p<0.05$,
[b.] Statistically significant when compared to basal ointment at $p<0.05$.

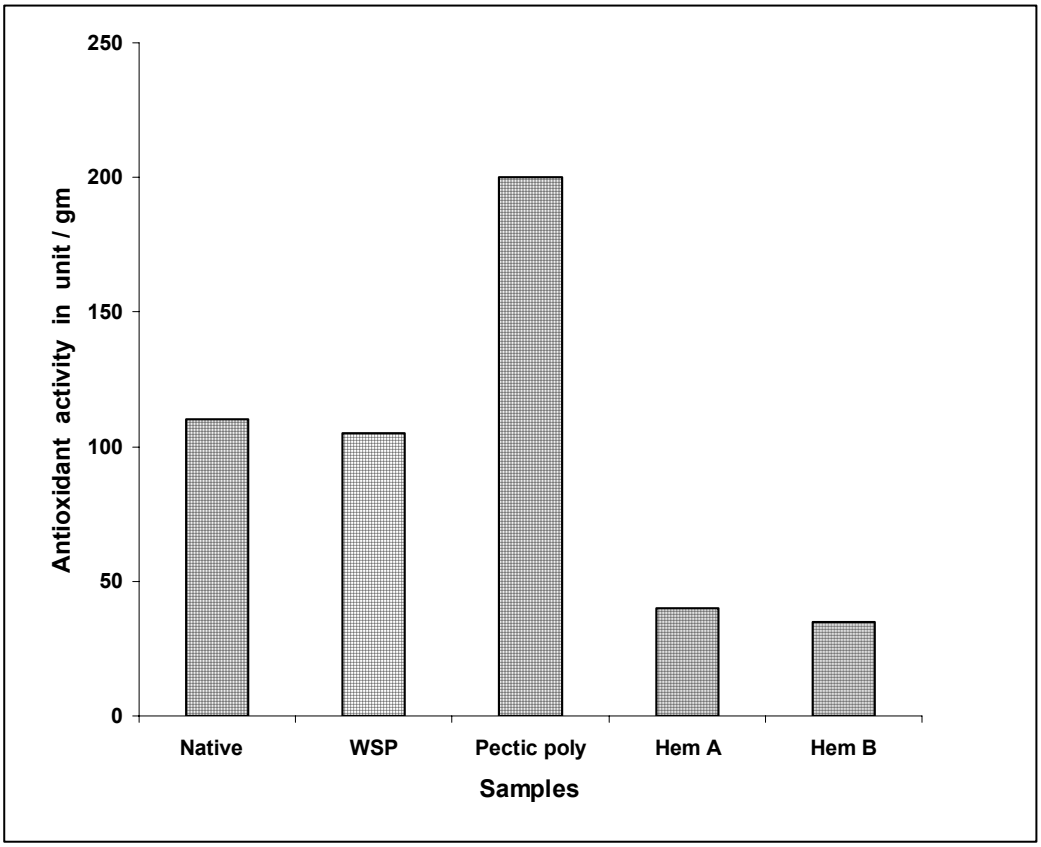


Fig. 13. Antioxidant activity of native, water-soluble polysaccharide (WSP), pectic polysaccharide, Hemicellulose A (Hem A), and Hemicellulose B (Hem B) fractions from *Emblica officinalis*.

Since ammonium oxalate extracted pectic fraction exhibited good wound healing and antioxidant property, it was taken for detailed investigation.

5.3 Fractionation of pectic fraction

DEAE Cellulose column chromatography

Ion exchange column chromatography has been applied by many investigators to fractionate pectins[287,288]. Based on their net charge and degree of esterification, pectic fractions can be eluted from the anion exchange column[288]. Ammonium salts[289] and NaOH[165] were used to elute polysaccharide fractions from DEAE- cellulose column.

Pectic fraction extracted with ammonium oxalate was fractionated using ion exchange chromatography on DEAE cellulose column (Fig. 14). Fractions were eluted with water, 0.1 - 0.5 M ammonium carbonate and 0.1 - 0.3 M alkali. Composition of these polysaccharides is shown in Table 28. Water eluted fraction was rich in carbohydrates and did not contain uronic acid. The sugar profile indicated it to be rich in galactose and glucose and contained small amounts of arabinose and xylose. The 0.1 M eluted fraction was rich in carbohydrate content and had 48% uronic acid. Compositional analysis indicated galactose to be the principal sugar (56.7%) and contained other sugars like glucose, arabinose, rhamnose/fucose. The 0.2 M eluted fraction was also rich in carbohydrates, but the content of uronic acid was 37%. The polysaccharide was rich in galactose and contained glucose, arabinose and rhamnose/fucose. Branched pectins are reported in pectins from apple and sugar beet[290]. Presence of rhamnose at the branches is

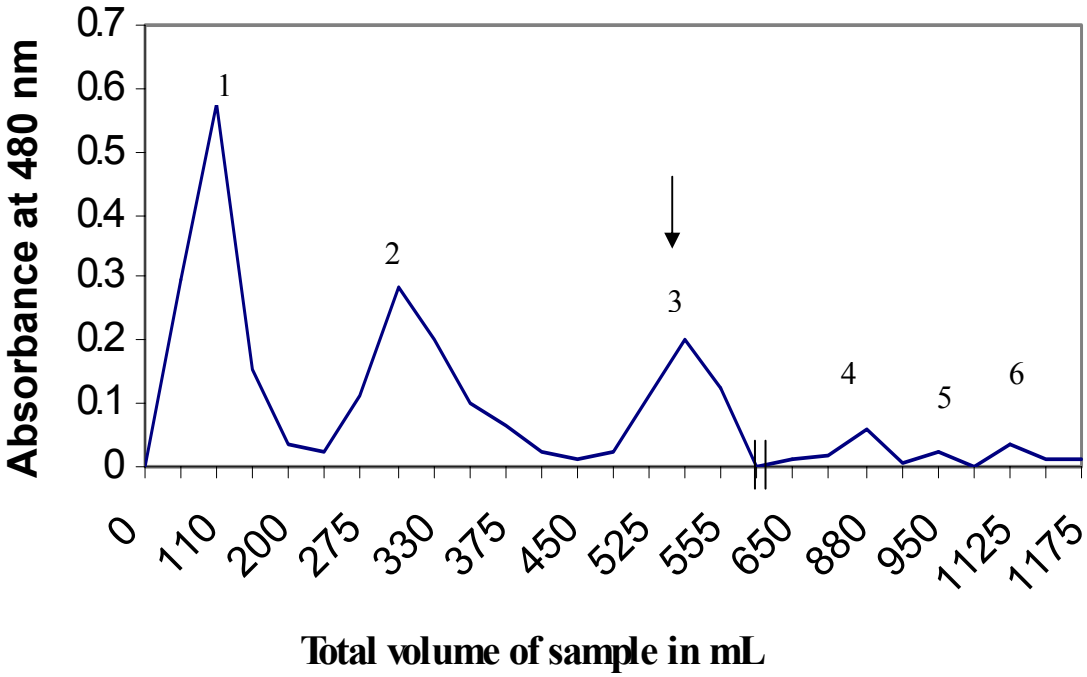


Fig. 14. DEAE cellulose column chromatography of pectic polysaccharide.

Fraction	Peak numbers
Water	1
0.1M $(NH_4)_2 CO_3$	2
0.2M $(NH_4)_2 CO_3$	3 (ACF)
0.1M Alk	4,5
0.2M Alk	6

reported in rhamnogalacturonans[291,292]. The alkali eluted fractions did not contain uronic acid and were rich in carbohydrates. The 0.1 M NaOH eluted fraction was rich in glucose and contained galactose and xylose and may be a mixture of complex polysaccharides. The 0.2 M NaOH eluted fraction contained high amounts of galactose along with glucose, arabinose, rhamnose/fucose and mannose and may be a mixture of arabinogalactan type of polysaccharides. The 0.3 M NaOH eluted fraction was rich in galactose and glucose and contained varying amounts of rhamnose/fucose, arabinose and xylose. Homogalacturonans eluting earlier than heterogalacturonans on ion-exchange chromatography has been reported from kiwi, pear and bush buttel fruit pectins [287-289].

DEAE eluted pectic polysaccharide fractions were tested for antioxidant activity. Among water, 0.1 M and 0.2 M ammonium carbonate eluted fractions (Fig. 15), 0.2 M ammonium carbonate eluted fraction showed better antioxidant activity compared to native and water-soluble polysaccharides. Hence, 0.2 M **A**mmonium **C**arbonate eluted **F**raction (0.2 M, **ACF**) was taken for detailed structural analysis.

5.4 Homogeneity

Cellulose acetate electrophoresis

The 0.2 M **A**mmonium **C**arbonate eluted **F**raction (**ACF**) was rich in uronic acid (37%) and was checked for purity by cellulose acetate electrophoresis and the electrophorogram is presented in Fig. 16. Citrus pectin is used as standard (A) and two different concentrations (B), (C) of ACF were spotted. The ACF moved as single spot indicating its

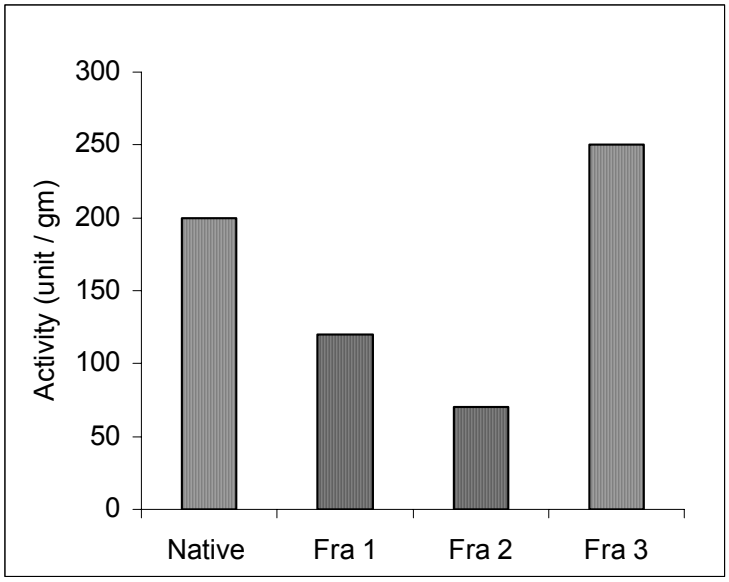


Fig. 15. Antioxidant activity of pectic polysaccharide (native) from *Emblica officinalis* and its DEAE cellulose eluted fractions.

Native : Pectic

Fra 1: Water eluted

Fra 2: 0.1M$(NH_4)_2CO_3$ eluted

Fra 3: 0.2M$(NH_4)_2CO_3$ eluted

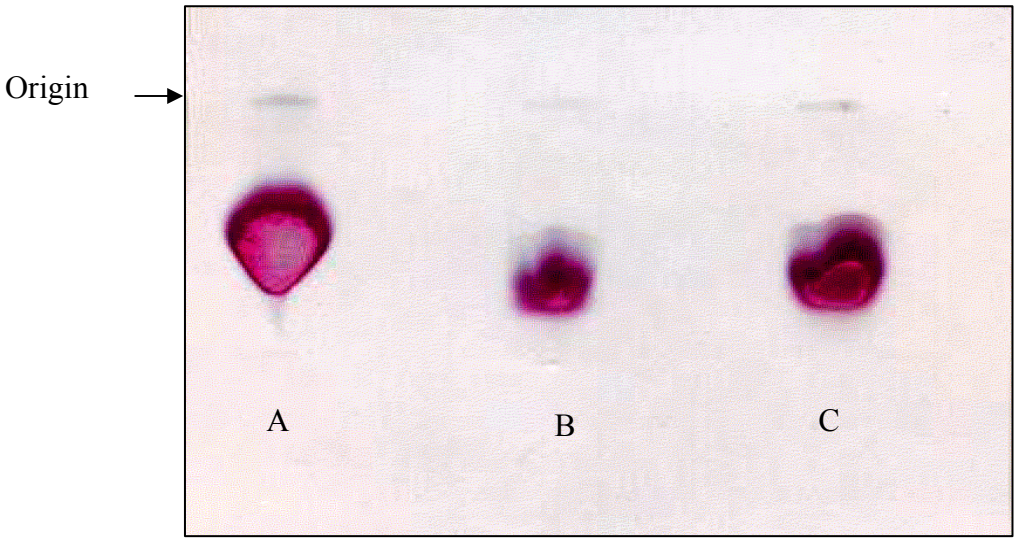


Fig. 16. Cellulose acetate electrophoresis of A - Citrus pectin with two different concentrations of ACF (B and C).

homogeneity on cellulose acetate electrophoresis. Cellulose acetate membrane electrophoresis has been used by many workers to check homogeneity of pectic polysaccharides[292].

Gel permeation chromatography

The DEAE eluted ACF eluted as a single peak on Sepharose CL-4B indicating its homogeneity and had a relative molecular weight of 55 Kda (Fig. 17). Pectic polysaccharide isolated from Kiwi had a molecular weight of 40 Kda [293].

High Performance Size Exclusion Chromatography (HPSEC)

Molecular weight and purity of ACF fraction was further substantiated by using E-Linear and E-1000 columns using HPSEC (Fig. 18). Single peak on HPSEC indicated homogeneity of fraction and retention time of the fraction was found to be 17.2 min. The molecular weight calibration on HPSEC was done using T_{70}-T_{40} dextrans. The molecular weight of ACF was found to be ~59 Kda. Pectic polysaccharides reported have ranges of molecular weights (250-40 Kda) [294,295].

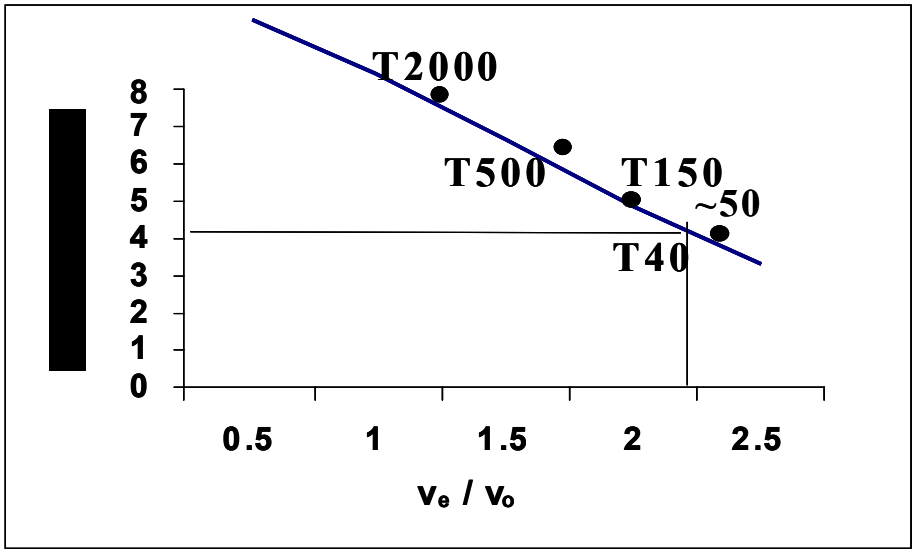


Fig. 17. Gel permeation chromatography on Sepharose CL - 4B of ACF.

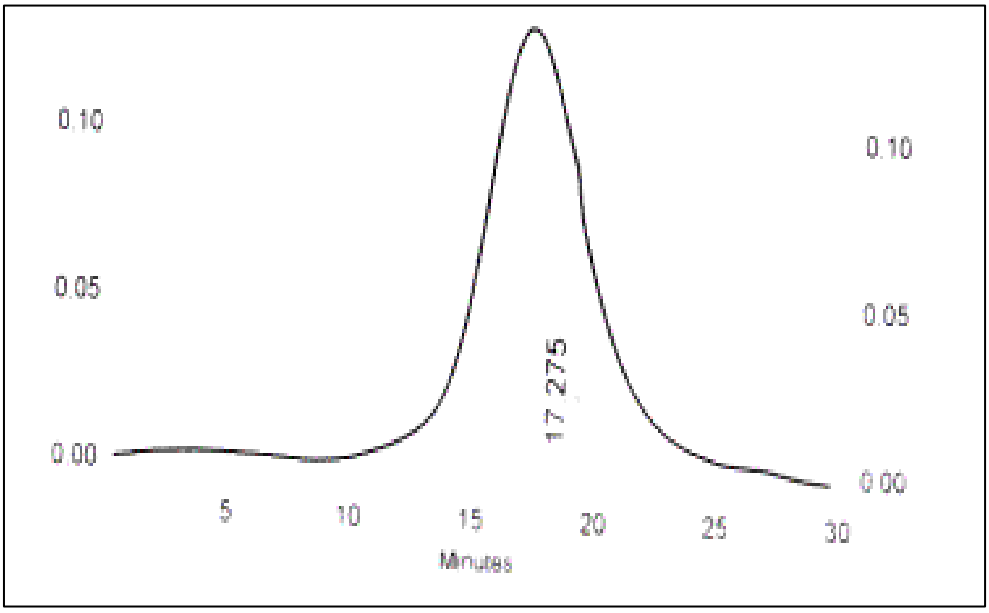


Fig. 18. High Performance Size Exclusion Chromatography (HPSEC) of ACF.

5.5 Structural elucidation

Reduction of carboxyl groups

The uronic acid content in 0.2 M ACF fraction was 37% and hence could not be used for the methylation directly. The ACF was subjected to carboxyl–reduction using carbodiimide and sodium borohydride. Carboxyl groups were reduced from 37% to 5% after 3 repeated treatments (Table 29). Similar method was used for carboxyl reduction of pectic polysaccharides and is reported by many workers [296,297].

The structural aspects on pectic polymers have been reported for a few fruits like apple[297,298], grapes [299,296], tomato[298,300] etc. Most of these studies on pectins are based on methylation, followed by GC-MS, FT-IR and ^{13}C NMR. A combination of methods were employed for structural analysis of ACF.

Methylation

Methylation analysis has been widely employed for linkage analysis of many of the polysaccharides [301,302]. In order to elucidate the linkage and substitution pattern, carboxyl reduced ACF was permethylated according to the method of Hakomori and the derivatives were analyzed by GLC-MS on SP-2330 column (Fig. 19). 2,3,4,6-Me_4 Glucose was used as reference standard for determining relative retention time. Diagnostic fragments of sample is shown in Fig 20 (A-G) and Table 30. ACF showed about 86% 2,3,6-Me_3-Gal and indicated it to be in the backbone of the polysaccharide, which indicated 1,4-linkage. Since galacturonic acid content was 37% the backbone of the ACF could be a galacturonic acid polymer in 1$\rightarrow$4 linkage. The presence of 2,3-Me_2-Gal

Table 29. Carbohydrate composition (%) of native and carboxyl reduced ACF

Polysaccharide	Rha	Ara	Xyl	Gal	GalA
Before reduction	7.5	6.1	1.8	51.0	37
After reduction	6.3	3.6	1.2	84.9	4

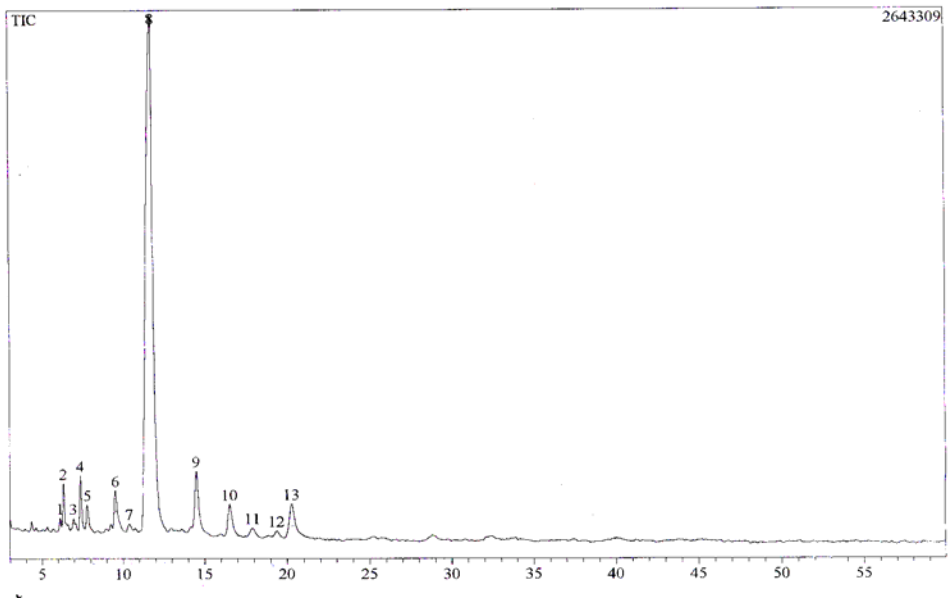
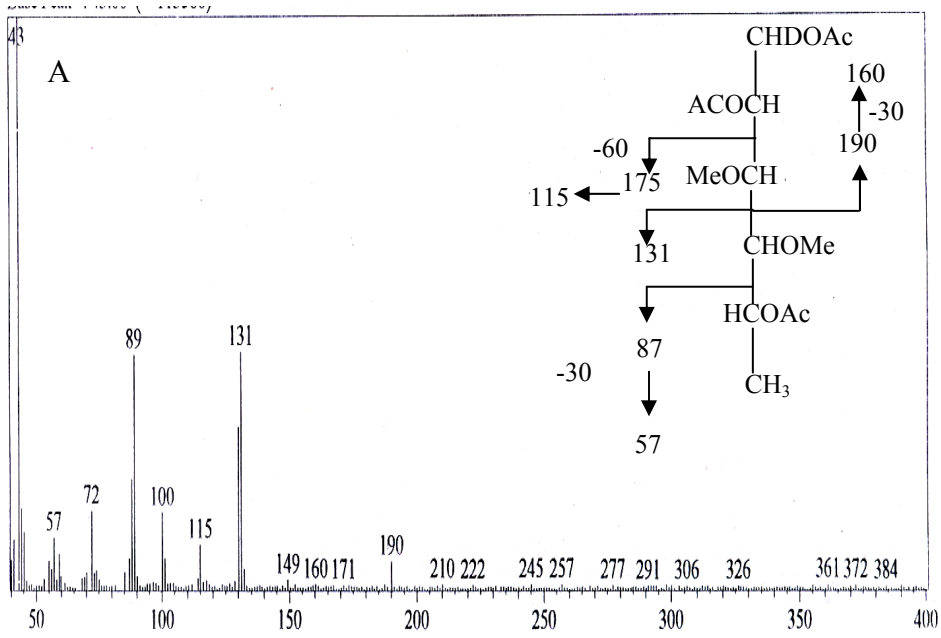
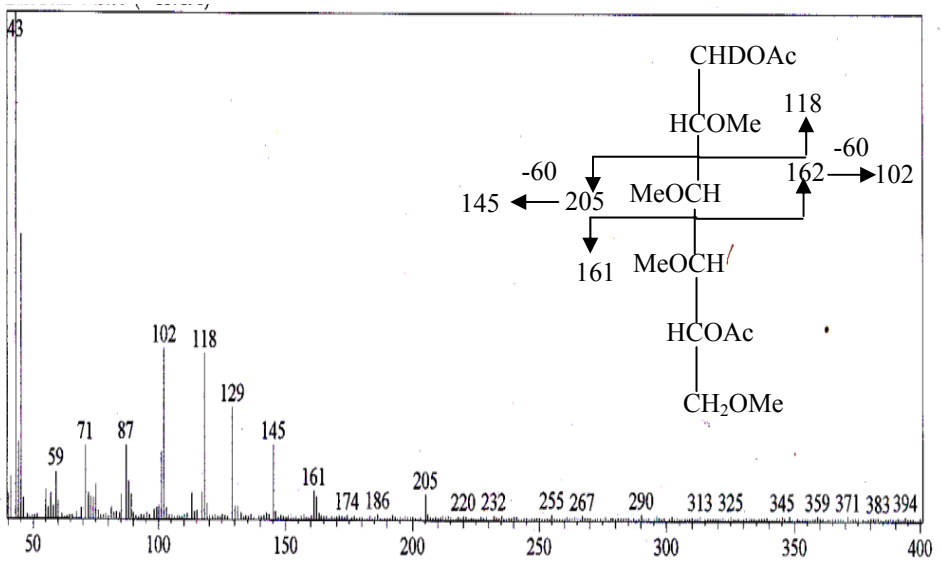


Fig. 19. GLC profile of permethylated ACF of *Emblica officinalis*.

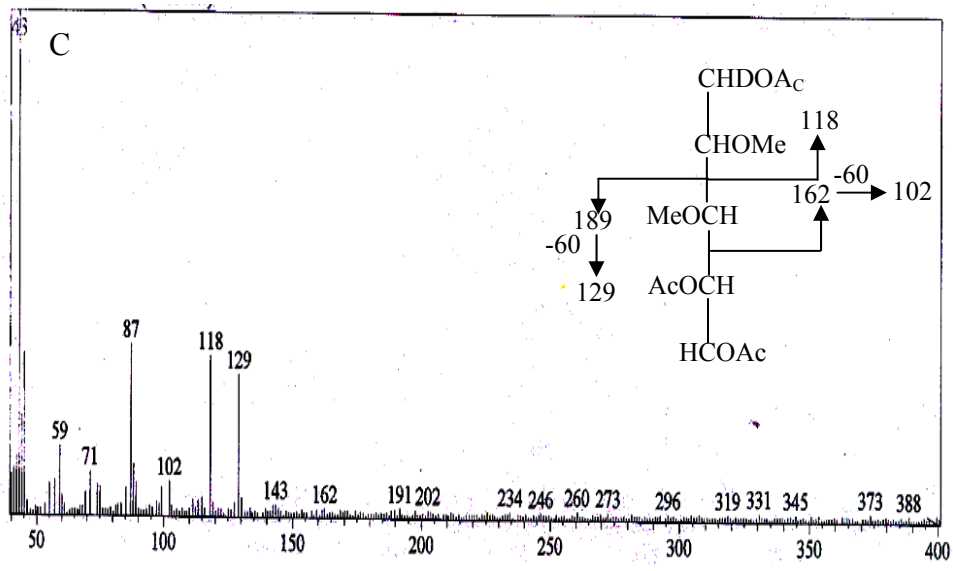


3,4-Me$_2$-Rha (2)

Fig. 20. Mass spectra and fragmentation scheme of partially methylated alditol acetates.

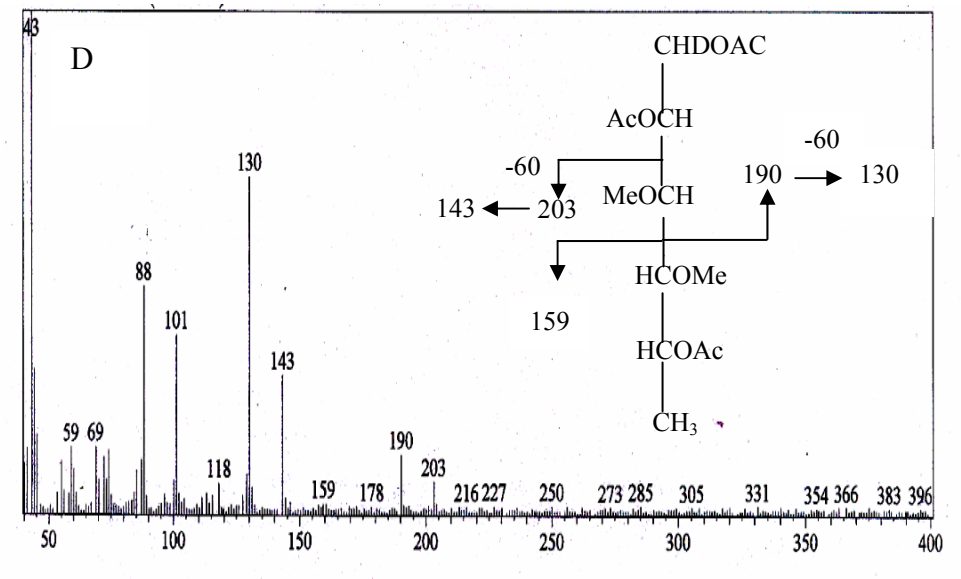


2,3,4,6 -Me_4-Gal (4)

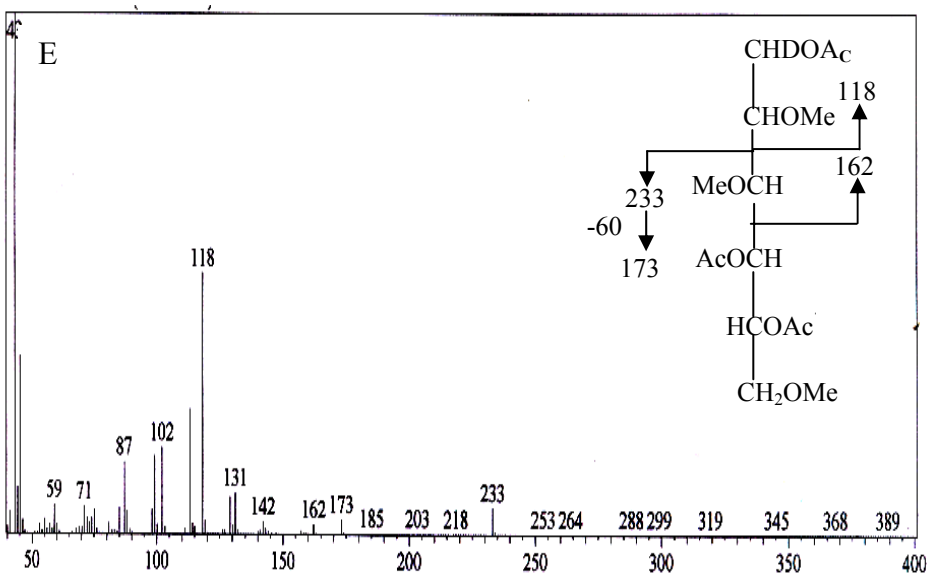


2,3-Me_2-Ara (5)

Fig. 20. Mass spectra and fragmentation scheme of partially methylated alditol acetates.

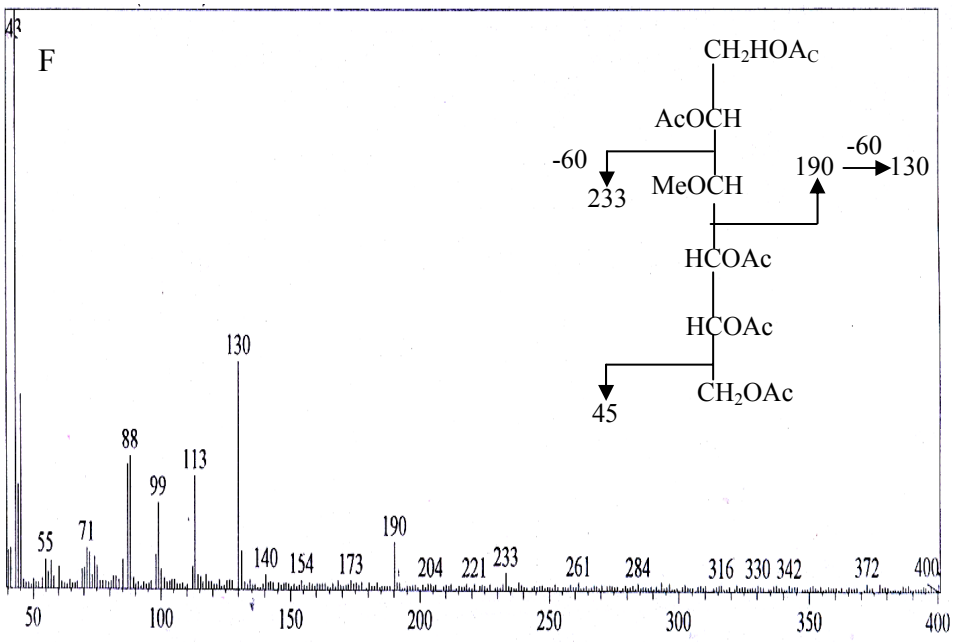


3-O-Me-Rha (6)

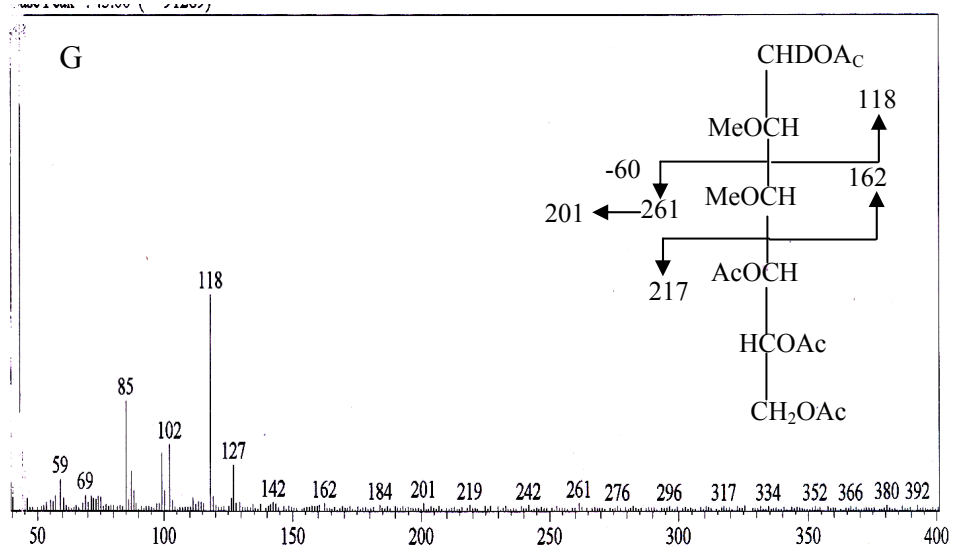


2,3,6-Me$_3$-Gal (8)

Fig. 20. Mass spectra and fragmentation scheme of partially methylated alditol acetates.



3-Me-Ara (10)



2,3-Me$_2$-Gal (13)

Fig. 20. Mass spectra and fragmentation scheme of partially methylated alditol acetates.

indicated branching of the polysaccharide. 2,3,4,6 Me_4-galactose indicated terminal galactose. Arabinose was present as 2,3-Me_2-arabinose and 3-Me-arabinose. 2,3-Me_2-arabinose would arise from the 1→5 linked chains and may be present as side chains and may be in furonose ring form. 3-Me-arabinose indicates branching of the 1→4 linked arabinose at position 5 - thus confirming furanose ring form of arabinose. Rhamnose was present as 3-Me-rhamnose and 3,4-Me_2-rhamnose. 3,4-Me_2-rhamnose indicate chains of rhamnose in 1→2 linkage and 3, Me-rhamnose indicates further branching through O-3. Rhamnose and arabinose would hence be attached to galacturonic acid backbone. Rhamnogalacturonans containing side chains composed of arabinose and galactose are reported for other fruits like kiwi[293], grapes[296] and apple[296,303]. Thus the ACF was rhamnogalacturonan-type having galacturonic acid back bone with galactose branching and had branches of rhamnose and arabinose, in turn attached to galactose at terminal positions. Tentative structure of the polysaccharide (ACF) is presented in Fig. 23 (page 184).

Optical rotation

High positive specific rotation (+155) of ACF indicated that the anomeric configuration of the polysaccharide is principally in α-linkage. Positive specific rotation (+230^o) is reported for citrus pectin [304,305]. Specific rotation of +175^o is reported for pectic polysaccharides from tansy[306].

Periodate oxidation

Periodate oxidation studies were carried out using sodium meta periodate (0.02 M) to estimate degree of substitution. The amount of periodate consumed became constant after 20 hours of incubation at 4^{o}C in dark and was found to be 71.9 mM and revealed high amount of substitution.

Smith degradation

Product of periodate oxidation was further used for hydrolysis and Smith degradation products were analysed by GLC. The sample yielded erythritol (85.1%) and glycerol (14.9%) indicating presence of galacturonic acid, galactose, arabinose and rhamnose.

NMR Studies

^{13}C NMR signal assignments were based on published spectra on plant pectins[33,37,307,308]. ^{13}C NMR is a promising tool in structural elucidation of polysaccharides. It gives detailed information on linkage, and confirmation of polysaccharides. This technique has been widely employed in elucidating structures of pectic polysaccharides from fruits[37,307,306]. The structural details as well as the nature of anomeric carbon rings in the isolated ACF was further established unequivocally by ^{13}C NMR spectral analysis, which showed signals characteristic of pectic type polysaccharide. The spectral quality of the native polysaccharide (Fig. 21A) was poor probably due to viscous nature of the sample. Similar spectra for native polysaccharides have been reported[309,310]. The galacturonic acid main chain occurring as back bone of the polysaccharide is too large and rigid to yield detectable signals

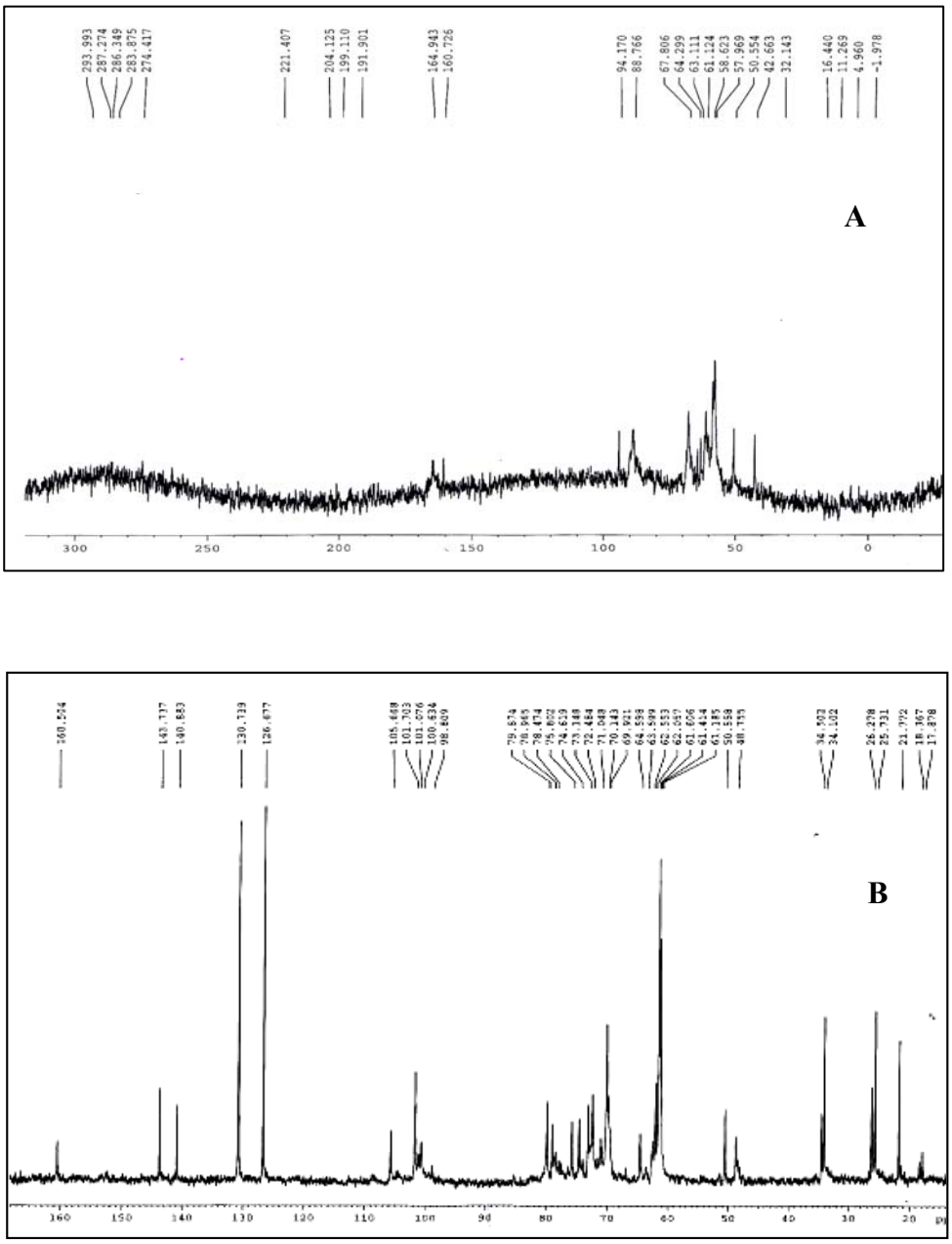


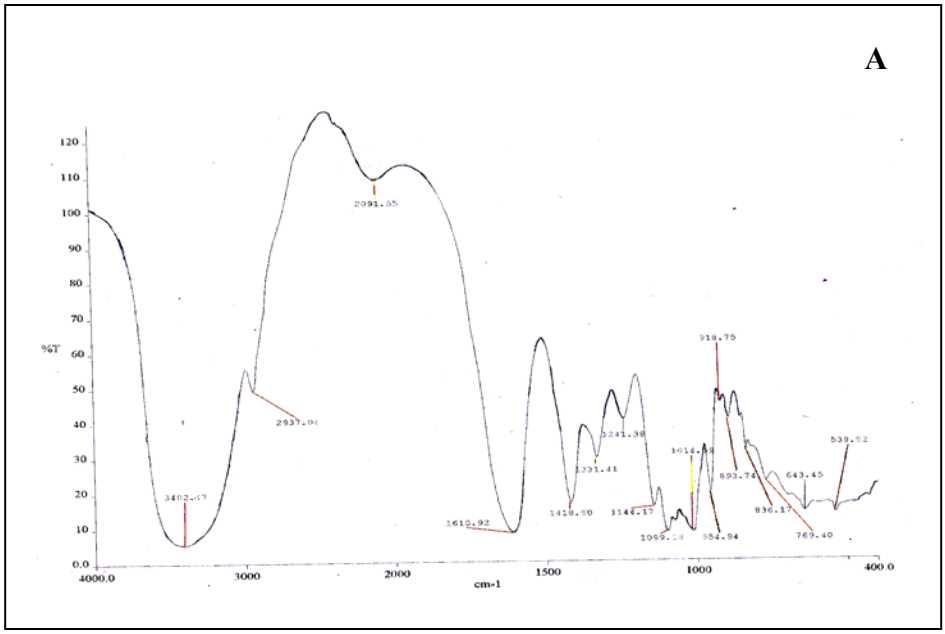
Fig. 21. ^{13}C NMR Spectrum of ACF of *Emblica officinalis.*
A – Native, B - reduced ACF.

and the signals are probably as a result of highly flexible chain. However, this was overcome by reducing the sample with sodium borohydride. Figure 21B shows the ^{13}C - NMR spectra of reduced pectic polysaccharide. The resonances at around 100.8 and 160 ppm are chemical shifts characteristic of galacturonan. The intense signals at 50.5 and 48.75 are assigned to $(CO)OCH_3$ of esterified galacturonic acid residues and the resonances at 160.5, 26.2, 25.7 ppm arises from O-$COCH_3$ of O-acetyl groups of galacturonic acid residues, respectively. Polysaccharide containing more than one O-acetyl group is not unusual in plant pectic polysaccharides[311]. O-Acetyl groups, most probably are attached to glycosyl uronic acid residues and the tri-O-methyl resonance, observed could indicate that there is availability for the location of O-acetyl groups in more than one position. The peak around 105.6 represents C-1 of rhamnosyl and at 101.7 indicates C-1 of galactosyl residue and were observed in the ratio 6.3 : 84.3 which corresponds closely to the relative amounts of rhamnose and galactose residues as measured by GLC. Spliting of galactosyl C-1 carbon atom (101.07, 100.6 and 98.8) indicates different galactosyl residue environments. The strong intense signal, at around 62 ppm indicates rhamnose is furonose ring form in (1→5) linkage in α configuration. Other signals 79.8, 75.8, 72.4 and 69.9 ppm supports the linkages of (1→4)-α-D galactopyronsyl residues and (1→5) linked α-L rhamnofurnosyl residues. Further signals at 18.3 and17.8 were indicative of methyl groups at C-6 of rhamnose. Some what unexpectedly additional peaks (very intense) were observed at 126,130,140 and 143 ppm and may represent chemical shifts of

oxamide residues[312]. This may explain the presence of unusually bound phenolics to the pectic polysaccharides (ACF).

FT-IR Studies

FT-IR spectra of isolated native polysaccharides is presented in Fig. 22A and is recorded in the frequency range of 400-4000 cm^{-1} and showed characteristic absorbance of pectic polysaccharides. The spectra was compared with published data [313-315]. The native spectrum showed broad absorption peak at 3402 cm^{-1} indicative of bound hydroxyl groups with hydrogen bonding. Signals around 1610 cm^{-1} were huge and intense absorption is attributed to carbonyl group of uronic acids present. The region between 1200-850 cm^{-1} showed several absorption peaks characteristic of polysaccharides[313,314,316]. Further, absorption at 1410 cm^{-1} was indicative of the presence of pectic methyl ester group ($O\text{-}CH_3$). The presence of 1331 and 1241 cm^{-1} absorption peaks indicate the presence of acetyl groups. The absorption around 836 cm^{-1} was indicative of α-configuration[316], which also correlated with high positive specific rotation for the polysaccharide and peak at 893 cm^{-1} may be due to β glycosidic linkage between the sugar residue in the galactan which coincides with its higher relative ratio of galactose. Similar spectrum of FT-IR is reported for rhamnogalacturonan type of polysaccharide[316]. Further, spectra of the reduced polysaccharide was taken and is shown in Fig. 22B. After reduction there was tremendous decrease in carbonyl group [C=O] around 1640 cm^{-1} compared to native, which was confirmed by increased sharp intense peak appearing around 2932 cm^{-1} due to CH_2 asymmetric absorption. From a combination of compositional, methylation, periodate oxidation, NMR



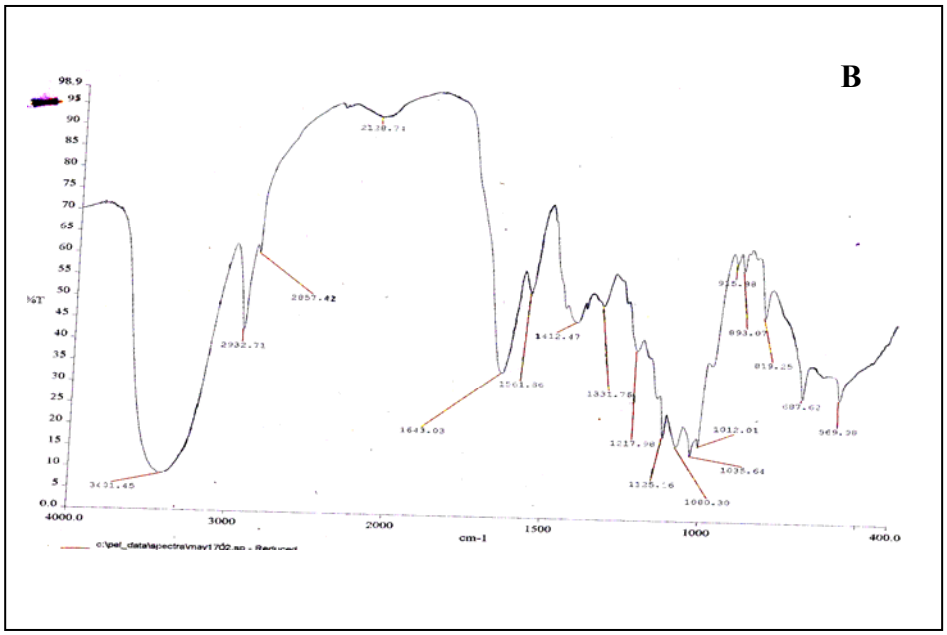


Fig 22. FT-IR Spectra of ACF of *Emblica officinalis*.
A – Native, B - Reduced polysaccharide.

and FT-IR,tentative structure of ACF of *Emblica officinalis* is presented in Fig 23. The polysaccharide had a backbone of 1→4-linked galacturonic acid residues is α linkages and are occasionally branched through O-6. The branches have galactosyl residues in α-1,4 linkages and also contain rhamnose and arabinose, and galactose was the terminal sugar.

3.2.1 Summary and conclusions

Initially studies were carried out on the effect of *Emblica officinalis* on diabetic status in streptozotocin induced diabetic rats. Since, *Emblica officinalis* is used in Ayurvedic prepartions, and that in recent years biological functions of polysaccharides are receiving much attention, an attempt was made to isolate various polysaccharide fractions of *Emblica officinalis* and their biological activity was tested in terms of wound healing property and antioxidant activity. A pectic polysaccharide showing potent biological activity was studied for detailed structural analysis. The results are summarized below:

1. Effect of *Emblica officinalis* on diabetic status was tested in streptozotocin induced diabetic rats. *Emblica officinalis* dry powder was tested at 1.0, 0.5, 0.1 and 0.025% concentrations. Feeding diabetic rats with *Emblica officinalis* at 1.0% concentration resulted in severe diarrhea and hence the experiment was terminated. *Emblica officinalis* at 0.5,0.1 and 0.025% did not show beneficial effect in streptozotocin induced diabetic rats, interms of urine volume, urine sugar, water intake and fasting blood glucose. Diabetic nephropathy status was measured as glomerular filtration rate and *Emblica officinalis* at 0.5, 0.1 and 0.025% concentrations did not show beneficial effect.

2. Since *Emblica officinalis* is used in Ayurvedic preparations, an attempt was made to isolate non-starch polysaccharide fractions and study if they have any biological activities. Water-soluble polysaccharide, pectic polysaccharide, hemicellulose A and

hemicellulose B were isolated from *Emblica officinalis* and biological activities were tested and were chemically analysed.

3. Carbohydrate composition of *Emblica officinalis* and its isolated fractions was studied. The water-soluble polysaccharide was mainly galactan type. The pectic polysaccharide was a complex mixture of pectic polysaccharides containing high amount of galacturonic acid. Hemicellulose A and B were xyloglucan type. The alkali insoluble residue was cellulosic in nature.
4. Antioxidant activity was tested in native, water soluble, pectic polysaccharide, hemicellulose A and B fractions. Maximum antioxidant activity was observed in pectic polysaccharide fraction.
5. Wound healing property was tested in water-soluble and pectic polysaccharide fractions. Best wound healing activity was observed in the pectic fraction. Wound healing property was also measured as a measure of the content of collagen formed.
6. Since pectic polysaccharide fraction showed good wound healing and anti-oxidant activity, this fraction was taken for detailed structural analysis.
7. The pectic polysaccharide was fractionated on DEAE-cellulose column. Water eluted fraction was neutral in nature. Ammonium carbonate eluted fractions (0.1 to 0.5 M) were acidic in nature and contained galacturonic acid along with galactose, arabinose, and rhamnose/fucose. NaOH eluted fractions had varying amounts of galactose, glucose, arabinose, rhamnose/fucose.

8. Antioxidant activity was tested in DEAE-cellulose fractionated polysaccharide fractions and 0.2M **A**mmonium **C**arbonate eluted **F**raction (**ACF**) showed maximum antioxidant activity and hence was taken for detailed structural analysis.

9. ACF was pure as ascertained by gel permeation chromatography on Sephoarose CL-4B, High Performance Size Exclusion Chromatography (HPSEC) and cellulose acetate electrophoretic profile.

10. Structural elucidation of ACF was done by a combination of methylation, GC-MS, periodate oxidation, Smith degradation, optical rotation, NMR and FT-IR.

11. Methylation analysis indicated $1\rightarrow4$ linked galacturonic acid backbone, with branches through O-6. The side chains contained galactose, rhamnose and arabinose.

12. ^{13}C-NMR spectra showed characteristic signals in anomeric region and signals due to carboxyl groups were present at 178 ppm. Signals at 126,130,140,143 are attributed phenolic group cross linkages in ACF.

13. Presence of uronic acid was observed in FT-IR at 178.4 cm^{-1} and these signals were reduced after carboxyl reduction.

14. Biological activity of pectic polysaccharide could be due to the structural complexity of the polysaccharide and/or due to the covalently linked phenolic acids to ACF (also see sections 3.3 and 3.3.1).

5.3 Phenolic acids of *Curcuma longa* and *Emblica officinalis*

Dietary constituents such as fruits, vegetables and spices are shown to possess health beneficial components of which phenolic metabolites have aroused much interest[317,318]. In recent years health beneficial effects of fruits, vegetables and spices are particularly being attributed to the phenolic compounds having antioxidant properties[319,320]. Phenolic components have been known to act as antioxidants not only because of their ability to donate hydrogen or electron but also because of their stable radical intermediates, which can effectively prevent the oxidation at cellular and physiological level [78,321-324].

Several investigations have determined the efficacy of both *Emblica officinalis* and *Curcuma longa* as health beneficial since they have been demonstrated to be antiatherosclerotic[325], antimutagenic[21,326-329], anticancer[330,331], hyperchlosterolemic[280], immunomodulatory[332], etc. Oxidative stress plays an important role in above mentioned disorders. Notably, ascorbic acid and curcumin have been shown to be the major bio-active components offering health beneficial properties in *Emblica officinalis* and *Curcuma longa,* respectively. However, precise phenolic acids and their antioxidant potency if any, needs to be addressed in depth to understand their array of health attributes.

5.3.1 Phenolic content

Different proportions of free and bound phenolics were observed in *Emblica officinalis* and *Curcuma longa*. The free phenolic content of

Emblica officinalis (EOFP) was 126.0 mg/g, 4.8 fold higher when compared to that of *Curcuma longa* free phenolics (CLFP, 28.3 mg/g) as measured by Folin-Ciocalteu method (data not shown). Also 1.8 fold higher bound phenolics were observed in *Emblica officinalis* bound phenolics (EOBP, 3.0 mg/g) relative to that of *Curcuma longa* bound phenolics (CLBP, 1.6 mg/g).

Antioxidant activity, reducing power ability, free radical scavenging activity as well as protection to DNA against oxidative stress induced DNA damage were evaluated in the free and bound phenolics of *Emblica officinalis* and *Curcuma longa.*

5.3.2 Antioxidant activity in various phenolic fractions of *Emblica officinalis* and *Curcuma longa*

Reducing power ability

The antioxidant activity (AOA) of free and bound phenolics of amla (*E.officinalis*) and turmeric (*Curcuma longa*) was investigated. Fig. 24 and 25 indicate a dose dependent increase in antioxidant activity of free and bound phenolic fractions of *Emblica officinalis* and *Curcuma longa*. As indicated in Fig. 23, at > 20 μg concentration of *Emblica officinalis* free phenolics (EOFP), saturation in the activity was observed. Activity was therefore compared at 10 μg gallic acid equivalent (GAE), where approximately 5 fold increase in activity was observed in EOFP than CLFP (Fig. 24). This correlates well with the total phenolic concentration in them, suggesting that *Emblica officinalis* is a better source of phenolics and possess better antioxidant activity than turmeric. This difference could be due to their constituent phenolic

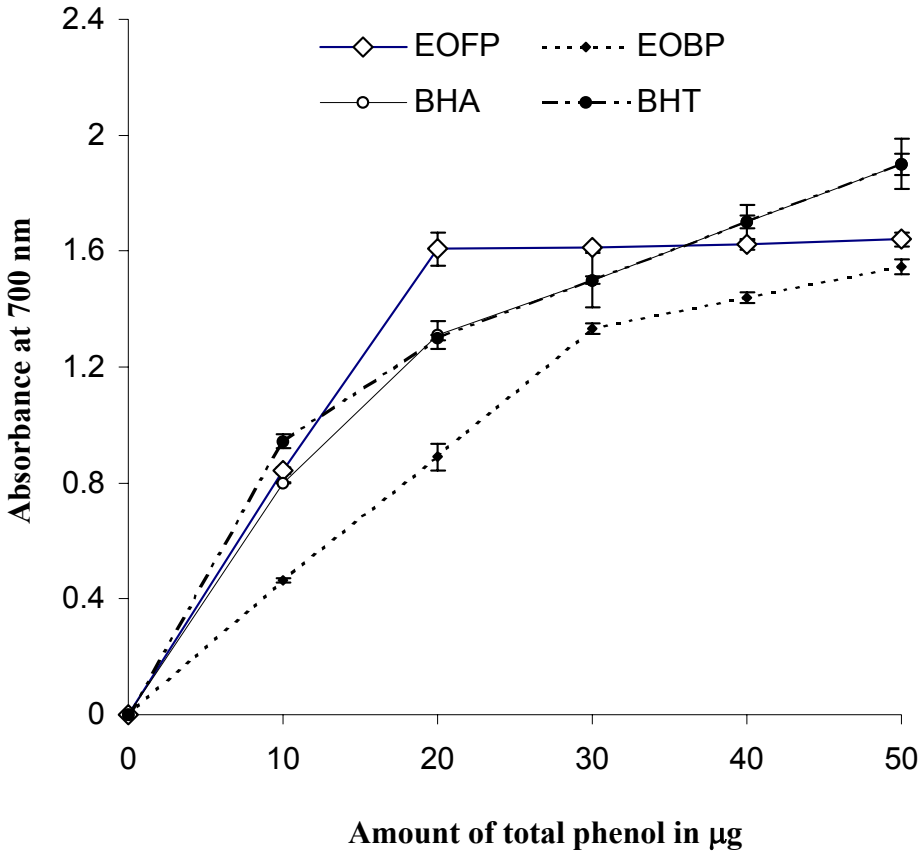


Fig. 23. Reducing power ability of free and bound phenolics of *Emblica officinalis.*

EOFP - *Emblica officinalis* free phenolics,
EOBP - *Emblica officinalis* bound phenolics.

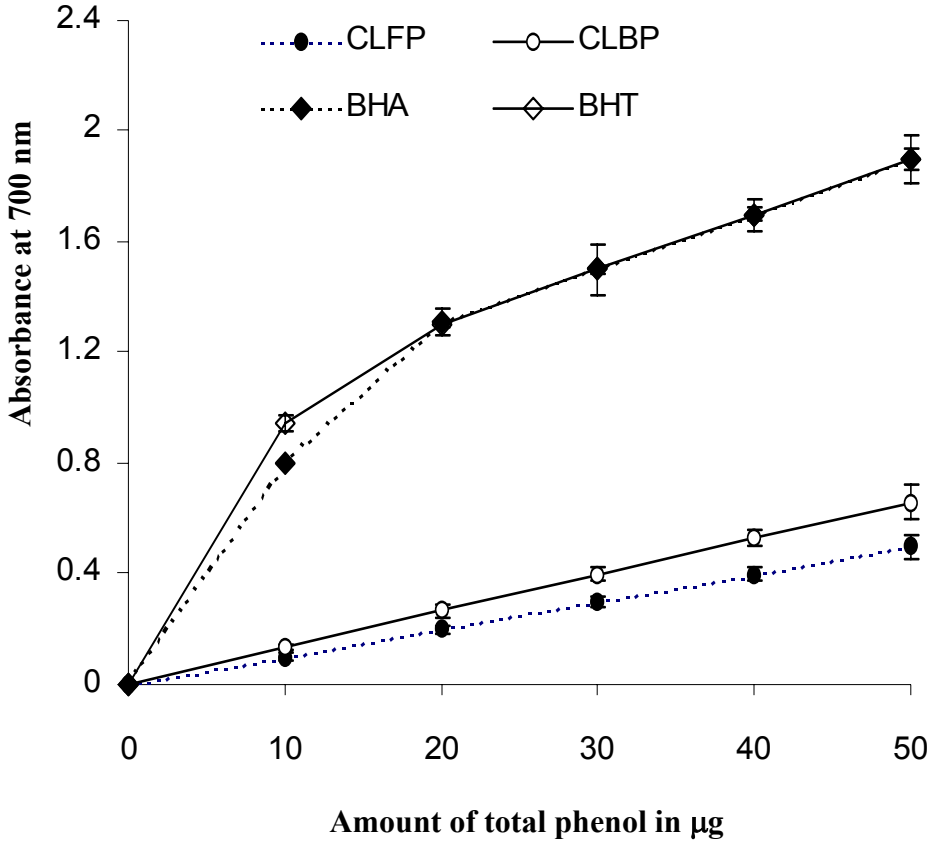


Fig. 24. Reducing power ability of free and bound phenolics of *Curcuma longa.*

CLFP – *Curcuma longa* free phenolics,
CLBP – *Curcuma longa* bound phenolics.

acids. Similarly EOBP also showed higher antioxidant activity than CLBP.

Free radical scavenging effect of free and bound phenolics of Emblica officinalis and Curcuma longa

Table 31 shows differential free radical scavenging ability of *Emblica officinalis* and *Curcuma longa* in addition to standard phenolic acids as evaluated by scavenging of DPPH radicals by the test solution, which was monitored by the characteristic absorption of DPPH radical at 517 nm. Both EOFP and EOBP showed an IC_{50} of 1.30 and 1.70 μg, respectively, as against that of 14.2 and 7.0 μg of CLFP and CLBP. The known antioxidants - BHA and BHT exhibited an IC_{50} of 7.8 and 17.0 μg, respectively. Results, therefore, suggest that *Emblica officinalis* phenolics are more potent than synthetic antioxidants - BHA and BHT, while *Curcuma longa* phenolic fractions are not as potent antioxidants compared to BHA and BHT. The EOFP and EOBP showed between 4-10 fold higher levels of antioxidant activity as evaluated by both free radical scavenging and reducing power assays compared to that of CLFP and CLBP.

DNA protection activity of free and bound phenolics of Emblica officinalis and Curcuma longa

Fig. 25 indicates that Fenton's reagent caused DNA damage as visualized by increased electrophoretic mobility. A dose dependent protection was observed by both free and bound phenolics of *Emblica officinalis* and *Curcuma longa* at 1.5 and 3.0 μg **G**allic **A**cid **E**quivalent

Table 31. Comparative IC_{50} values (listed in ascending order of activity) of EOFP, EOBP, CLFP and CLBP compared with standard antioxidants

Sl. No.	Sample / Standard	Std	IC_{50} (in μg)
1	Gallic acid	Standard	0.9 ± 0.02
2	*Emblica officinalis* free phenolics (EOFP)	Sample	1.30 ± 0.09
3	*Emblica officinalis* bound phenolics (EOBP)	Sample	1.70 ± 0.07
4	Tannic acid	Standard	1.98 ± 0.08
5	Ascorbic acid	Standard	2.10 ± 0.09
6	Curcumin	Standard	3.20 ± 0.05
7	Ferulic acid	Standard	4.80 ± 0.13
8	*Curcuma longa* bound phenolic (CLBP)	Sample	7.00 ± 0.13
9	Butylated hydroxy anisole (BHA)	Standard	7.80 ± 0.10
10	*Curcuma longa* free phenolics (CLFP)	Sample	14.25 ± 0.21
11	Butylated hydroxy toluene (BHT)	Standard	17.00 ± 0.35
12	Pro-coumaric acid	Standard	62.50 ± 0.61

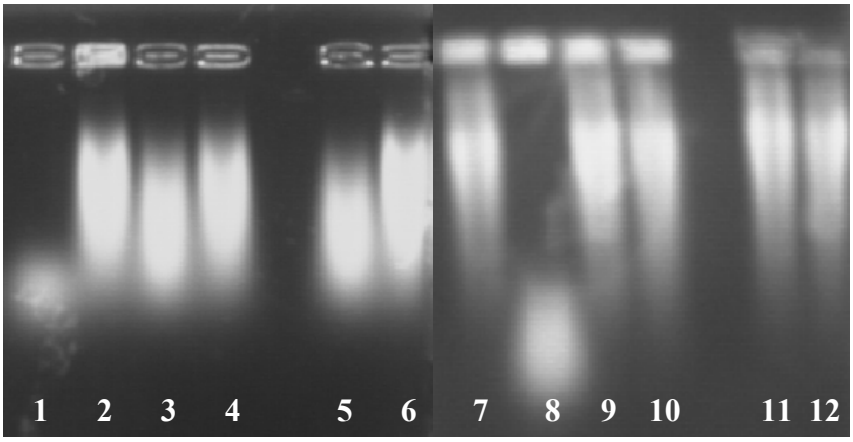


Fig. 25. DNA protection ability of free and bound phenolics of *Emblica officinalis* and *Curcuma longa.*

Lanes 1& 8 – Oxidized DNA; Lanes 2 & 7 – Native DNA; Lanes 3 & 4 – EOFP (1.5 μg and 3.0 μg), Lanes 5 & 6 – EOBP (1.5 μg and 3 μg), Lanes 9 & 10 – CLFP (1.5 μg and 3.0 μg), Lanes 11 & 12 – CLBP (1.5 μg and 3 μg).

Abbreviations as in Figs. 23 & 24.

(**GAE**). A significant (> 80 %) protection to native DNA during oxidation in the presence of these fractions was observed. Results therefore suggest that free and bound phenolics of *Emblica officinalis* and *Curcuma longa* can protect DNA against oxidative stress induced damage during chronic disorders.

5.3.3 Profile of phenolic acids in free and bound phenolic fractions of *Emblica officinalis* and *Curcuma longa* by HPLC analysis

The elution profiles of EOFP and EOBP coincided very well with the amount of gallic/tannic acid indicating that the major aqueous phenolic acid in *Emblica officinalis* is gallic acid/tannic acid. Ascorbic acid was absent in *Emblica officinalis* phenolic fraction. Determination of total phenol content as free and bound phenolics of *Emblica officinalis* revealed that in *Emblica officinalis*, a major fraction (4 fold over bound form) was contributed from free phenolic acids, which is constituted by gallic/tannic acid, as revealed by HPLC (Fig. 26B). Further, the bound fraction also showed the same gallic/tannic acid (Fig. 26C) exhibiting equivalent activity at similar concentration of phenol. Hence, the results suggested that gallic/tannic acid may exist in free form and approximately 2% may be present in bound fraction and this may be residual. However the binding of the gallic/tannic acid to various other components of *Emblica officinalis* may not be ruled out, since these phenolic acids having higher amounts of hydroxyl group, appear to bind to proteins and carbohydrates due to their binding property[333,334]. Nevertheless, their bioavailability need not be questioned since the bound fraction needs to be released into circulation.

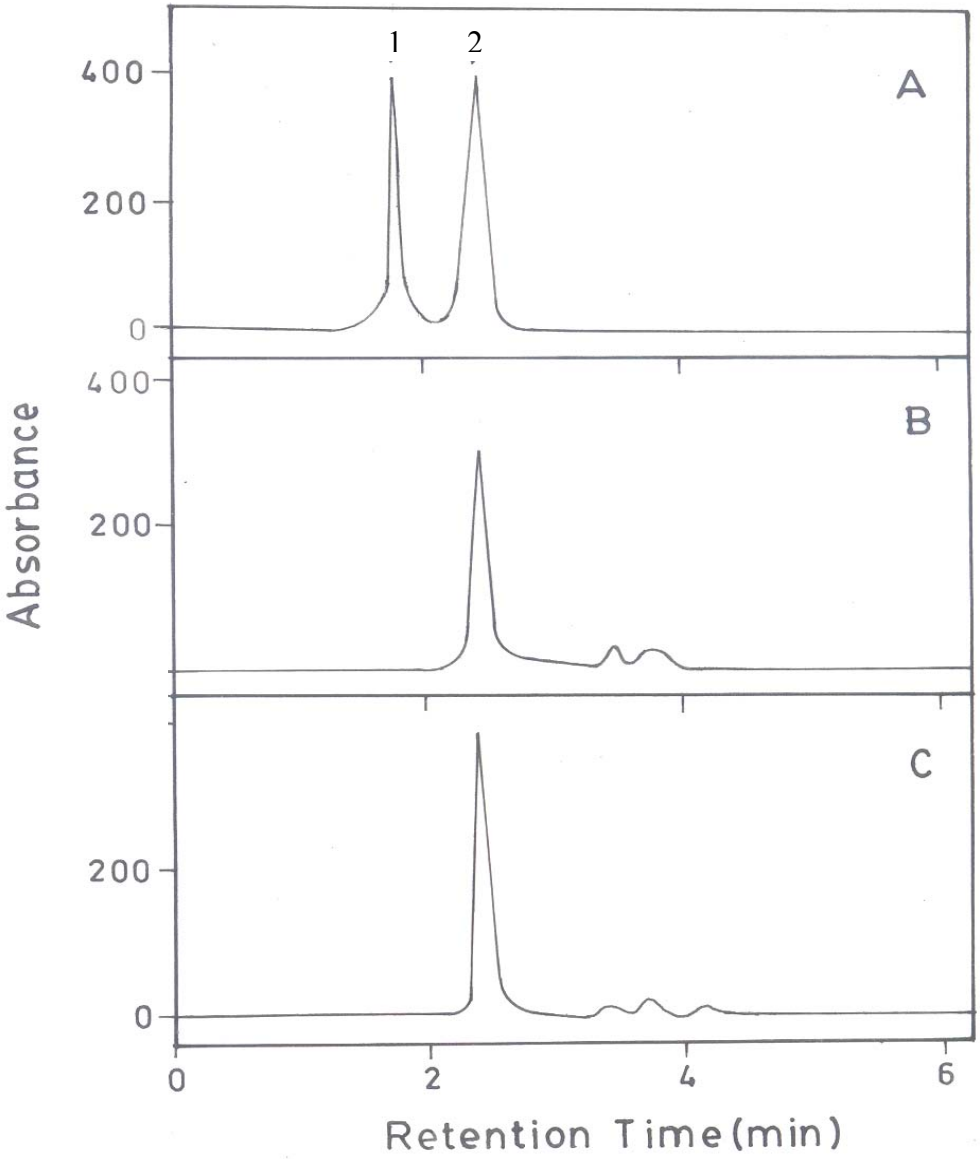


Fig. 26. HPLC profile of *Emblica officinalis* free and bound fractions compared with standards.

A - Standard Ascorbic acid – 1 (RT – 1.74), Tannic/Gallic acid – 2 (RT – 2.360);
B - EOFP; C - EOBP
Abbreviation as in Fig. 24.

Our results indicated that gallic/tannic acid is the only phenolic acid present in both free and bound form in *Emblica officinalis* contributing to the antioxidant activity. Hence, it is possible that tannoid derivatives reported earlier[87] may be constituted by enriched amount of gallic acid residues.

Further, it is very pertinent to address the question of the possibility of interference from ascorbic acid. So far only ascorbic acid has been reported to be the antioxidant in *Emblica officinalis*[335]. In order to differentiate this, we prepared water extract of *Emblica officinalis* also in addition to free and bound phenolic extracts and analyzed for the presence of ascorbic acid in each fraction by HPLC in addition to establishment of antioxidant activity. It was reported that both gallic acid and ascorbic acid were present in water extract[336]. Ethyl acetate extracted EOFP, had a negligible amount of ascorbic acid and was totally absent in EOBP. The results clearly suggested that the contribution of ascorbic acid is not significant indicating a predominant contribution from phenolic acids in *Emblica officinalis* for antioxidant activity. This can also be substantiated by similar levels of free radical scavenging activity in EOFP and EOBP at GAE concentration of phenols. On the other hand, in case of *Curcuma longa*, at equal concentration of phenol, CLBP showed at least 2 fold increase in activity than CLFP. This can be correlated to the presence of curcumin exclusively in CLFP (Fig. 27B), and the presence of p-coumaric acid (1.31 mg/g) and ferulic acid (0.29 mg/g) in CLBP (Fig. 27C). Hence the results indicated contribution of free radical scavenging ability of p-coumaric acid and ferulic acid in addition to curcumin. Free and bound

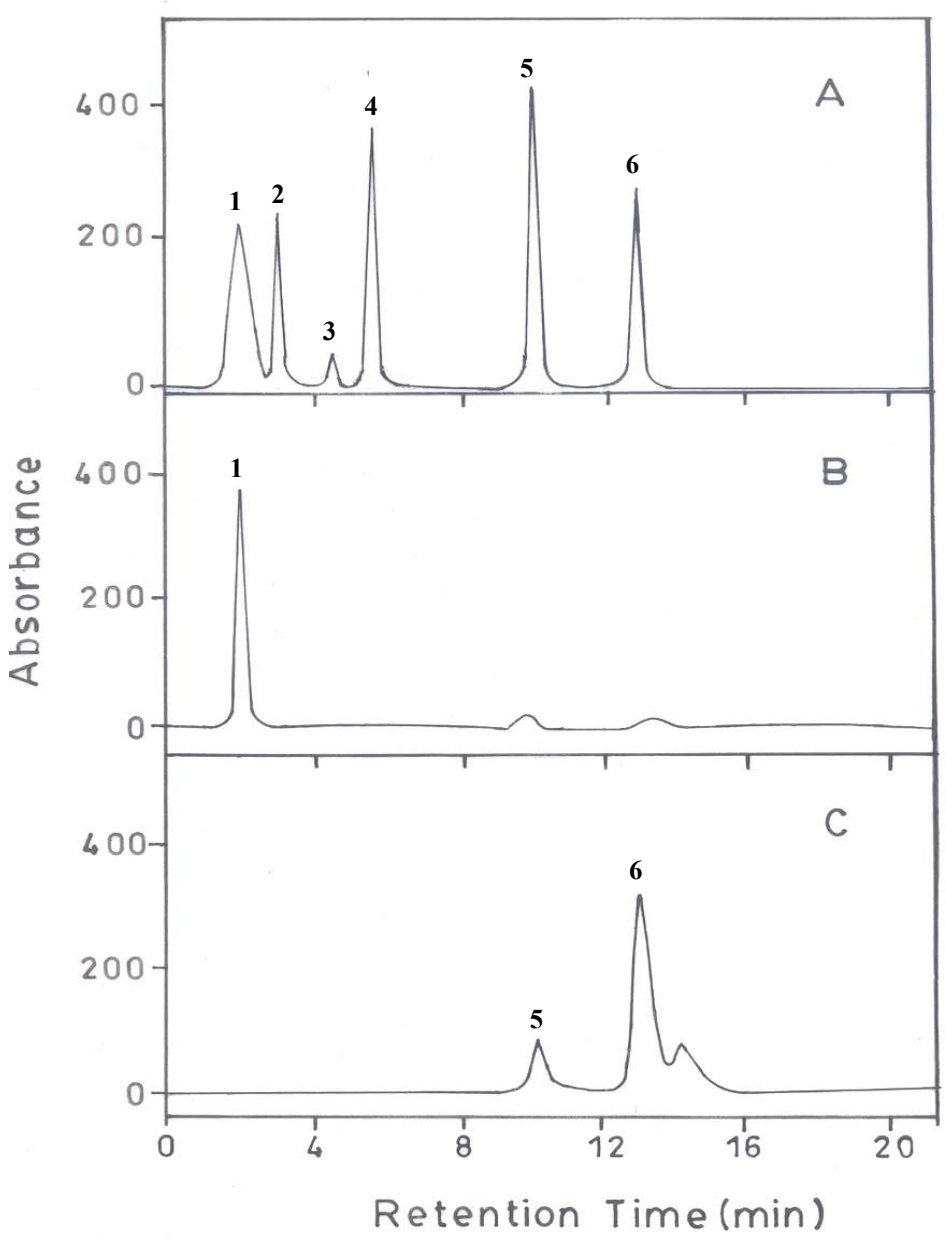


Fig. 27. HPLC profile of *Curcuma longa* free and bound fractions compared with standards.

A - Standard phenolic acids, 1 - Standard curcumin (RT – 1.92); 2 - protocatechuic acid (RT – 3.04), 3 - gentesic acid (RT – 4.49), 4 - caffeic acid (RT – 5.631), 5 - p-coumaric acid (RT – 10.024) and 6 - ferulic acid (RT –12.874);
B - CLFP; C - CLBP.

Abbreviation as in Fig. 24.

phenolics thus have the ability to donate electrons to free radicals and converting them to more stable radicals thus terminating the oxidative stress caused by free radical chain reaction. These results were further confirmed (Table 31) by determining the free radical scavenging ability of standard curcumin, p-coumaric acid and ferulic acid, where they exhibited an IC_{50} of 3.2, 62.5 and 4.8 μg, respectively.

Phenolics bound to dietary fibres were identified many years ago and have been the subject of considerable study in recent years[15,337-339]. From experiments with rats fed with ^{14}C-labeled bound phenolics in spinach, it was determined that 19% of the label was excreted in feces, 20% was excreted in urine, and 34% of the label was retained in body tissues after 18 hours[340]. Phenolic acids covalently bound in ester linkage to the arabinose side chains of arabinoxylans is reported in cell walls[337,341]. Insoluble antioxidants have not been completely identified, but it is reasonable to assume a significant part from phenolic acids esterified to dietary fiber and tannins, may exhibit antioxidant activity[342] and it is known that antioxidants are released in the colon by microbial fermentations and they are absorbed[343]. These antioxidants are bioavailable and can function as free radical scavengers in the body. In addition, these compounds may also act by reducing platelet adhesion, vasorelaxation, cell apoptosis, and induction of detoxification enzymes[344-349]. Antioxidants in foods also have potential to react with nitrites and free radicals and perform different biological functions. Covalently bound phenolics are resistant to digestion in the stomach and small intestine, but enzymatic hydrolysis is reported in the colon[241,.340,350]. Release of free phenolics in microbial fermentation is

reported from arabino-ferulates. Corn bran contains high levels of ferulate and may contain small amount of highly active compounds such as tannins[342]. Water-soluble antioxidants can be absorbed by small intestine. Antioxidants covalently bound to cell walls are transported into the large intestine after being released by microbial fermentation. Antioxidants released in this manner are available to scavenge free radicals present in the colon. Epithelial cells of the colon absorb free phenolics and may release them in to circulation to perform vital biological functions.

3.3.1 Summary and Conclusions

The content of phenolics, their identification and antioxidant activity were studied in *Emblica officinalis* and *Curcuma longa* and the results are summarized below:

1. Total phenolic content of *Emblica officinalis* was higher and was 129 mg/g (free phenolics 126 mg/g, bound phenolics 3 mg/g), compared to C*urcuma longa*, which was 29.9 mg/g (free phenolics 28.3 mg/g, bound phenolics 1.6 mg/g).
2. Antioxidant activity of free and bound phenolics of *Emblica officinalis* and C*urcuma longa* clearly indicated that *Emblica officinalis* is a better source of phenolics and showed higher antioxidant activity than *Curcuma longa*. Both free and bound phenolics of *Emblica officinalis* and C*urcuma longa* showed DNA protection ability against stress induced by ferric chloride in *in vitro* studies.
3. Separation and identification of phenols was carried out in free and bound phenlics of *Emblica officinalis* and *Curcuma longa* using HPLC. Though the content of ascorbic acid was high in *Emblica officinalis, g*allic acid was found in both free and bound phenolics fraction, but there was no ascorbic acid in the phenolic fraction. In C*urcuma longa,* curcumin was found to be present in the free phenolics and p-coumaric acid and ferulic acid were present in the bound phenolics. These phenolics may be responsible for the antioxidant activity of *Emblica officinalis* and *Curcuma longa*.

Emblica officinalis showed better antioxidant activity than C*urcuma longa*, and may be due to high phenolic content. It is quite likely that these phenolics are bound to dietary fibres *in situ* and the biological activities studied as wound healing and antioxidant activity of ACF could be due to phenolics bound to the pectic polysaccharide (ACF).

Chapter IV

Bibliography

1. Notkins AL. *Sci Am*, **241** (1979) 56.
2. Ranjan C, Rajagopalan R. *Current Science*, **83** (2002) 1533.
3. Teng CT. *Arch Bichem Biophys,* **48** (1954) 409.
4. Taylor R, Agius L. *Biochem J*, **250** (1988) 625.
5. Singh BM, Palma MA, Nattras M. *Diabetes*, **36** (1987) 740.
6. Owen OE, Reichard GA. *J Clin Invest*, **50** (1971) 1536.
7. Michael B. *Nature,* **414** (2001) 813.
8. Nishimura C, Siato T, Ito T, Omori Y, Tanimoto T. *Diabetologia,* **37**(1994) 328.
9. Thornalley PJ. *Biochem J*, **269** (1990) 1.
10. Suzuki K, Koh YH, Mizuno H, Hamaoka R, Taniguchi N. *J Biochem*, **123** (1998) 353.
11. Schmidt AM, Hori O, Chen JX, Li JF, Crandall J, Zhang J, Cao R, Yan SD, Brett J, Stern D. *J Clin Invest,* **96** (1995) 1395.
12. Xia P, Inoguchi T, Kern TS, Engerman RL, Oates PJ, King GL. *Diabetes*, 43 (1994) 1122.
13. Portilla, D Dai G, Peters JM, Gonzalez FJ, Crew MD, Proia AD. *Am J Physiol Renal Physiol*, **278** (2000) 667.
14. Keogh RJ, Dunlop ME, Larkins RG. *Metabolism,* **46** (1997) 41.
15. Ishii H. Michael R, Jiroue K, Koya D, Takagi C, Xia P, Allen C, Bursell S, Timothy SK, Lawrenee MB, William FH, Lawrence ES, Edward PF, George LK. *Science*, **272** (1996) 728.
16. Studer RK, Craven PA, De Rubertis FR. *Diabetes,* **42** (1993) 118.
17. Koya D, Jirousek MR, Lin YW, Ishii H, Kuboki K, King GL. *J Clin Invest*, **100** (1997) 115.
18. Kolm-litty V, Sauer U, Nerlich A, Lehmann R, Schleicher, ED. *J Clin Invest,* **101** (1998) 160.

19. Brownlee M, Cerami A. *Ann Rev Biochem,* **50** (1981) 385.
20. Ruderman NB, Williamson JR, Brownlee M. *FASEB Lett,* **6** (1992) 2905.
21. Maurice BL, **1982** In: Cecil text book of medicine, J.B. Wyngaarden and L.H. Smith, (Eds.), Vol.II, 16th edition , W.B. Saunders company, Philadelphia, USA.,pp 2297.
22. Dyck PJ, **1982** In: Cecil text book of medicine, J.B. Wyngaarden and L.H. Smith, (Eds.), Vol.II, 16th edition , W.B. Saunders company, Philadelphia, USA.,pp 2153.
23. Mogensen CE, Christensen CK, Vittinghus E. *Diabetes*, **28** (1983) 6.
24. Bojestig M, Amqvist HJ, Hermansson G, Karlberg BE, Ludvigsson J. *N Engl J Med*. **330 (**1994) 584.
25. Maver SM, Steffes NW, Brown DM. *Am J Med,* **70** (1981)603.
26. Seyer-Hansen K, Gundersen HJG. *Diabeteologia,* **18** (1980) 501.
27. Mogensen CEF, Andersen MJF. *Diabetes,* **22** (1973) 706.
28. Nyengaard JR, Rasch R. *Diabetologia*, **36** (1993) 189.
29. Nyengaard JR, Flyvbjerg A, Rasch R**.** *Diabetlogia,* **36** (1993) 1126.
30. Maver SM, Steffs MW, Michael AF, Brown DM. *Diabetes*, **25** (1976) 850.
31. Arison RN, Ciaccio EL, Glitzer MS, Cassaro JA, Pruss MP. *Diabetes*, **16** (1967) 51.
32. Nyengaard JR, Bendtsen TF. *Anat Rec,* **232** (1992) 194.
33. Osterby R, Gundersen HJG. *Diabetologia*, **18 (**1980) 493.
34. Rosenblum ND. *Kidney Int,* **45(**1994) 73.

35. Beavan LA, Davies M, Couchman JR, Williams MA, Mason RM. *Arch Biochem Biophys*, **269** (1989) 576.

36. McLennan SV, Death AK, Fisher EJ, William PF, Yue DK, Turtle JR. *Cell Mol Biol*, **45** (1999) 123.

37. Myers BD. Winetz JA, Chui F, Michaels AS. *Kidney Int*, **21**(1982) 633.

38. Goode NP, Shires M, Crellin DM, Davison AM. *Diabetologeia,* **38** (1995) 1455.

39. Rohrbach DH, Martin GR. *Ann New york Acad Sci,***40** (1982) 1203.

40. Tamsma JT, van den Born J, Bruijn JA, Assmann KJ, Weening JJ, Berden JH, Wieslander J, Schrama E, Hermans J, Veerkamp JH, et al.. *Diabetologia***, 37** (1994) 313.

41. Klein L, Butcher DL, Subilovsky O, Kikkawa R, Miller M. *Diabetes,* **24** (1975) 1057.

42. Kim Y, Kleppel MM, Butkowski R, Mauer SM, Wieslander J, Michael AF. *Am J Pathol*, **138** (1991) 413.

43. Sheinman JI, Brown DM, Michael AF. *Biochem Biophys Acta*, **542** (1978) 128.

44. Haralson MA, Jacobson HR, Hoover RL. *Lab Invest,* **57** (1987) 512.

45. Mott JD, Khalifah RG, Nagase H, Shield CF, Hudson JK, Hudson BG. *Kidney Int*, **52** (1997) 1302.

46. Dixon AJ, Burns J, Dunnil MS, McGee JO. *J Clin Pathol,* **33**(1980) 484.

47. Nerlich A, Schleicher E. *Am J Pathol,* **139** (1991) 889.

48. Jackle-Meyer I, Szukics B, Neubauer K, Metze V, Petzoldt R, Stolte H. *Eur J Clin Chem Clin Biochem*, **33** (1995) 211.
49. Werle E, Diehl E, Hasslacher C. *Metabolism,* **47** (1998) 63.
50. Ziyade FN, Han DC, Cohen JA, Guo J, Cohen MP. *Kidney Int*, **53** (1998) 631.
51. Van Det NF, Verhagen NA, Tamsma JT, Berden JH, Bruijn JA, Daha MR, van der Woude FJ. *Diabetes*, **46** (1997) 834.
52. Cohen MP, Wu VY, Cohen JA. *Biochem Biophys Res Commun,* **239** (1997) 91.
53. Cohen MP, Hud E, Wu VY, Ziyadeh FN. *Mol Cell Biochem,* **151** (1995) 61.
54. Roden L, **1980**. Structure and metabolism of connective tissue proteoglycans. In: Lennarz.W.J. (Ed.) The Biochemistry of Glycoproteins and Proteoglycans. Plenum Publishing Corp. New York. pp. 267-371.
55. Hascall VC, **1986**. In: Evered. D. Whelan. J. (Ed.) Functions of the Proteoglycans. John Wiley & Sons. New York. pp. 1-8.
56. Raab G, Klagsbrun M. *Biochim Biophys Acta,* **1333** (1997) 179.
57. Casu B, Oreste P, Torri G, Zoppetti G, Choay J, Lormeau JC, Petitou M, Sinay P. *Biochem J,* **197** (1981) 599.
58. Dietrich CP, Sampio LO, Toledo OMS. *Biochem Biophys Res Commun,* **71** (1976) 1.
59. Dietrich CP, Sampio LO, Taledo OMS, Cessaro CMF. *Biochem Biophys Res Commun*, **75** (1977) 329.
60. Parthasarathy N, Spiro R. *Diabetes*, **31** (1982) 738.
61. Kanwar YS, Rosenzweig LJ, Linker A, Jakubowski ML. *Proc Natl Acad Sci*, **80** (1983) 2272.

62. Groffen AJ, Ruegg MA, Dijkman H et al. *J Histochan Cytochem*, **46** (1998) 1.

63. Rosenberg RD. *J Clin Invest*, **99** (1997) 2062.

64. Kofoed EA. *Kidney Int,* **41 (**1992) 1021.

65. Tappel AL, **1973**. In: Dingle. J.T. Fell. H.B. (Ed.) Lysosomes in biology and pathology. Vol 2. North Holland. Amsterdam. pp.207.

66. Liebel A. *Diab Metab Res Rev*, **18** (2002) 336.

67. Lebovitz HE, **1995**. In: Leslic. R.D. Robins.D.C. (Ed.) Diabetes Clinical Science in practice. Cambridge University press. New York. pp.450-464.

68. American Diabetes Assoc. *Diab Care,* **18** (1995) 1510.

69. Satyavati GV, Tandon N, Sharma M. *Diabetes Bulletin*, **10** (1989)181.

70. Platel K, Srinivasan K. *Nahrung,* **39** (1995) 262.

71. Nagarajan S, Jain HC, Aulakh GS, **1982**. In: Atal. C.K. Kapur. B.M. (Ed.) Cultivation and Utilization of Medicinal plants RRL. Jammu-Tawi. India. pp.584.

72. Shibib BA, Khan LA, Rahman R. *Biochem J*, **10** (1999) 477.

73. Bailey CJ, Day C. *Diabetes Care*, **12** (1989) 553.

74. Sureshbabu P, Srinivasan K. *J Nutr Biochem*, **10** (1999) 477.

75. Sureshbabu P, Srinivasan, K. *Mol Cell Biochem*, **175** (1997) 49.

76. Temple NJ. *Nutr Res,* **20** (2000) 449.

77. Willett WC. *Science,* **296** (2002**)** 695.

78. Doll R. *Proc Nutr Soc,* **49** (1990) 119.

79. Sabu MC, Kuttan R. *J Ethnopharmacol*, **81** (2002) 155.

80. Cuvelier M E, Richard H, Berset C. *Biosci Biotehnol Biochem,* **56** (1992) 324.

81. Thakur CP, Thakur B, Sinha PK, Sinha SK. *Indian J Cardiol*, **21** (1988) 167.

82. Tripathi SN, Tiwari CM, Upadyay BN, Singh RS. *J Res Indian Med Yoga Homoep,* **14** (1979) 159.

83. Nagabhushan M, Bhide SV. *Nutr Cancer,* **8** (1986) 201.

84. Polasa K, Sesikaran B, Krishna TP, Krishnaswamy K. *Food Chem Toxicol,* **29** (1991) 699.

85. Aggarwal BB, Kumar A, Bharti AC. *Anticancer Res*, **23** (2003) 363.

86. Jose JK, Kuttan G, Kuttan R. *J Ethnopharmacol,* **75** (2001) 65.

87. Sharma N, Trikha P, Athar M, Raisuddin S. *Hum Exp Toxicol*, **19** (2000) 377.

88. Selvam R, Subramanian L, Gayathri R, Angayarkanni N. *J Ethnopharmacol,* **47** (1995) 59.

89. Bhattacharya A, Chatterjee A, Ghosal S, Bhattacharya SK. *Indian J Exp Biol*, **37** (1999) 676.

90. Yu ZF, Kong LD, Chen Y. *J Ethnopharmacol*, **83** (2002) 161.

91. Englyst HN, Kingman SM, Cummings JH. *Eur J Clin Nutr*, **46** (1992) 533.

92. Trowell HC, Southgate DAT, Wolever TMS, Leeds AR, Grassull MA, Jenkins DJA. *Lancet*, **1** (1976) 967.

93. Komalavilas P, Mort AJ. *Carbohydr Res*, **189** (1989) 261.

94. O.Neill M, Albersheim P, Darvill A, **1990**. The pectic polysaccharides of primary cell wall. In: Dey.D.M. (Ed.) Methods in plant Biochemistry. Vol 2.Acdemic press. London.pp.415-441
95. Srivastava R, Kulasherstha DK. *Phytochem,* **28** (1989) 2877.
96. Yamada H. *Carbohydr Polym*, **25** (1994) 269.
97. Whistler RL, Bushway AA, Singh PP. **1976** In. Adv in cabohydr Chem and Biochem, Vol.32. Ed.Tipson, S. and Horton D. Acad Press, New York. pp235.
98. Franz G. *Planta Med*, **55** (1989) 493.
99. Wagner H. *Pure Appl Chem*, **62** (1990) 1217.
100. Yanada H, Kiyohara H, Cyong JC, Otsuka Y. *Mol Immun*, **22** (1985) 295.
101. Matsumato T, Cyong JC, Kiyohara H, Matsui H, Abe A, Gurabi M, Danbara H, Yamada H. *Int J Immunopharam*, **15** (1993) 683.
102. Yamada H, Ra KS, Hutigaram H, Cyong JC, Otsuka Y. *Carbohydr Res*, **189** (1989) 209.
103. Yamada,H, Sun XB, Matsumoto T, Ra KS, Hirano M, Kiyohara H. *Planta Med*, **57** (1991) 555.
104. Takashi M, Hazime S, Tsuyoshi N, Hirokazu K. *Food Rev Intl*, **11** (1995) 23.
105. Macfarlane GT, Macfarlane S. *Scand J Gastroenterol Suppl,* **32** (1997) 3.
106. Trowell H, Burkitt D, Heaton, **1985**. Dietary fiber – Fiber - depleted food and disease. Academic press. London.
107. Rowland IR, Gangolli SD, **1999**. Role of gastrointestinal flora in the metabolic and toxicological activities of xenobiotics. In: Ballantyne. B. Marrs. T.C, Syverson. T. (Ed.) General and

applied toxicology. Macmillan publisher Ltd. London. pp.561-576.

108. Gibson GR, Wang X. *J Appl Bacteriol,* **77** (1994) 412.
109. Mortensen E, Neilsen H, **1995**. *In vivo* and *In vitro* effects of short-chain fatty acid on intestinal blood circulation. In: Cummings. J. Rombeaus. J. Sakata T. (Ed.) Physiological and clinical aspects of short-chain fatty acids. Cambridge University press. Cambridge. pp391-400.
110. Cummings JH, **1985**. In: Trowell. H. Burkitt. D. Heaten K. (Ed.) Dietary fiber, fiber depleted foods and diseases. Academic Press. London. pp 161.
111. Cummings JH, Englyst HN. *Am J Clin Nutr*, **45** (1987) 1243.
112. Von Engelhardt W, Bartels J, Kirschberger S, Meyer zu Duttingdorf HD, Busche R. *Vet Q*, **20** (1998) 52.
113. Katoh K, **1995**. Effects of short-chain fatty acids on exocrine and endocrine pancreatic secretion. In: Cummings. J. Rombeaus. J. Sakata T. (Ed.) Physiological and clinical aspects of short-chain fatty acids. Cambridge University press. Cambridge. pp223-231.
114. Ohbo M, Katoh K, Saraki Y. *J Comp Physiol,* **13** (1996) 166.
115. Wolever T, Spaadafora P, Eshuis H. *Amer J Clin Nutr***, 53** (1991) 681.
116. Ichikawa H, Sakata T. *J Nutr,* **128** (1998) 843.
117. Mainous MR, Block EF, Deitch EA. *New Horiz*, **2** (1994) 193.
118. Leschelle X, Delpal S, Goubern M, Blottiere HM, Blachier F. *Eur J Biochem*, **267** (2000) 6435.
119. Peters SG, Pomare EW, Fisher CA. *Gut*, **33** (1992) 1249.

120. Cummings JH, Pomare EW, Branch WJ, Naylor CP, Macfarlane GT. *Gut,* **28** (1987) 1221.
121. Livesey G, Elia M, **1995**. Short chain fatty acids as an energy source in the colon: metabolism and clinical implications. In: Cummings. J. Rombeau. J. Sakata T. (Ed.) Physiological and clinical aspects of short-chain fatty acids.. Cambridge University press. Cambridge. pp427-481
122. Roediger R, **1995**. The place of short-chain fatty acids in colonocyte metabolism in health and ulcerative colitis: the impaired colonocyte barrier. In: Cummings. J. Rombeau. J. Sakata. T. (Ed.). Physiological and clinical aspects of short-chain fatty acids. Cambridge university press. Cambridge. pp337-351.
123. Gostner A, Dusel G, Kelber E, Scheppach W, Bartram HP. *Eur J Cancer Prev*, **9** (2000) 205.
124. Woolard HR. *Develop Biol*, **68** (1979) 360.
125. Waterborg JH, Mathews HR. *Eur J Biochem***, 14** (1984) 329.
126. Boffa LC, Gruss RJ, Allfrey VG. *J Biol Chem* **, 256** (1981) 9612.
127. Rastl E, Swetley P. *J Biol Chem,* **253** (1978) 4333.
128. Kruh J. *Mol Cell Biochem* ,**42** (1982) 65.
129. Hatch GM, Cao SG, Angel A. *Biochem J,* **306** (1995) 759.
130. Miller GL. *Anal Chem*, **31** (1989) 426.
131. Dahlqvist A. *Anal Biochem*, **7** (1964) 18.
132. Folin , WU. *J Biol Chem*, **38** (1919) 81.
133. Yakozawa, T, Chung, HY, He, LQ, Oura, H. *Biosci Biotech Biochem,* **60** (1996) 1000.
134. Allalouf D, Ber A, Sharon N. *Biochem Biophys Acta*, **83** (1964) 278.

135. McKelvy JF, Lee YC. *Arch Biochem Biophys*, **132** (1969) 99.

136. Dische Z. *J Biol Chem.,* **167** (1947) 189.

137. Ludowieg J, Benmanan JD. *Anal Biochem,* **19** (1967) 80.

138. Dodgson KS. *Biochem J,* **78** (1961) 312.

139. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. *J Biol Chem*, **193** (1951) 262.

140. Farndale AW, Buttle DJ, Barrett AJ. *Biochem Biophys Acta*, **883** (1986) 173.

141. John MW, Peacock EE. *Surgery*, **64** (1968) 288.

142. Scott JE. *Meth Biochem Anal,* **8** (1960) 145.

143. Mikuni-Takagaki Y, Toole BP. *J Biol Chem* **254** (1979) 8409.

144. Van de Lest CHA, Versteeg EMM, Veerkamp JH, Van Kuppevelt TH. *Anal Biochem,* **221** (1994) 356.

145. Dahlquist A. *Anal Biochem,* **22** (1968) 99.

146. Pogell GM, Gryder RMJ. *J Biol Chem,* **228** (1957) 701.

147. Kawai Y, Anno K. *Biochem Biophys Acta,* **242** (1971) 428.

148. Snedecor GW, Cocharan WG (Ed.) Statistical Methods **1976.** 6th edn. Ames. Iowa State University Press. USA. pp298.

149. Raghuramulu N, Madhavan Nair K, Kalyanasundaram S, **1983**. A manual of laboratory techniques. National Institue of Nutrition. Indian Council of Medical Research. Hyderabad. pp 246-247.

150. Brancrft J, Stevens A, **1982.** Theory and practice of histological tenchiques . 2nd (Ed). Churcill Livingstone, New York. pp 194-198.

151. Brancrft J, Stevens A, **1982**. Theory and practice of histological tenchiques., 2nd Ed. Churcill Livingstone. New York. pp188-190.

152. Zeisber M, Ericksen MB, Hamano Y, Neilson EG, Ziyadeh F, Kalluri R. *Biochem Biophys Res Commun*, **295** (2002) 401.
153. Lachman L, Libermann HA, **1991.** Semi-solid dosage forms. In: The theory and practice of Industrial pharmacy. Varghese. Bombay. pp245-259.
154. Morton, Melone MH. *Ach Int Pharmacodyn,* **176** (1972) 117.
155. Hassid WZ, Neufeld EF, *Methods Carbohydr Chem*, **4** (1964) 33.
156. Asp NG, Johansson CG, Hallmer H, Siljestrom M. *J Agric Food Chem*, **3** (1983) 476.
157. Suhasini AW, Muralikrishna G, Malleshi NG. *Food Chem*. **60** (1997) 537.
158. Paramahans SV, Tharanathan RN. *Cereal Chem,* **59** (1982) 430.
159. Selvendran R R, O'Neill MA. *Meth Biochem Anal*, **32** (1987) 25.
160. Partridge SM. *Nature*, **164** (1949) 443.
161. Trevelyan WE, Procter DP, Harrison JS. *Nature,* **166** (1950) 444.
162. Hawk PB, **1965.** Hawk's Physiological chemistry (Ed.) 14th edition, McGraw Hill. New York. pp1214.
163. Sawardekar JS, Slonekar LS, Jeanes A. *Anal Chem,* **37** (1967) 1602.
164. McGinnis GD, Fang P. *Methods Carbohydr Chem*. **8** (1980) 33
165. Siddiqui IR, Wood PJ. *Carbohydr Res,* **16** (1971) 452.
166. Anderson DMW, Stoddart JF, *Carbohydr Res*, **2** (1966) 104.
167. Anderson DMW, Hendrie A, Millar JRA, Munro AC. *Analyst,* **96** (1971) 870.
168. Taylor RL, Conrad HE. *Biochem,* **11** (1972) 1383.
169. Hakomori SI. *J Biochem*, **55** (1964) 205.

170. Waeghe TJ, Darvill AG, McNeil M, Albersheim P. *Carbohydr Res*, **123** (1983) 281.
171. Jansson PE, Kenne L, Leidgren H, Lindberg B, Longren J. *Univ. Stockholm Chem Commun*, **8** (1976) 1.
172. Fleury PF, Lange J. *J Pharm Chem,* **17** (1933) 196.
173. Brown, F, Hallsall TG, Hirst EL, Jones JKN. *J Chem Soc,* **1** (1948) 27.
174. Abdel-Akher M, Hamilton JK, Montgomery R, Smith F. *J Amer Chem Soc,* **74** (1952) 4970.
175. Kacurakova M, Ebringerova A, Hirsch J, Hromadkova Z. *J Sci Food Agric,* **66** (1994) 423.
176. Dill K, Bermon E, Pavia AA. *Adv Carbohydr Chem Biochem*, **43** (1985) 1.
177. Ayumi H, Masaptsune M, Seiichi H. *Food Sci Technol Res*, **5** (1999) 74.
178. Nordkvist E, Salomonsso A, Aman P. *J Sci Food Agric*, **35** (1998) 657.
179. Singleton VL, Joseph AR. *Am J Enol Vitic,* **16** (1965) 144.
180. Lih-Shiuh L, Su-Tze C, Wen-Waw C. *J Agric Food chem,* **49** (2001) 963.
181. Yen GC, Chen HY. *J Agric Food Chem*, **43** (1995) 27.
182. Rodriguez H, Akman S. *Free Rad Res,* **29** (1998) 499.
183. Nandini CD, Sambaiah K, Salimath PV. *Nutr Res*, **20** (2000) 1301.
184. Plaami SP. *Food Rev Intl,* **13** (1997) 29.
185. Khatter R, Mehan SP, Joshi UM, Gujral SJ. *Arogya*, **16** (1990) 20.

186. Smith DC, Hibito R, Barnett M, Collier GR. *J Nutr,* **127** (1997) 359.
187. Bourquin LD, Titgemeyer EC, Garleb KA, Fahey GC. J Nutr **122**(1992) 1508.
188. Cummings JH, Macfarlane GT. *J Appl Bacteriol,* **70** (1991) 443.
189. Chethankumar M, Rachappaji K, Nandini CD, Sambaiah K, Salimath PV. *J Nutr Biochem,* **13** (2002) 522.
190. Olsen WA, Rogers L. *J Lab Clin Med,* **27** (1971) 838.
191. Arvanitakis C, Olsen WA. *Gastroenterology,* **64** (1973) 854.
192. Taylor DG, Price RG, Robinson D. *Biochim Biophys Acta,* **273** (1972) 237.
193. Yukisumi I, Eiichi S, Noburu H, Hiroshi N. *Nippon Yakuri gaku Zasshi*, **94** (1989) 129.
194. Irwin EV, Fred Kern JR, Karl ES. *J Lab Clin Med,* **66** (1965) 131.
195. Felix O, Zahola A. *Ann Nutr Metab,* **37** (1993) 14.
196. Khokhar S. *J Nutr Biochem,* **5** (1994) 176.
197. Elsenhans B, Sufke U, Blume R, Caspary WF. *Clin Sci,* **59** (1980) 373.
198. Sakata T, Yajima T. *Q J Exp Phys,* **69** (1984) 639.
199. Chethankumar M. Salimath PV, Sambaiah K. *Nahrung,* **46** (2002) 345.
200. Zhang H, Osada K, Sone H, Farukawa Y. *J Nutr Sci Vitaminol,* **43** (1997) 271.
201. Reddi AS. *Clin Chim Acta,* **189** (1990) 211.
202. Kjellen L, Bielefeld D, Hook M. *Diabetes,* **32** (1983) 337.
203. Malathy K, Kurup PA. *Diabetes* **21** (1972) 1162.

204. Mohanam S, Suda M. *Acta Diabetol Lat,* **21** (1984) 203.

205. Hikino M, Mikami T, Faissner A, Vilela-Silva AC, Pavao MS, Sugahara K. *J Biol Chem,* **278** (2003) 43744.

206. Deepa SS, Umehara Y, Higashiyama S, Itoh N, Sugahara K. *J Biol Chem* **277** (2002) 43707.

207. Nandini CD, Sambaiah K, Salimath PV. *J Nutr Biochem,* **14** (2003) 203.

208. Poole RA. *Biochem J,* **236** (1986) 1.

209. Sharon N, Lis H. *Chem Eng News,* **59** (1981) 21.

210. Srinivasan SR, Berenson GS, Radhakrishnamurthy B. *Diabetes,* **19** (1970) 171.

211. Shimomura H, Spiro RG. *Diabetes,* **36** (1987) 374.

212. Salmivirta M, Lindholt K, Lindahl U. *FASEB J*, **10** (1996) 1270.

213. Varki A. *Proc Natl Acad,* **91** (1994) 7390.

214. Vijayagopal P, Srinivasan SR, Radhakrishnamurthry B. Berenson GS. *J Biol Chem,* **256** (1981) 8234.

215. Premkumari K, Kurup PA. *Ind J Biochem Biophys*, **20** (1983) 163.

216. Sartor G, Carlstrom S. *Acta Med Scand*, **656** (1981) 51.

217. Royall D, Wolever TM Jeejeebhoy KN. *Am J Gastroenterol,* **85** (1990) 1307.

218. Ritz E, Stefanski A. *Am J Kidney Dis,* **27** (1996) 167.

219. Hasslacher C, Ritz E, Whahl P, Michael C. *Nepphrol Dialysis Transpl,* **9** (1994) 1097.

220. Anderson JW, Geil GB, **1994.** In: Shills. M.E, Olson. J.A. Shike. M.A. (Ed.) Modern Nutrition in health and disease. 8th edition. Lea and Febiger. Philadelphia. USA. pp1259.

221. Christiansen JS, Gammelagaard J, Frandsen M, Parving HH. *Diabetologia*, **20** (1981) 451.
222. Seyer-Hansen K. *Clin Sci Mol Med,* **51** (1976) 551.
223. Spiro RG. *Diabetologia,* **12** (1976) 1.
224. Berkow R, **1982**. (Ed.)The Merck manual of diagnosis and therapy) 14th edition. Merch Sharp and Dahme Research Laboratories. USA.
225. Stackhouse S, Miller PL, Park SK, Meyer TW. *Diabetes*, **39** (1990) 989.
226. Nerlich AG, Sauer U, Kolmitty V, Wagner E, Koch M, Schleicher ED. *Diabetes,* **47** (1998) 170.
227. Spiro MJ. *Diabetologia,* **26** (1984) 70.
228. Serrano MA, Cabezas JA, Reglero A. *Comp Biochem Physiol*, **80** (1985) 629.
229. Fushimi H, Tarui S. *J Biochem,* **79** (1976) 265.
230. Rao GM. *Biomed,* **35** (1981) 159.
231. Bose MS, Sudha M. *Acta Diabetol Lat,* **21** (1984) 203.
232. Vijaykumar ST, Kurup PA. *Atherosclerosis*, **21** (1995) 245.
233. Kumari SKS, Devi KS. *Ind J Exp Biol,* **31** (1993) 595.
234. Kasinath BS. *Arch Biochem Biophys*, **318** (1995) 286.
235. Gupta D, Raju J, Baquer NZ. *Ind J Exp Biol,* **37** (1999) 196.
236. Rohrbach DH, Wagner CW, Star VL, Martin GR, Brown KS, Yoon JW. *J Biol Chem,* **258** (1983) 11672.
237. Rathi AN, Vishwanathan A, Shanmugasundaram KR. *Ind J Exp Biol,* **19** (1981) 715.
238. Menon PVG, Kurup PA. *Artery*, **2** (1976) 219.
239. Menon PVG, Kurup PA. *J Nutr*, **106** (1976) 555.

240. Scandling JD, Myers BD. *Kidney Int,* **41** (1992) 840.
241. Deen, WM, Bridges CR., Brenner BM, Myers BD. *Am J Physiol*, **249** (1985) 374.
242. Farqshar MG, **1964**. In: Siperstein. M.D, Colwell. A.R, Sr., Meyer K. (Ed) Small blood vessel involvement in diabetes mellitus. American institue of biological science. Washington. pp31.
243. Farqeshar MG, Lemkin MC, Srow JL, **1985.** Role of proteoglycans In: glomerular function and pathology in nephrology. Ed. Robinson RR. New York. Springer-Verlag. pp580.
244. Kanwar YS, Liu ZZ, Kashihara N, Wallner EI. *Semin Nephrol,* **11** (1991) 390.
245. Kanwar YS, Farquhar MG. *J Cell Biol,* **81** (1979) 137.
246. Parthasarathy N, Spiro RG. *J Biol Chem***, 256** (1981) 507.
247. Saraswati S, Vasan NS. *Biochem Biophys Acta*, **755** (1983) 237.
248. Kanwar YS, Hascall VC, Farquhar MG. *J Cell Biol,* **90** (1981) 527.
249. Mahdi GS, Naismith DJ, Price RG, Taylor SA, Risteli J, Risteli L. *Ann Nutr Metab,* **38** (1994) 61.
250. Lee Stanley M. *Diabetologia*, **22** (1982) 349.
251. Kubosawa H, Kondo Y. *Nephron,* **76** (1997) 445.
252. Yavuz DG, Ersoz O, Kucukkaya B, Budak Y, Ahiskali R, Ekicioglu G, Emerk K, Akalin S. *J Hypertens*, **17** (1999) 1217.
253. Lavik OJ, Aukland K. *Ren Physiol,* **8** (1985) 136.
254. Lopes CV, Pereira-Lima JC, Hartmann AA. *Histopathology,* **45** (2004) 198.

255. Artico M, De Santis S, Tranquilli Leali FM, Cavallotti D, Celestini A, Cavallotti C. *J Diabetes Complications*, **18** (2004) 119.

256. Kimmelstiel P, Kim O.J, Beres J, *Am J Clin Pathol,* **38** (1962) 280.

257. Paul JB, Robert GS. *Science,* **168** (1970) 596.

258. Piez KA. *Ann Rev Biochem,* **37** (1968) 547.

259. Ziyadeh FN, Sharma K, Ericksen M, Wolf GJ. *Clin Invest,* **93** (1994) 536.

260. Holloway WD, Tasman-Jones C, Lee SP. *Am J Clin Nutr,* **31** (1978) 927

261. Holloway WD, Tasman-Jones C, Maher K. *Am J Clin Nutr,* **37** (1983) 253.

262. Vanderhoof JA. *Nutr*, **14** (1998) 595.

263. Cummings JH, Trowel H, Burkitt D, Heaton K, (Ed.) **1985**. In: Dietary fibre, Fibre depleted foods and diseases, Academic Press. London. pp. 161.

264. Shah S, Lance P, Smith TJ, Berenson SN, Cohen SA, Horvath, PJ, Lau TTY, Baumann H. *J Biol Chem,* **267**(1992) 10652.

265. Jocobsson KG, Reisenfeld J, Lindahl U. *J Biol Chem*, **260** (1985) 12154.

266. Juan AR, Samuel EA. *Biochem Biophys Res Commun,* **248** (1998) 664.

267. Karlsen AE, Fijimoto WY, Rabinovitch P, Dube S, Lernmark A. *J Biol Chem,* **266** (1991) 7542.

268. Pedersen O, Hjollund E, Lindskov HO, Helms P, Sorensen NS, Ditzel J. *Diabetes care,* **5** (1982) 284.

269. Nandini CD, Salimath PV. *Food Chem*, **73** (2001) 197.

270. Swamy NR, Ramakrishnaiah N, Kurien PP, Salimath PV. *J Sci Food Agric,* **57** (1991) 379.

271. Fry SC. *J Exper Botany,* **40** (1989) 1.

272. Wang SL, Zao YL, Li XY, Li YC, Li H. *Acta Botanica Yunnanica,* **18** (1996) 345.

273. Gonda R, Tomoda M, Ohara N, Takada K. *Biol Pharm Bull,* **16** (1993) 235.

274. Gonda R, Tomoda M, Takada K, Ohara N, Shimizu N. *Chem Pharm Bull*, **40** (1992) 990.

275. Gonda R, Tomoda M, Shimizu N, Kanari M. *Chem Pharm Bull,* **38 (**1990) 482.

276. Swanston-Flatt SK, Flatt PR, Day C, Bailey CJ. *Proc Nutr Soc,* **50** (1991) 641.

277. Grover JK, Yadav S, Vats V. *J Ethnopharmacol*, **81 (**2002) 81.

278. Oubre AY, Carlson TJ, King SR, Reaven GM. *Diabetolgia,* **40** (1997) 614.

279. Satyavati GV, Raina MK, Sharma M **1976**. Medicinal Plants of India. New Delhi: Indian Council of Medical Research.

280. Thakur CP. *Experientia*, 41 (1985) 423.

281. Ramesh HP, Tharanathan RN. *Carbohydr Res,* **308** (1998) 239.

282. Liang C, Paul K K. *Carbohydr Res*, **301** (1997) 205.

283. Swamy NR, Salimath P V, *Carbohydr Res*. **197** (1990) 327.

284. Aspinall GO, **1980**. Chemistry of cell wall polysaccharides In: Priess. J. (Ed) The Biochemistry of plant. Academic press. New York. Vol 3. pp.473-500.

285. Chindambara Murthy KN, Vittal Reddy K, Jyothi M, Veigas, Uma D Murthy. *J Med Food,* **7** (2004) 256.
286. Haynes E, Tomokins G, Washburn, Winters M. *Proc Soc Exptl Biol Med,* **36** (1937) 839.
287. Dick AJ, Labauitch JM. *Plant Physiol*, **89** (1989) 1394.
288. Fares K, Renard CMGC, R'zina Q, Thiboult JF. *Int J Food Sci Technol,* (2001) 36.
289. Cheng L and Kindel PK. *Anal Biochem,* **228** (1995) 109.
290. Renard CMCG, Thibault JF. C*arbohydrate Res*, **244** (1993) 99.
291. McNeil M, Darvill AG, Fry S, Albersheim P. *Plant Physiol*, **66** (1980) 1128.
292. Kikuchi A, Satoh S, Fujii T. *Biosci Biotech Biochem,* **56** (1992) 1144.
293. Redgwell RJ, Melton L, Melton LD, Brasch DJ. *Plant Physiol,* **98** (1992) 71.
294. Jimenez A, Rodriguez R, Fernandez-caro I, Guillen R, Fernandez-Bolanos J, Heredia A. *J Agric Food Chem,* **49** (2001) 409.
295. Huisman MMH, Schols HA, Voragen AGJ. *Carbohydr Polym,* **31** (1996) 123.
296. Saulnier L, Brillouet JM, Joseleau JP. *Carbohydr Res,* **181** (1988) 63.
297. Schols HA, Posthumus MA, Voragen AGJ. *Carbohydr Res*, **206** (1997) 117.
298. Pressey R, Himmelsbach DS. *Carbohydr Res,* **127** (1984) 356.
299. Nunan KJ, Sims IM, Bacic A, Robinson SP, Fincher GB. *Plant Physiol* **118** (1998) 783.

300. Seymour GB, Colquhoun IJ, Dupont MS, Passley KR, Selvendran RR. *Phytochem*, **26** (1990) 1871.

301. Tharanathan RN, Reddy CG, Murlikrishna G, Susheelamma NS. *Carbohydr Polym*, **23** (1994) 121.

302. Vidal S, Williams P, O'Neill MA, Pellerin P. *Carbohydr Polym,* **45** (2001) 315.

303. De Vries JA, Voragen AGJ, Rombouts FM, Pilnik W. *Carbohydr Polym* **3** (1983) 193.

304. Kertesz ZI **1951**.The pectic substances, Interscience Publishers Inc. New York

305. Pilnik W, Voragen AGJ, **1970.** Pectic substances and other uronides In: Hulme. A.C (Ed.) The Biochemistry of fruits and their products. Vol 1 Academic press. London. pp 53.

306. Woolard GR, Novellie L, Vander walt. *Cereal Chem*, **53** (1976) 601.

307. Davis EA, Derouet C, Du Penhoat CH. *Carbohydr Res*, **197** (1990) 205.

308. Keenan MHJ, Belton PS, Matthew JA, Howson SJ. *Carbohydr Res*, **138** (1985) 168.

309. Bushneva OA, Ovodova RG, Shashkov AS, Ovodov YS. *Carbohydr Polymer,* **49** (2002) 471.

310. Polle AY, Ovodova RG, Shashkov YS. *Carbohydr Polym,* **49** (2002) 337.

311. Stewart D, Iannetta PP, Davies HV. *Phytochem*, **56** (2001) 423.

312. Andrzej T, Romana A, Iwona W, Tadeusz MK, Boguslawa PB, Magdalena R. *Carbohydr Res*, **334** (2001) 71.

313. Coimpra MA, Barros A, Rutledge DN, Delgadillo I. *Carbohydr Res,* **317** (1999) 145.

314. Coimbra MA, Barros A, Barros M, Rutledge DN, Delgadillo I. *Carbohydr Polym,* **37** (1998) 241.

315. Math IM, Kornig JL. *Adv* Carbohydr *Chem Biochem*, **44** (1986) 7.

316. Kacurakova M, Capek P, Sasinkova V, Wellner N, Ebringerova A. *Carbohydr Polym,* **43** (2000) 195.

317. Ames BM, Shigenaga MK, Hagen TM. *Proc Natl Acad Sci,* **90** (1993) 7915.

318. Hertog MG, Feskens EJ, Hollman PC, Katan MB, Kromhout D. *Lancet,* **342** (1993) 1007.

319. Croft KD. *Ann N Y Acad Sci,* **854** (1998) 435.

320. Formica JV, Regelson W. *Food Chem Toxicol,* **33 (**1995) 1061.

321. Maillard MN, Soum MH, Boivia P, Berset C. *Lebensm-wiss Technol,* **3** (1996) 238.

322. Galli RL, Shukitt-Hale B, Youdim KA, Joseph JA. *Ann N Y Acad Sci,* **959** (2002) 128.

323. Middleton E, Kanakaswami C, Theoharides TC. *Pharmacol Rev,* **52** (2000) 673.

324. Ferguson CR. *Muta Res,* **475** (2001) 89.

325. Thakur CP, Thakur B, Singh S, Sinha PK, Sinha SK. *Indian J Cardiol*, **21** (1988) 167.

326. Nagabhushan M. Mutagens, **1988**. Antimutagens from Indian diet with special reference to spices. *PhD, thesis University of Bombay, India*.pp1-308.

327. Nagabhushan M, Amonkar AJ, Bhide SV. *Food Chem Toxicol,* **25** (1987) 545.

328. Nagabhushan M, Amonkar AJ, De'Souza AV, Bhide SV. *Indian Drugs,* **25** (1987) 91.

329. Frei B, England L, Ames BN. *Proc Natl Acad Sci,* **86 (**1989) 6377.

330. Nagabhushan M, Bhide SV. *J Am Coll Nutr,* **11** (1992) 192.

331. Nagabhushan M, Bhide SV. *J Nutr Growth Cancer,* **4** (1987) 83.

332. Ojha JK, **1985**. In: Chyvanaprash a Scientific Study, Ist edn. Indigenous drugs of India, VN Dhar & Sons. Published . Tara Books Agency, Varanasi, India, pp506.

333. Fraizer RA, Papadopoulou A, Mueller-Harvey I, Kissoon D, Green RJ. *J Agric Food Chem,* **51** (2003) 5189.

334. Wroblewski K, Muhandiram R, Chakrabartty A, Bennick A. *Eur J Biochem,* **268** (2001) 4384.

335. Khopde SM, Indira PK, Mohan H, Gawandi VB, Satav JG, Yakhmi JV, Banavaliker MM, Biyani MK, Mittal JP. *Current Science*, **81** (2001) 185

336. Duke James A, **1992**. Handbook of phytochemical constituents of GRAS herbs and other economic plants. Boca Raton, FL. CRC Press.

337. Fulcher RG, **1986**. Morphological and chemical organization of the oat kernel. In: Webster. (F.H. Ed.) Oat chemistry and Technology. American Association of Cereal Chemists. St. Paul. Chap 3.

338. Kroon PA, Faulds CB, Ryden P, Robertson JA, Williamson G. *J Agric Food Chem*, **45** (1997) 661.

339. Kroon PA, Williamson G. *J Sci Food Agric*, **79** (1999) 355.

340. Buchanam CJ, Wallace G, Fry SC, Eastwood MC. *J Sci Food Agric*, **71 (**1996) 459.

341. Andreasen MF, Chriistensen, LP, Meyer AS, Hansen A. *J Agric Food Chem,* **48** (2000) 2837.

342. Hagerman HE, Riedl KM, Jones A, Sovik KN, Ritchard NT, Hartzfeld BW. *J Agric Food Chem,* 46 (1998) 1887.

343. Ohta T, Semgboku N, Kuchi A, Egashira Y, Sanada H. *J Agric Food Chem,* **45** (1997) 1664.

344. Ursini F, Tubaro F, Rong J, Sevanian A. *Nutr Rev,* **57** (1999) 241.

345. Bravo L. *Nutr Rev,* **56** (1998) 317.

346. Slavin JL, Martini MC, Jacobs DR, Marquart L. *Am J Clin Nutr*, **70**, (1999) 459.

347. Ferguson LR, Harris PJ. *Eur J Cancer Prev*, **8** (1999) 17.

348. Zhu M, Phillipson JD, Greengrass PM, Bowery NE, Cai Y. *Phytochem*, **44** (1997) 441.

349. Kamal-Eldin A, Frank J, Razdan A, Tengblad S, Basu S, Vessby B. *Lipids*, **35** (2000) 427.

350. Chesson A, Provan GJ, Russell WR, Scobbie L, Richardson AJ, Stewart C. *J Sci Food Agric*, **79** (1999) 373.