STUDIES ON FERMENTATION OF DIETARY FIBERS TO SHORT CHAIN FATTY ACIDS AND EFFECT OF BUTYRIC ACID ON EXPERIMENTALLY INDUCED DIABETIC RATS

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in

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By

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Dedicated to

Parents,

Relatives,

Teachers & Friends…

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DECLARATION

I hereby declare that the thesis entitled "Studies on fermentation of dietary fibers to short chain fatty acids and effect of butyric acid on experimentally induced diabetic rats" submitted to the University of Mysore for the award of the degree of Doctor of Philosophy in Biochemistry is the results of research work carried out by me under the guidance of Dr. P.V. Salimath, Head, Department of Biochemistry & Nutrition, Central Food Technological Research Institute, Mysore during the period 2000-2007.

I further declare that these results have not been submitted for any other degree or fellowship.

Place: Mysore (K.S. Rachappaji)
Date: 10-12-2007

DATE: 10 -12-2007

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CERTIFICATE

This is to certify that the thesis entitled "Studies on fermentation of dietary fibers to short chain fatty acids and effect of butyric acid on experimentally induced diabetic rats" submitted by Mr. K.S. Rachappaji for the award of the Degree of Doctor of Philosophy in Biochemistry, to the University of Mysore is the result of research work carried out by him in the Department of Biochemistry and Nutrition under my guidance during the period 2000-2007.

(Dr. P.V. Salimath)
Guide

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K.S. Rachappaji

CONTENTS

LIST OF SYMBOLS AND ABBREVIATIONS

DM	Diabetes mellitus	mL	Milliliter (s)
SCFA	Short chain fatty acid	L	Liter (s)
DF	Dietary fiber	$[\alpha]_D$	Specific rotation
AA	Acetic acid	min	Minutes (s)
BA	Butyric acid	hr	Hour (s)
PA	Propionic acid	TFA	Trifluroacetic acids
SEM	Standard error of means	N	Normal
GAG	Glycosaminoglycan	TCA	Trichloroacetic acids
GBM	Glomerular basement membrane	M	Molar
		HCl	Hydrochloric acid
DMMB	1, 9- Dimethylmethylene blue	mM	Millimolar
		H_2SO_4	Sulphuric acid
Ucr	Urine creatinine	eV	Electron volts
C-4-SO_4	Chondroitin sulphate	meq	Millieqivalents
%	Percent	V	Volts
^oC	Degree celcius	v/v	Volume/volume
dil	Dilute	w/v	Weight/volume
h	hours	nm	Nanometer
k Da	Kilo Dalton	rpm	Revolutions per minute
OD	Optical Density	PC	Paper chromatography
RI	Refractive index	HPLC	High performance liquid chromatography
TR	Retention time		
RT	Room Temperature	Rha	Rhamnose
Ve/Vo	Elution volume/Void volume	Fuc	Fucose
		Ara	Arabinose
µg	Micrograms (s)	Xyl	Xylose
mg	Milligram (s)	Man	Mannose
g	Gram (s)	Gal	Galactose
mm	Millimeter (s)	Glc	Glucose
cm	Centimeter (s	GlcA	Glucuronic acid
µL	Microliter (s)	AX	Arabinoxylans

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SYNOPSIS

Diabetes mellitus is the major metabolic disorder that affects around 30 million people all over the world. Main characteristic feature of this disorder is high blood sugar level (hyperglycemia) and alteration of carbohydrate, lipid, and protein metabolism. Long duration of uncontrolled blood glucose leads to major complications such as diabetic nephropathy, neuropathy and retinopathy. Among them diabetic nephropathy is a cause of the serious concern. It affects more than one-third of patients with type I diabetes and an ever-increasing proportion of patients with type II diabetes. During diabetic nephropathy, kidney basement membrane becomes thicker making glomeruli more porous to the macromolecules. Analysis of GBM has shown alteration of basement membrane components with decreased heparan sulphate content and laminin and increased type IV collagen.

Diabetes therapy aims at controlling blood glucose to normal levels and control complications of diabetes. Drug therapy includes glucose lowering agents as well as medications in order to treat or prevent secondary complications of the disease. However, nutrition is the cornerstone of diabetes care. Hence, the goal of nutritional management is optimal metabolic control through diet. In this direction, many dietary components are being used to control diabetes and subsequently delay the diabetic complications. One such dietary component is dietary fiber, which is well established for their beneficial effects on diabetes. *Moringa oleifera* commonly known as drumstick and *Syzygium jambolana* commonly called as jamun are rich sources of dietary fiber. The present investigation was aimed to study the effects of these plant materials on diabetic nephropathy status with particular emphasis on kidney heparan sulphate.

Beneficial role of dietary fiber during diabetes is brought about either by acting as insoluble matrix thereby facilitating slow absorption of glucose or through their fermented products. Dietary fiber fermentation leads to production of short chain fatty acid and is receiving a lot of interest in recent years. Butyric acid, one of the short chain fatty acids is receiving much attention due to its role on many physiological processes. Many butyric acid derivatives are under clinical trails for their role in disease conditions.

Even though, butyric acid modulates different physiological conditions in many diseases, its beneficial effect on diabetes is not well understood. Hence, antidiabetic property of butyric acid and butyric acid producing ability of dietary fibers and their sources needs to be evaluated.

Various studies have shown that diabetes mellitus is associated with increased formation of free radicals and decrease in antioxidant potential. Due to these events, the balance normally present in cells between radical formation and protection against them is disturbed. In both insulin-dependent (Type I) and non-insulin-dependent diabetes (Type II), there is increased oxidative stress. Plant polysaccharides are gaining lot of interest for their biological role such as antioxidant, anti-cancer, anti-ulcer, anti-diabetic, anti-microbial and immunomodulatory activities. Hence, the investigation was carried out on;

1. Effect of butyric acid on experimentally induced diabetic rats.
2. *In vivo* and *In vitro* fermentation of dietary fibres to short chain fatty acids.
3. Effect of *Moringa oleifera* (drumstick) pulp and *Syzygium jambolana* (jamun) seed on diabetic and diabetic nephropathy state with particular emphasis on heparan sulphate.
4. Carbohydrate profile of *Syzygium jambolana* and study of phenolic acids and their antioxidant potential.

The thesis is presented in 4 chapters;

I General Introduction, II. Materials and Method, III. Results and Discussion and IV. Bibliography.

Chapter I: It deals with general introduction on diabetes, diabetic nephropathy, glomerular basement membrane thickening during diabetes, including involvement of heparan sulphate, dietary management, dietary fiber fermentation and short chain fatty acids. Importance of drumstick and jamun on diabetes has been brought out. Biological activities of non-starch polysaccharides and phenolic acids in diabetes have been evaluated. These aspects are cited with proper literature. The first chapter ends with the scope of present investigation.

Chapter II: This chapter deals with Materials and Methods that are employed during this investigation. Diet preparation, diabetes induction, dietary treatment, management of animals, collection of biological samples and analysis of samples for nutritional studies (animal experiments) are described. Mixed and pure culture preparation, substrate preparation, media preparation, incubation (both aerobic and anaerobic), short-chain fatty acid isolation and analysis were employed in microbiology studies. Isolation of polysaccharides, analyses of sugars, biological activity employed are detailed

Chapter III: This chapter details the results obtained and are discussed with appropriate references. This is divided into four sub-sections. Each sub-section has a brief introduction. The results obtained are detailed followed by discussion of the results and concludes with conclusions.

Investigations were carried out on;

III.1. Effect of butyric acid on experimentally induced diabetic rats

Butyric acid, one of dietary fiber fermentation byproduct is examined for antidiabetic property in streptozotocin induced diabetic rats. Diabetes was induced with STZ at 55 mg/kg body weight and animals were given butyric acid at different dosages (250, 500 and 750 mg/kg body weight). Diabetic status was assessed by measuring food intake, water intake, urine sugar, urine volume, body weight and fasting blood sugar. During diabetes there was a significant increase in urine volume, urine sugar and fasting blood glucose. Dietary fibres given at wheat bran (5%) and guar gum (2.5%) along with butyric acid at 500 mg/kg body weight showed maximum beneficial effect on diabetes and diabetic nephropathy state.

III.2. *In vivo* and *in vitro* fermentation of dietary fibres to short chain fatty acids

This section deals with the studies on fermentation of dietary fibers to short chain fatty acids. Faecal and caecal microbes are used for aerobic and anaerobic *in vitro* degradation of different dietary fiber sources. Substrate concentration and incubation

time were standardized in the beginning. Incubation for 48 h and 1% substrate concentration were better in production of butyric acid and used for further experiments. Fermentation of different sugars yielded different SCFA profile and among them rhamnose was propiogenic in nature. Among the polysaccharides tested, hemicelluloses proved to have butyrogenic property. Microbes isolated from butyric acid-treated groups were tested for their butyrogenic proeprty. Among them, isolate number 3 showed better butyric acid producing property among the substrates studied. However, wheat bran and spent turmeric rich in insoluble dietary fibers were found to be best butyrate producing substrates. Fermentation of fenugreek, bitter gourd and drumstick with faecal and cercal mixed culture produced different profile of SCFA indicating that microbial profile could vary with sources (faecal/ caecal/ intestine). Some of the dietary fiber rich sources were tested for butyric acid producing ability by using rat faecal and caecal mixed cultures**.** Both soluble and insoluble dietary fibers were studied for their butyrogenic properties by *in vivo* and *in vitro* experiments. *In vitro* fermentation of wheat bran was butyrogenic in nature, whereas guar gum was propiogenic in nature. In *in vivo* study, higher faecal out put was noticed in wheat bran-fed group compared to guar gum-fed group.

III.3. Effect of *Moringa oleifera* (drumstick) pulp and *Syzygium jambolana* (jamun) seed on diabetic and diabetic nephropathy state with particular emphasis on heparan sulphate

Drumstick, a commonly consumed vegetable and jamun, a tropical fruit material were examined. 10% of drumstick and 2.5% of seed powder were incorporated to AIN diet and animals were maintained for 7-8 weeks. Diabetic symptoms such as polyphagia, polydipsia, polyuria and body weight were studied on weekly basis. Both plant materials were found effective in improving diabetic symptoms. Diabetic nephropathy status was assessed by measuring glomerular filtration rate (GFR) in terms of creatinine clearance. Both drumstick pulp and jamun seeds controlled these parameters. Comparatively, jamun was found to be better.

Activities of disaccharidases such as maltase, sucrase and lactase were increased in the intestine during diabetes. These were alleviated to various extents by both

drumstick pulp and jamun seed. All the three enzyme activities were decreased in kidney during diabetes and were controlled by both drumstick pulp and jamun seed in the diet.

Improved GFR by feeding both the plant materials indicated their beneficial effect on kidney. Activities of enzymes involved in glycosaminoglycan-synthesizing and degrading enzymes such as β-glucuronidase, N-acetyl glucosaminidase (NAG) and glutamine fructose-6-phosphate amino transferase (GFAT) were studied. Activities of GFAT and NAG that increased during diabetes were ameliorated to various extents by drumstick pulp and jamun seed in the diet. β-glucuronidase, which showed marginal decrease during diabetes, was ameliorated by the presence of drumstick pulp and jamun seed in the diet.

Kidney tissues were examined for their total carbohydrates, total protein, uronic acid, sulphate and amino sugar. All the above-mentioned components were increased in diabetic animals and were decreased to various extents by the feeding of drumstick pulp and jamun seed. To study the effect of drumstick pulp and jamun seed on kidney GAG, kidney tissues were pooled and kept for drying in acetone for a month. Isolated glycosaminoglycans (GAGs) were analyzed for their components such as total sulfated GAGs, total sugar, uronic acid, sulphate content and amino sugar. Total GAG in SFD decreased which was controlled by feeding drumstick pulp and jamun in the diet. Total sugar, uronic acid, amino sugar increased during diabetes (SFD) and were prevented to various extents by drumstick pulp and jamun in the diet. The decreased sulfate content indicated decreased GAG and both drumstick pulp and jamun seed in the diet significantly controlled it. Further, total GAGs were fractionated to heparan sulfate and chondroitin sulfate by their sensitivity to chondroitinase ABC. Presence of different GAGs was revealed based on their mobility on agarose gel following electrophoresis. Heparan sulfate was the major GAG present, followed by chondoitin sulfate, which was in a minor quantity. Results clearly indicated that decreased heparan sulfate content during diabetic nephropathy was ameliorated by incorporating drumstick powder at 10% (w/w) level and jamun at 2.5% (w/w) level.

III.4. Carbohydrate profile of *Syzygium jambolana* and study of phenolic acids and their antioxidant potential

This section deals with isolation and carbohydrate profile of non-starch polysaccharides from *Syzygium jambolana* and their antioxidant activity. Non-starch polysaccharides such as water-soluble, pectic, hemicellulose A, hemicellulose B and AIR were isolated sequentially and their sugar profiles were identified by GLC. Water-soluble polysaccharides are rich in arabinose and glucose, whereas, pectic polysaccharides were rich in arabinose and galactose. Both Hem A and Hem B of seed were found to have higher amounts of glucose indicating them to be β-glucan type of polysaccharides. Seed coat hemicelluloses contained higher amount of xylose and glucose indicating them to be xylan or xyloglucan type of polysaccharides.

Anti-oxidant activity of water-soluble polysaccharides, pectic, Hem A and Hem B were studied using reducing power ability and free radical scavenging activity. Total phenolic acid content was found to be higher in water-soluble poysaccharide and pectic polysaccharides. Results clearly showed that both water soluble and pectic polysaccharides are good antioxidants with respect to DPPH assay and reducing power. Content of free and bound phenolics of *Syzygium jambolana* seed, seed coat and pulp were separated and identified by HPLC. Gallic acid was found to be major phenolic acid present in bound form in seed and pulp.

Chapter IV: The thesis concludes with a collective bibliographic citation of all the chapters.

1. GENERAL INTRODUCTION

Diabetes mellitus, a major health problem, is responsible for substantial personal and economical costs and has become the main public health challenge for the 21st century (Zimmet, 2000). The past two decades have seen an explosive increase in the number of people diagnosed with diabetes worldwide (Amos et al. 1997; King, 1998). An estimated 30 million people worldwide had diabetes in 1985. By 1995, this number had shot up to 135 million. The latest WHO estimate for the number of people with diabetes, worldwide, in 2000 is 177 million. This is likely to increase to about 300 million by 2025. Diabetes and its complications cost an estimated $132 billion annually in the United States alone in terms of healthcare costs and lost productivity. Diabetes comes from a Greek word that means to siphon. The most obvious sign of diabetes is excessive urination. Water passes through the body of a person with diabetes as if it were being siphoned from the mouth through the urinary system out of the body. Mellitus comes from a Latin word that means sweet like honey. The urine of a person with diabetes contains extra sugar (glucose). In 1679, a physician tasted the urine of a person with diabetes and described it as sweet like honey. Diabetes mellitus is an ancient disease, earliest description of its symptoms is found in the Ebers Papyrus Egypt, dating back to 1500 B.C. In the second century AD Aretaeus of Cappa, named and described the disease 'diabetes'. In 1889 Oscar Minkowski and Baron Joseph von Mering, working in Strasbourg sought to determine the pancreas was essential to life.

Biochemical definition

Diabetes is a heterogeneous group of disorders characterized by high blood glucose levels (American Diabetes Association, 2001). This disorder is caused by insufficient or lack of production of insulin (a hormone) by the pancreas (a gland

in the abdomen). Insulin is responsible for absorbing glucose (a simple sugar) into the blood stream, where it is available for body cells to use for growth and energy.

Classifications of diabetes

The 1980 Expert Committee proposed two major classes of diabetes mellitus and named them, IDDM or Type I, and NIDDM or Type II.

Type I diabetes

Also known as insulin-dependent diabetes mellitus (IDDM). About 15% of diabetic patients suffer from this type and most often seen in children or young adults. Although this disorder can appear at any age, type I diabetes occurs when the body produces little or no insulin. Usually the cause of this type of diabetes is not known, but it can sometimes be due to a viral infection, injury of the pancreas or an immune system disorder.

Type II diabetes

Also known as non-insulin-dependent diabetes mellitus (NIDDM), it is the most common type of diabetes and about 90-95% of people with diabetes have this type. In type II diabetes the pancreas usually will be producing some insulin, but for some reason, the body cannot use the insulin effectively. It is most common in adults over the age of 40 and in people who are overweight or have high blood pressure. It has been linked with the western lifestyle, since it is most common among overweight people and those who do not get enough exercise.

Table 1: Main differences between Type I and Type II diabetes

	Type I(IDDM)	TypeII (NIDDM)
Age at onset	Usually under 40	Usually over 40
Body weight	Thin	Usually overweight
Symptoms	Appear suddenly	Appear slowly
Insulin produced	None	Toolittleor it is ineffective
Insulin required	Must take insulin	May requireinsulin
Other names	Juvenile diabetes	Adult onsetdiabetes

General symptoms of diabetes

People with type I diabetes usually develop symptoms over a relatively short period. Symptoms in people with type II diabetes are not as noticeable as in type 1. Type 2 symptoms are often diagnosed by chance through routine medical check-ups. Symptoms vary from person to person, but common symptoms include: increased urination, increased or loss of appetite, excessive thirst, visible weight loss, blurred vision, recurrent skin infections, fatigue, vaginal infections or infections of the foreskin in uncircumcised men, slowly healing sores. However, main features of diabetes mellitus can be illustrated as Fig. 1.

Metabolic changes during diabetes

The term diabetes mellitus also describes a metabolic disorder of multiple etiologies characterized by chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both. Metabolism consists of anabolism (the constructive phase) and catabolism (the destructive phase, in which complex materials are broken-down). The transformation of the macronutrients carbohydrates, fats, and proteins in food to energy and other physiological

processes are parts of the metabolic process. Metabolic changes during diabetes can be summarized as follows.

Fat metabolism

Abnormal lipid metabolism in diabetes increases the free fatty acids. This increase is related to severity of diabetes that is due to mobility of fatty acid from adipose tissues by lipolysis of triglyceride (Singh et al. 1987). Insulin inhibits the release of free fatty acid from adipose tissues. In diabetes, fatty acids in the liver are converted to acetyl Co-A, which gets converted in to ketone bodies and results in hyperketonemia (Owen et al. 1971). Glucose toxicity can result in abnormal fatty acid metabolism, namely autoxidation of glyceraldehydes, which generates hydrogen peroxide and α-ketoaldehydes, leading to chronic oxidative damage. Fat intolerance is an inherent feature of diabetic dyslipidemia (Lewis et al. 1991).

Fig. 1: Main features of diabetes mellitus

Protein metabolism

Proteins form the cellular structural elements, biochemical catalysts, and are important regulators of gene expression. Body proteins are broken down, when dietary supply of energy is inadequate during illness or prolonged starvation. The proteins in the liver are utilized in preference to those of other tissues such as brain. The gluconeogenesis pathway is present only in liver cells and in certain kidney cells. Negative nitrogen balance and muscle wasting manifest the impaired metabolism of proteins in diabetic humans as in animals with experimental diabetes. There is increased catabolism and decreased utility of amino acids for protein synthesis (Teng, 1954). *In vivo* studies have shown that insulin enhances short side-chain amino acid intracellular uptake, stimulates transcription and translation of RNA, increases the gene expression of albumin and other proteins and inhibits liver protein breakdown enzymes. In IDDM patients most of the whole-body protein turnover studies have shown that insulin deficiency increases protein breakdown and increases amino acid oxidation and that these effects are reversed by insulin treatment. Recent studies have demonstrated that a substantial increase in leucine transamination during insulin deprivation contributes to leucine catabolism in IDDM patients. Protein synthesis in the insulin-deprived state is also increased although to a lesser extent than protein breakdown, and this increased whole-body protein synthesis is reduced with an insulin infusion thus the effects of insulin are largely mediated through its effects on protein breakdown (Haitham et al. 1996).

Carbohydrate metabolism

Carbohydrate metabolism plays an important role in both types of diabetes mellitus. The entry of glucose into most tissues-including heart, muscle, and adipose tissue-is dependent upon the presence of insulin. Insulin controls the

uptake and metabolism of glucose in these cells and plays a major role in regulating blood glucose concentration. The reactions of carbohydrate metabolism cannot take place without the presence of B vitamins, which function as coenzymes. Phosphorous, magnesium, iron, copper, manganese, zinc and chromium are also necessary as cofactors. In normal condition insulin promotes the utilization of glucose by tissues and lowers the blood glucose. During diabetes due to defects in insulin function abnormal carbohydrate metabolism occurs. In liver and kidney there is increase in gluconeogenesis resulting in acidosis condition. In nervous tissues, there is a decreased utilization of glucose and diversion to sorbital pathways, which is one of the consequences of hyperglycemia (Taylor et al 1988). Overall metabolic changes during diabetes can be summarized as shown in Fig. 2.

Diagnosis

Diabetes is diagnosed by examining glucose levels in blood or urine samples using one or more of the following tests: Random glucose test (RGT), Fasting blood glucose (FGT) and Glucose tolerance test (GTT).

Complications of diabetes

Both type 1 and type 2 forms of diabetes are characterized by chronic hyperglycemia and complications are more likely, if diabetes has not been well controlled. Late-stage complications do not usually develop for 10-15 years in type 1 diabetic patients. In type 2 diabetes, symptoms can appear close to the time of diagnosis because the disease may have been undetected for a long time. Complications can include development of diabetes specific microvascular pathology in the retina, renal glomerulus and peripheral nerve. As a consequence of its microvascular pathology, diabetes is a leading cause of blindness, end stage renal disease and a variety of debilitating neuropathies. Diabetes is also associated with accelerated atherosclerotic microvascular disease affecting arteries that

supply to heart, brain and lower extremities. As a result patient with diabetes have a much higher risk of myocardial infraction, stroke and limb amputation. Large prospective clinical study shows a strong relationship between glycemia and diabetic microvascular complications in both type 1 and type 2 diabetes (UK Prospective Diabetes Study, 1998; Wei et al 1998; Ebara et al. 2000; Ginsberg et al. 2000).

The long-term effects of diabetes mellitus include progressive development of specific complications of retinopathy with potential blindness, nephropathy that may lead to renal failure, and/or neuropathy with risk of foot ulcers, amputation, and features of autonomic dysfunction, including sexual dysfunction. People with diabetes are at increased risk of cardiovascular, peripheral vascular and cerebrovascular disease. Both IDDM and NIDDM are vulnerable to specific diabetic late complications such as retinopathy neuropathy and nephropathy, which cause serious morbidity (Deckert, 1978).

Diabetic neuropathy

Diabetic neuropathies are among the most frequent complication of long-term diabetes. It is estimated that 60% to 70% of diabetics have mild to severe forms of nervous system damage. Cramping, tenderness and muscle weakness also occur but atrophy is rare. Advanced femoral nerve disease is a major contributing cause of lower extremity amputations. Nerves in the arms, abdomen and back may also be affected. Symptoms may include impaired heart function, slowed digestion, reduced or loss of perspiration, severe oedema, carpal tunnel syndrome, alternating bouts of diarrhoea and constipation, bladder atony, urinary and faecal incontinence and impotence. Since, diabetes is a metabolic disease with vascular and nervous system complications and an erection involves all levels of the nervous system from the brain to the peripheral nerves, lesions anywhere along the

path may be responsible for erectile failure. It has been estimated that close to 50% of diabetic males have some degree of erectile dysfunction. Neuropathies usually improve with the control of the diabetes. Severe or chronic changes may require several weeks or months to show maximum improvement. The major risk posed by peripheral neuropathy is of foot trauma and diabetic ulcers (Esper Boel et al. 1995).

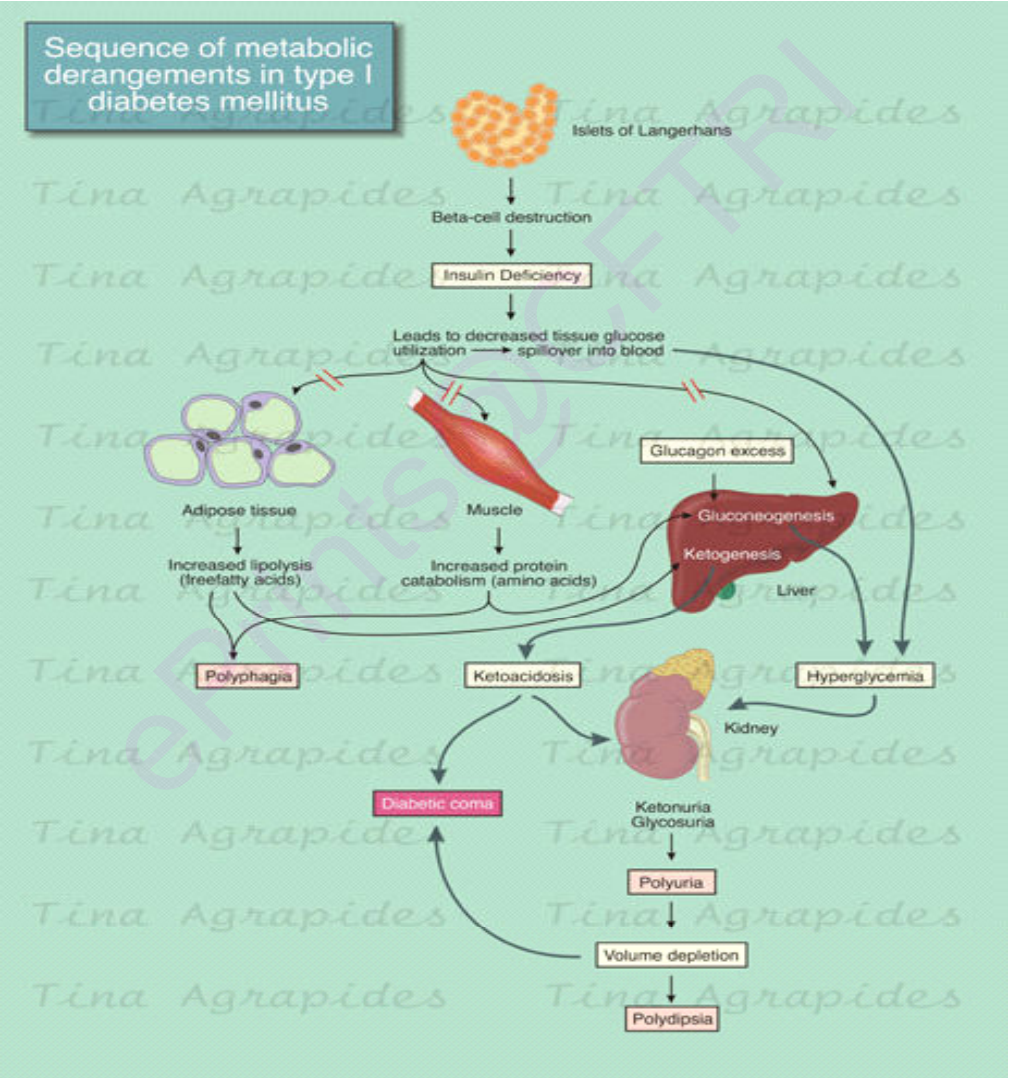


Fig. 2: Metabolic changes during diabetes

Diabetic retinopathy

Changes occurring in the eye, which are distinctive of diabetes, involve the narrowing, hardening, bulging, hemorrhaging or severing of the veins and capillaries of the retina. This is a serious complication known as retinopathy and may lead to loss of vision. Visual changes in the earlier stages may include diminished vision, contraction of the visual field, changes in the size of objects or photophobia. In the more advanced stage, termed proliferative retinopathy, hemorrhages, retinal detachment and other serious forms of deterioration are observed. When the disease progresses to this late stage, total blindness may occur. The risk of retinopathy increases with the severity and duration of hyperglycemia. After 20 years, nearly more than 60% of patients with type II diabetes have some degree of retinopathy. Retinopathy is a long-term complication of diabetes marked by changes is the blood vessels in the retina. When damage has occurred the blood vessels may leak and can eventually develop scar tissue that result in a blurring or distortion of visual images transmitted to the brain. It is well known that insulin dependent diabetic patients with clinical nephropathy have a high prevalence of proliferative retinopathy (Groop et al. 1986) and there seems to be an association between the development of diabetic nephropathy and proliferative retinopathy.

Diabetic nephropathy

Damage to the kidney called "nephropathy" is a common long-term complication of diabetes and is now one of the leading causes of renal failure in the western world. About 40% of type 1 diabetic patients develop diabetic nephropathy and there is a wide variation in the type and degree of renal damage. Nephropathy is less frequent than retinopathy and when it occurs is a consequence of long standing diabetes. It can start as minor damage causing poor filtration, but

can progress, especially, if untreated, to chronic kidney failure (renal failure), requiring blood dialysis or kidney transplant. Kidney failure can ultimately cause death. Diabetes is the most common cause of kidney failure, accounting for more than 40 percent of new cases. Even when drugs and diet are able to control diabetes, the disease can lead to nephropathy and kidney failure. Most people with diabetes do not develop nephropathy that is severe enough to cause kidney failure. About 16 million people in the United States have diabetes, and about 1 million of them have kidney failure as a result of diabetes. Diabetic nephropathy is one of the most problematic renal diseases because of the exponentially increasing number of patients entering chronic dialysis programs with renal failure resulting from diabetes, and the high mortality rates of these patients receiving dialysis (Ritz et al. 1999). This can develop in up to one third of patients with type 1 patients and 25% of patients with type II diabetes. Several studies have shown that the progression of diabetic nephropathy may be reduced or delayed by strict metabolic control (UKPDS, 1998).

Diabetic complications by glucose toxicity

Four main hypotheses of hyperglycemia can be summarized (Fig 3) into;

1. Increased polyol path way flux
2. Increased Advanced Glycation End products (AGE)
3. Activation of protein kinase C
4. Increased hexosamine

Large amount of data emphasize four key metabolic pathways as being major contributors to hyperglycemia induced cell damage (Bennett et al 1995; Robertson, 2004). Hyperglycemia results in increased enzymatic conversion of glucose to the polyalcohol-sorbitol, with concomitant decrease in NADPH and glutathione. The resulting loss of antioxidant reducing equivalents result in

enhanced sensitivity to oxidative stress associated with intracellular ROS. Hyperglycemia-induced overproduction of superoxide significantly inhibits glucose-6-phosphate dehydrogenase, the rate-limiting enzyme of hyperglycemia-induced ROS generation and consequent activation of pathological pathways - GlcNAc, O-linked N-acetylglucosamine; plasminogen activator inhibitor; TGF-β, Pentose phosphate pathway that is required for providing reducing equivalents to the antioxidant defense system. The product, NADPH, is the cell's principal reductant and is required for providing reducing equivalents to the glutathione peroxidase, and sorbitol is metabolized to fructose by sorbitol dehydrogenase, increasing the ratio NADH/ NAD^+. This results in oxidized triose phosphates with *de novo* synthesis of diacylglycerol (DAG). Increased DAG content activates protein kinase C responsible for several pathologies of diabetic complications (Brownlee, 2004). AGEs have been implicated in the pathogenesis of the major microvascular complications of diabetes mellitus: nephropathy, neuropathy, and retinopathy. Glucose, although one of the least reactive reducing sugars, can react with a free amino group to form an adduct, commonly referred as Schiff's base. In Schiffs base, the aldehydic carbon-oxygen double bond of the sugar is converted to a carbon-nitrogen double bond with the amine. Formation of the Schiff's base is relatively fast and highly reversible. Subsequent rearrangement of the Schiff's base originates an Amadori product. This reaction, which is believed to occur via an intermediate, open chain enol form, is much faster than the reverse reaction. Consequently, the Amadori glycation product tends to accumulate on proteins, initiating the processes of advanced glycation. AGEs arise from auto-oxidation of glucose to glyoxal, decomposition of the Amadori product to 3-deoxyglucosone, and fragmentation of glyceraldehyde-3-phosphate and dihydroxyacetone phosphate to methylglyoxal. Glyoxal, methylglyoxal, and 3-deoxyglucosone are reactive intracellular dicarbonyls that react with amino groups of intracellular and

extracellular proteins to form AGEs. Production of intracellular AGEs precursors interferes with target cell integrity by modifying protein function or by inducing receptor-mediated production of reactive oxygen species, which have been shown to cause changes in gene expression. The hexosamine biosynthesis pathway is an additional pathway of glucose metabolism that may mediate some of the toxic effects of glucose (Du et al. 2000). Under usual metabolic conditions, 2-5% of glucose entering cells is directed into the hexosamine pathway, beginning with the conversion of fructose 6-phosphate to glucosamine 6-phosphate by the rate-limiting enzyme glutamine fructose-6-phosphate aminotransferase (GFAT). Because hyperglycemia-induced overproduction of superoxide significantly inhibits glyceraldehyde-3-phosphate dehydrogenase activity, this inhibition would activate all the pathways of hyperglycemic damage by diverting upstream glycolytic metabolites into these signaling pathways. During hyperglycemia, because of the increased nutrient availability, much of the excess glucose is shunted into the hexosamine pathway. The end product of this pathway, UDP-N-acetyl glucosamine, is the substrate for the glycosylation of important intracellular factors including transcription factors, thereby affecting the expression of many genes including plasminogen activator inhibitor-1 (PAI-1) and leads to the development of the microvascular complications of diabetes. Until recently, there was no unifying hypothesis linking these four mechanisms. It has been shown that hyperglycemia-induced overproduction of superoxide by mitochondria is the trigger that drives each of these pathways (Nishikawa et al. 2000).

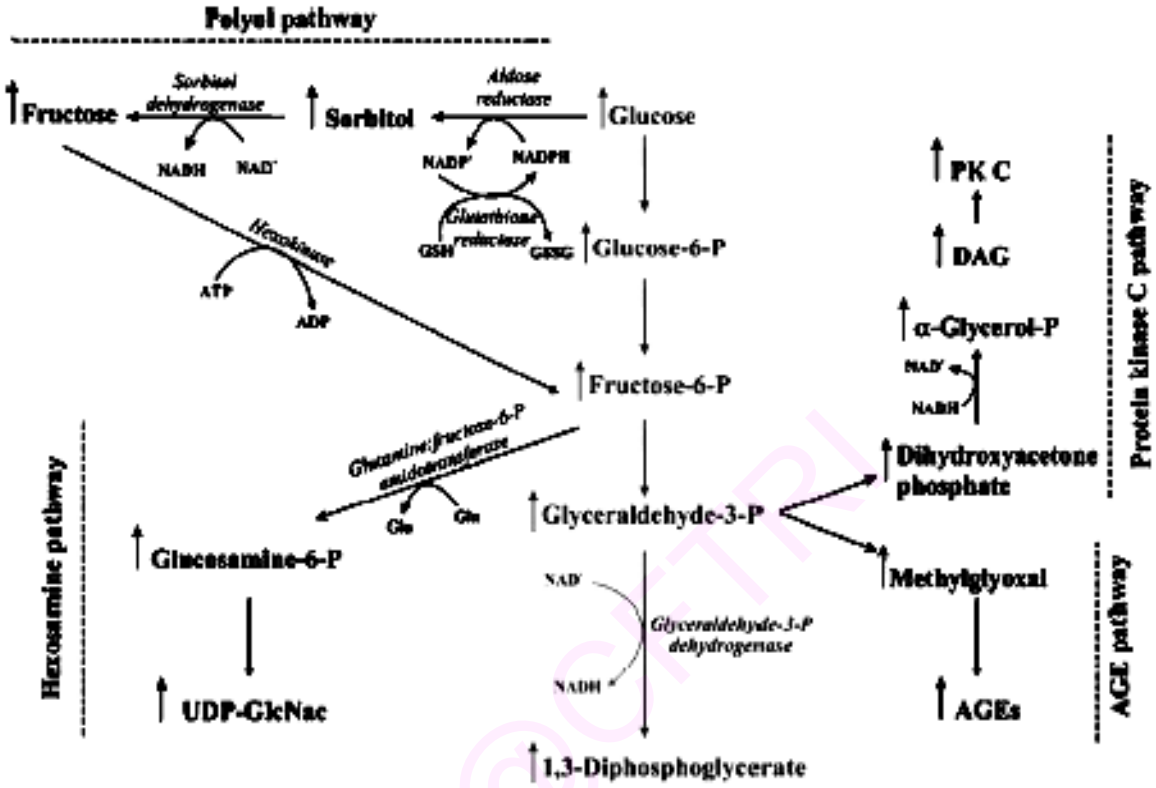


Fig. 3: Mechanisms of hyperglycemia-induced damage on advanced glycation end products (AGEs), DAG, diacylglycerol; PKC, protein kinase C.

Structural and functional changes of kidney during diabetic nephropathy

The function of the kidneys is to filter waste and excess fluid from the blood. Blood enters the kidney through an artery and then passes through the glomeruli (clusters of capillaries that act as filters). After filtration, the cleaned blood, and important proteins return to the body through a vein, while waste products and excess fluids are passed into the urine. The illustration below demonstrates this process. During diabetes this process of filtering the blood is hampered due to high blood glucose levels and high blood pressure, which damage the filtering system (glomeruli) and subsequently allows proteins to leak back into the urine. (Doublier et al. 2003).

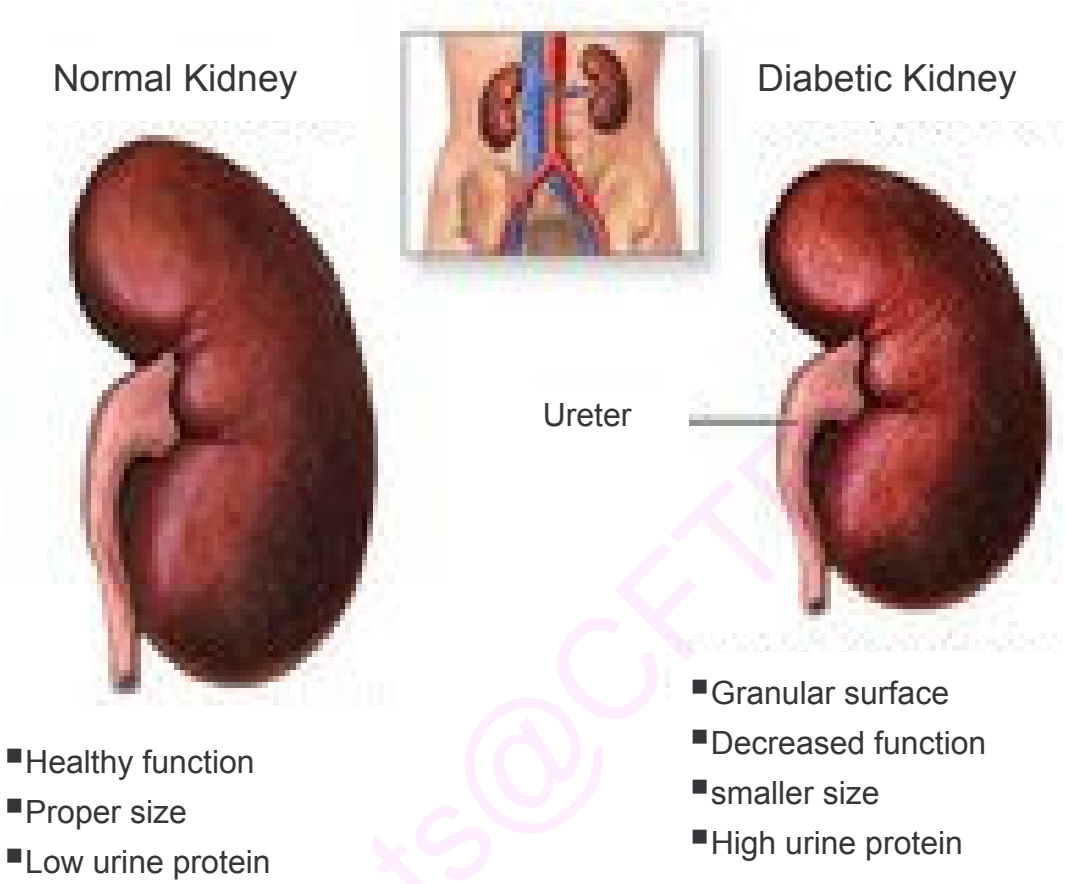


Fig. 4: Morphology of normal and diabetic kidney

Basement membrane and its components

Basement membranes are defined electron microscopically as characteristic sheet - like structure of extracellular matrix, which is formed in interface between parenchyma cells and their surrounding connective tissues. They are ubiquitously distributed in the body and found in nearly all multicultural animal species, while those in glomerular capillary loops or synaptic membranes are highly differentiated with their unique structure. The basement membrane serves as supports for cells and cell layers. Continuous basement membrane acts as passive selective molecular sieve between tissue compartments (Noonan et al. 1993). Only

tissues boundary on cells, which attach but also a filter with selective permeability or a highly specialized substrate for cellular differentiation and gene expression. The negative charge of the glomerular basement membrane (GBM) has been attributed to the presence of heparan sulphate proteoglycan called perlican (Tsen et al. 1995). Basement membrane is formed from glycoproteins and proteoglycan promoters, which interact with each other to produce defined supramolecular assemblies.

Glycoproteins

Glycoproteins result from covalent association of carbohydrate moieties (glycan) with proteins. Glycoproteins are widely distributed in animals, plants and microorganisms. The main glycoproteins in the basement membrane are type IV collagen, laminin (Paulsson, 1992).

Type IV collagen

Collagens are the most abundant proteins in the body. It is a fibrillar protein that aggregates in to a polymeric mesh. Each type IV collagen monomer consists of three polypeptide chains folded onto a triple stranded helix (Hudson et al. 1993). The monomers aggregate by three types of homotypic interaction; the covalent association of the C-termini in to dimers, the covalent association of N-termini into tetramers and lateral interactions between adjacent collagenous segments. At least six different type of collagen are known (α1- α6) which are encoded by separate genes named *col4A6*. Only two stable collagen IV network have been characterized; the first composed of α1 and α2 chains and the second containing network is associated with a sustained expression of the α1 and α2 chains. During maturation of the GBM, the collagen IV composition is changed gradually from a α1 and α2 containing to an, α3, α4 and α5 containing net work. Studies in experimental animals have shown that the increase in renal extracellular

matrix proteins is accompanied by a decrease in synthesis of collagens (Nerlich et al. 1991).

Laminin

Laminin is assembled from three polypeptide chains by β and γ chains. For each individual chain, different isoforms are identified. They were designated as α1-5, β1-4 and γ1-3. While most variants are encoded by a different gene, truncated chains may also be produced by alternative splicing proteolytic processing. During nephrogenesis, the laminin composition of the GBM is altered. The α1 and α4 chains are replaced gradually by the α5 chains and the β1 chains by β2, thus yielding a predominating laminin isoform composed of α5 ß2 γ1 in the mature GBM. The laminin β2 chain seems to have an important function in the GBM. Mutant mice that lack this chain are shown to display a nephritic syndrome in the second week after birth. Interestingly, massive proteinuria is associated with fusion of the foot processes, resembling the phenotype of minimal changes and nephrosis. This suggests an important role for laminin-II in cell- matrix interaction. Such a function is conceivable since laminins interact with multiple components of the GBM, including agrin, nidogen and perlecan (Battaglia et al. 1992). Both receptors appear on the cell surface during development of nephron, after the mesenchymal transition, which corresponds to the onset of expression of laminin-1.

Entactin

Also called Nidogen is a 158 kDa large single chain glycoprotein first purified from the extracellular matrix produced by the EHS tumor. It is composed of three glomerular domains (G1, G2 and G3) separated by rod like segments. The C- terminal globule (G3) binds noncovalntly and in equimolar amounts to domain

III of the laminin γ chains. Domain G2 associated with collagen IV links the laminin network. This domain also has a perlecan binding function. Given these cross-linking properties, nidogen is an important component in the assembly of basement membranes (Yurchenco et al. 1990).

Proteoglycan

Proteoglycans are a heterogeneous and complex family of macromolecules composed of a core protein to which complex linear heteropolysaccharides called glycosaminoglycans (GAG) are covalently linked. The protein component of proteoglycans is the core protein, which directs the biosynthesis of proteoglycans to different molecular constructions and functions (Semiramis et al. 1996). So far more than 20 genetically different species of core proteins have been identified. The major structural component of proteoglycan is GAG.

These are a specific class of biological macromolecules distributed among all organisms. These are the major structural components of the extracellular matrix (Iozzo, 2001). Proteoglycans are not only structural components, but also participate in and regulate many cellular events and physiological processes, including cell proliferation and differentiation processes, including cell-cell and cell-matrix interactions (Schwartz, 2000). Their potential for being interactive in such a degree stems from the great structural diversity in their GAG chains i.e., the GAG's type, size and composition, as well as the degree of substitution and domain arrangement. At least six types of GAG chains have been identified so far in higher mammals.

GAGs are linear polysaccharides composed of variable number of repeating disaccharide units. Each disaccharide consists of one hexosamine (D-galactosamine or D-glucosamine) and one uronic acid (D-glucuronic acid or L-iduronic acid) or neutral hexose (D-galactose). According to the type of the

monosaccharide and glycosidic bonds between them, GAGs can be divided in to five major categories.

1. Hyaluronic acid
2. Chondroitin sulphate
3. Dermatan sulphate
4. Keratan sulphate
5. Heparan sulphate

Hyaluronic acid

Hyaluronic acid is the only non-sulphated GAG and therefore none of its hydroxyls are esterfied with a sulphate group. It is synthesized by hyaluronan synthase in the plasma membrane, where the elongation occurs by the alternate addition of UDP-glucuronic acid and UDP-N-acetyl-glucosamine from the reducing ends of the nascent chains.

Chondroitin sulphate

Exists in basement membrane in proteoglycanic form called chondroitin sulphate. The core protein of bamacan, 138 kDa (mouse) consists of five domains; the laminin- terminal globular head, the second rod attached with one each of CS chains and a N-linked oligosaccharides chain, the third globular and the fourth rod containing two oligosaccharide chains and fifth globular tail with two CS chains. The function of bamacan has not yet been well elucidated.

Keratan sulphate

Galactose residue of keratin sulphate is linked by β (1, 4) linkage to N-acetyl glucosamine. The glucosamine residue is sulphated at C-6. It is found in cartilage, intervertebrate discs and cornea.

Dermatan sulphate

It is an epimer of chondroitin sulphate. Uronic acid residue is epimerised to iduronic acid. The disaccharide is characterized by iduronic acid linked by α (1, 3) linkage to N-acetyl-D-galactosamine. The galactosamine residue is sulphated at C-4. It is found in skin and other tissues.

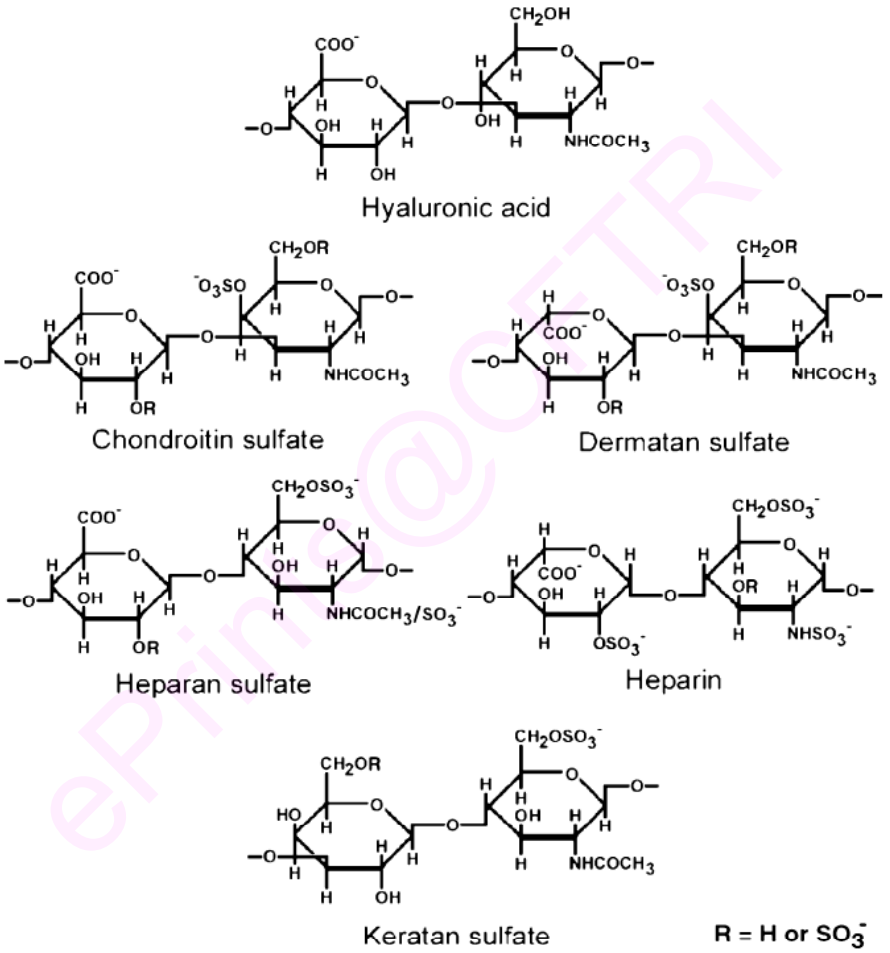


Fig. 5: Structure of disaccharides commomly present in glycosaminoglycans.

Source: Volpi and Meccari, 2006.

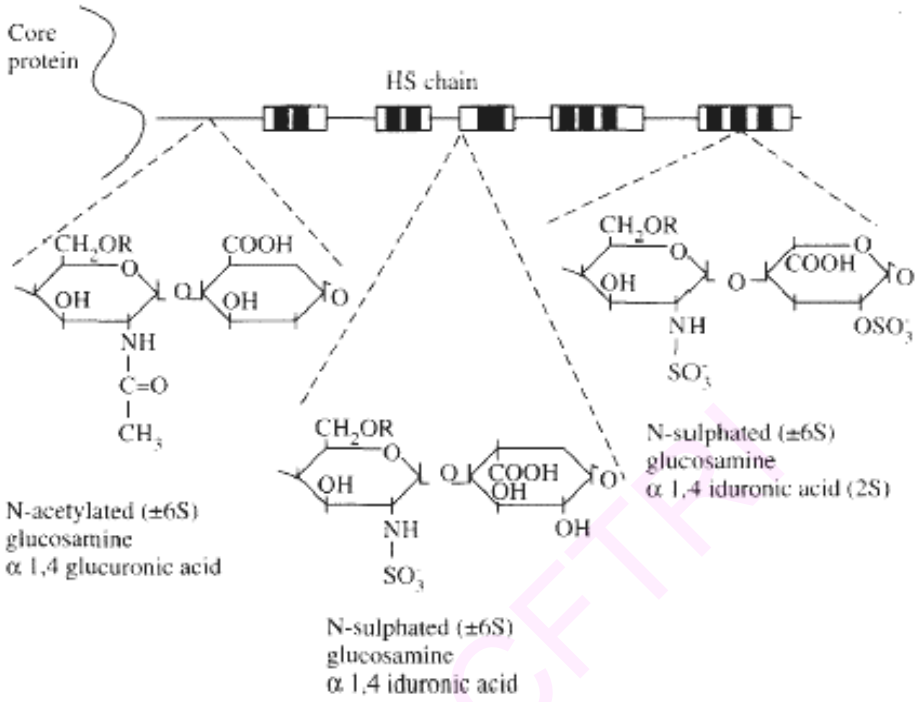


Fig. 6: Structure of heparan sulphate.

Source: Gallagher and Turnbull, 1992; Yanagishita and Hascall, 1992

General structure of heparan sulphate

The HS polysaccharide is composed of alternating hexuronic acid [D-glucuronic acid (GlcA) or L-iduronic acid (IdoA)] and D glucosamine (GlcN) residues, which may be N-sulphated or N-acetylated (Fig. 6). IdoA occurs in conjunction with GlcNSO, residues mainly within clusters of N-sulphated disaccharides (S-domains). 0-sulphate groups may be found at C-2 of IdoA residues and at C-6 and C-3 of $GlcNSO_4$. More rarely GlcNAc may be 6-0-sulphated when adjacent to an N-sulphated disaccharide and GlcA can also be sulphated at C-2. The spacing of the S-domains as well as the pattern of O-sulphation are characteristic of the cellular origin of the HS and may be critical for optimum binding to protein ligands (Stringer et al. 1997).

Structural and functional changes in glomerular basement membrane during diabetic nephropathy

The first obvious changes after manifestation of diabetes mellitus are the increase of the size of kidney and glomerular volume (Mauer et al. 1989; Mauer et al. 1984). During the early stage the hypertrophic glomeruli show normal structure. These changes are probably caused by hemodynamic factors. They are potentially reversible and are not reliable indicators for the development of diabetic nephropathy. Significant structural changes, particularly thickening of glomerular basement membrane and mesangial expansion occur only after several years of diabetes. Mogensen et al. observed that persistent urinary excretion of minor amounts of albumin are closely linked to the development of diabetic renal disease (Mogensen et al. 1983; Mogensen, 1987).

Electron microscopic studies show that the increase or thickening of glomerular extracellular matrix correlates well with the extent of microalbuminuria. This correlation is found in both type 1 and type 2 diabetic patients (Osterby et al. 1993; Osterby et al. 1993). Detailed immunohistochemical studies have shown that during the development of diabetic glomerulosclerosis, there is an increase of collagen species (Makino H etal. 1994). During the development of diabetic nephropathy new types of collagen appear gradually substituting the physiologically occurring collagen types (Nerlich et al. 1991; Mohan et al. 1990; Nerlich et al. 1991). Similar morphologic and structural changes can also be observed in renal interstitium and the glomerular arterioles (Ceol et al. 1996).

Studies in experimental animals have shown that the increase in renal extracellular matrix protein deposition is caused by an increased synthesis of collagens (Park et al. 1997). The increase of glomerular matrix proteins is accompanied by a decrease in basement membrane associated heparan sulfate

proteoglycan. Since this negatively charged proteoglycan represents the anionic barrier of the glomerular basement membrane decrease of this integral component of the glomerular filtration unit may explain the increased glomerular permeability of albumin (Fig. 8). However, this matrix component, which is present in all sub-endothelial vascular walls (Schleicher et al. 1989), is insoluble in physiological fluids and is therefore not readily useful for diagnostic purposes.

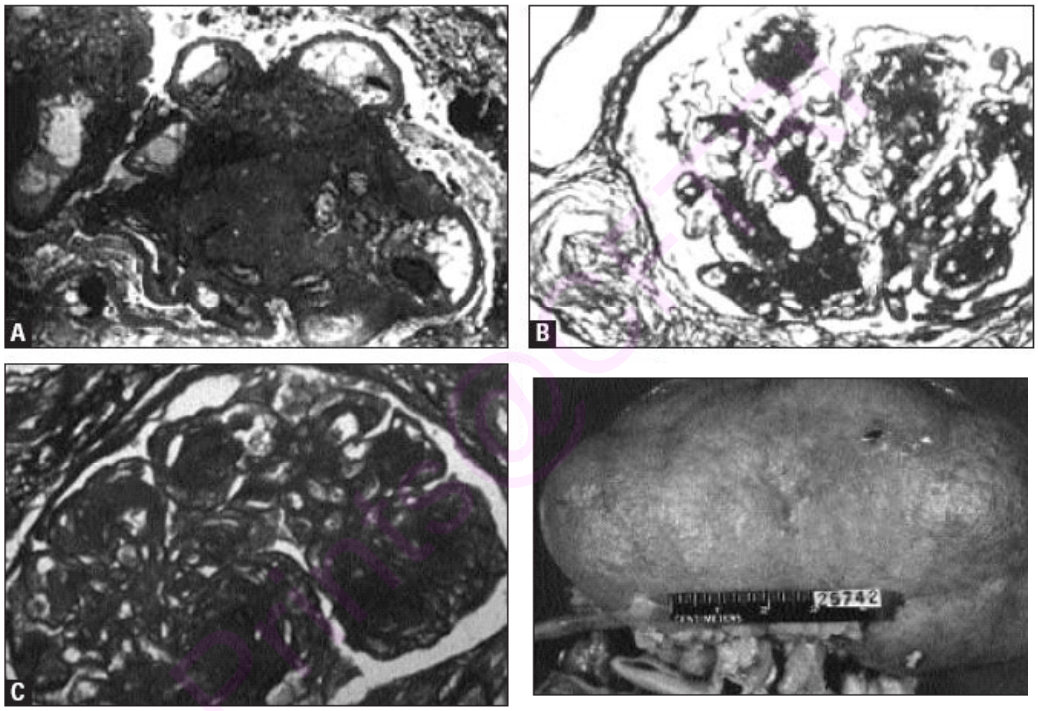


Fig. 7: Clinical features of diabetic kidney (Mesangial expansion)

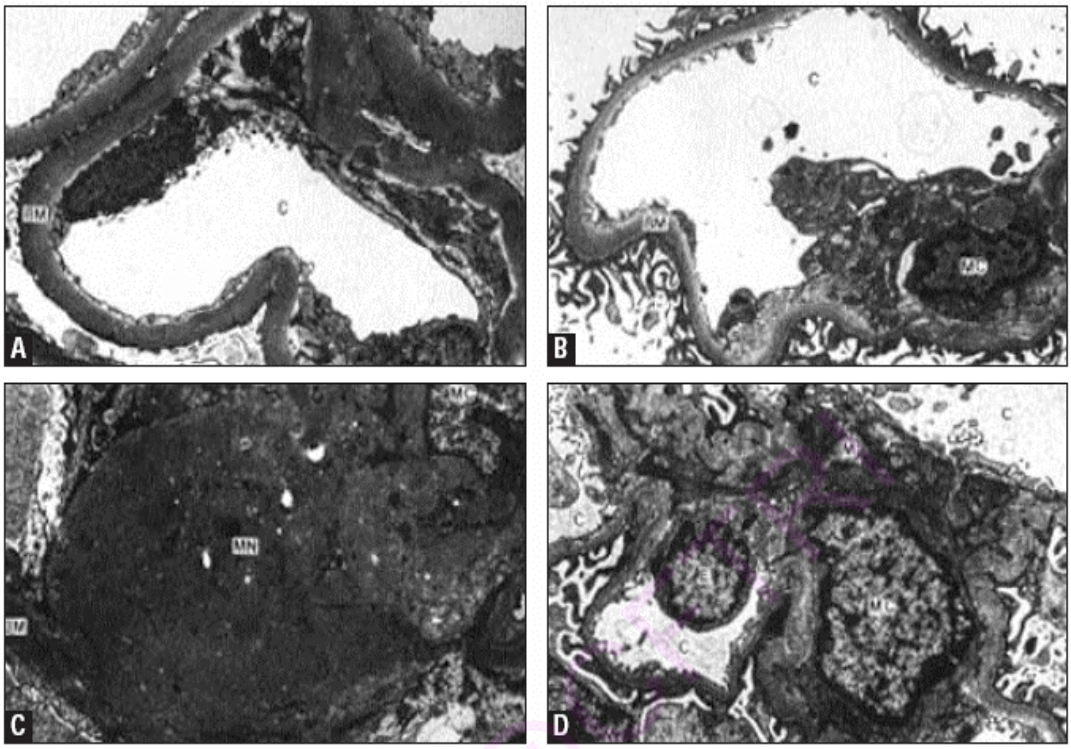


Fig. 8: Clinical features of diabetic kidney (GBM thickening)

Glomerular basement membrane thickening during nephropathy

This occurs due to deposition of glycoproteins. Studies on human glomerular basement membrane indicate that in diabetes there is an increase in the amount of basement membrane material. It is seen that there is an increase in hydroxlysine to which glucose and galactose units are attached. Increase in basement membrane material has been found in pancreatomised diabetic rats (Cohen et al. 1972). The enzymes involved in hydroxylation and sugar attachment have been found to be significantly increased in renal glomeruli from streptozotocin diabetic rats, which were restored to normal levels by insulin (Cohen et al. 1977). The activity of UDP galactose: N acetyl glucosamine glycoprotein galactosyl transferase was reported to be normal in kidney cortex of

diabetic rats. It is proposed that there is an increase in the hydroxylysine disaccharide units but not in asparagine linked heteropolysaccharide units (Bunn et al. 1978). The elevation of glucosyl transferase, glutamine fructose-6-phosphate aminotransferase and UDPG-dehydrogenase has been reported in diabetes (Spiro et al. 1971; Nerlich et al. 1998). All these together leads to increased accumulation of basement membrane constituents. Increase in the activities of the above enzymes in particular, enzymes of collagen biosynthesis has been shown to be dependent on the duration of diabetes and the resulting hyperglycemia. Activities of hydrolytic enzymes like N-acetyl glucosaminidase, glucuronidase involved in degradation of glycoproteins and proteoglycans have been reported to vary during diabetes (Serrano et al. 1976).

The changes in the activities of metabolic enzymes of glycoproteins and proteoglycans also have a bearing on proteoglycans of the kidney. Kidney is rich in heparan sulphate proteoglycans, which are in majority (88-90%) along with other chondroitin sulphate B (4-8%) and chondroitin sulphate C (<2%) and hyaluronic acid (Dietric et al. 1977). Heparan sulphate proteoglycans have been recognized as an important regulator of charge selective barrier of glomerular permselectivity (Parthasarathy et al. 1981). Studies have revealed several abnormalities in the glomerular proteoglycan metabolism. These include reduction in heparan sulphate content in glomerular basement membrane in hyperglycemic environment. The decrease in heparan sulphate in glomerular basement membrane has been reported in both diabetic rats and humans (Kanwar et al. 1983; Reddi, 1990; Brown et al. 1981; Shimomura et al. 1987). It has also been reported in *in vitro* system, that the glomerular epithelial cells in the environment of increased glucose synthesis has less heparan sulphate proteoglycans, which were restored to normal levels by insulin (Kasinath et al. 1995). Changes in heparan sulphate and

other mucopolysaccharides during diabetes have also been reported in kidney cortex. (Rohrbach et al. 1982).

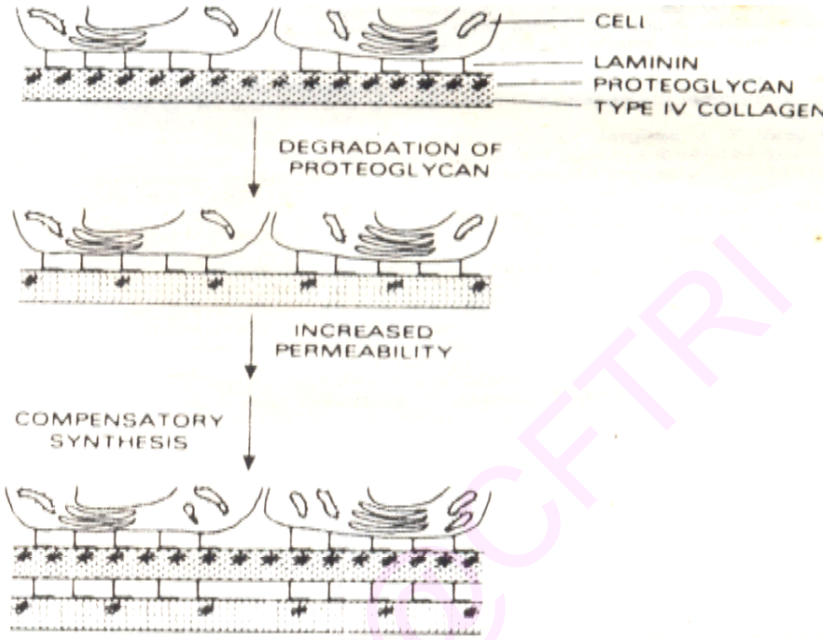


Fig. 9: The change in proteoglycan and glycoprotein during diabetes

Treatment and prevention

Prevention of complications is a key issue because of the huge premature morbidity and mortality associated with diabetes (Atkinson et al. 2001; Taylor et al. 1999; Cereghini, 1996). Glycemic control in both IDDM and NIDDM has been shown that hyperglycemia is a major determinant of progression of diabetic nephropathy. Several studies including the diabetes control and complications trial have indicated that intensified glycemic control retards the rate of development of both microalbuminuria and overt protienuria in patients with IDDM with normal albuminuria (Pier Ruggenenti et al. 2000). Hence, control of blood sugar is the best way to minimize the risk of complications.

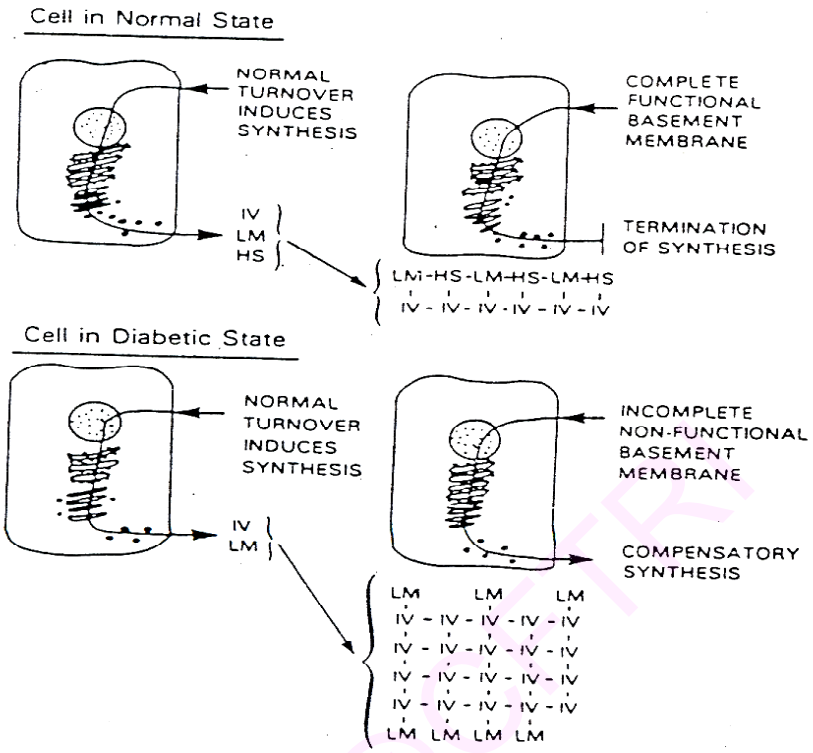


Fig 10: Schematic representation of possible events leading to the thickening of basement membrane in diabetes.

At the top of the figure 10, synthesis of the common constituents of basement membrane such as laminin, type IV collagen and heparan sulphate proteoglycan has been completed and the functional basement membrane is intact. In the diabetic state, the level of the heparan sulphate proteoglycan decreased due to increased degradation (middle) or inadequate synthesis (not depicted). As a result of the loss of proteoglycan, the basement membrane becomes more porous. We propose that a high porosity of the basement membrane triggers the compensatory synthesis of more basement membrane components, which leads to an increase in type IV collagen and laminin as a shown at the bottom.

Maintaining the blood glucose level (120- 130 mg/dL) is the primary aim in management of diabetes. Control of diabetes involves balancing the amounts of glucose and insulin in blood. In the past decade, several major studies have focused attention on the need for strict control of glycemia to prevent and or

reduce the risk of both the specific microvascular and the less specific macrovascular complications. The diabetes control and complications trial (DCCT) was a landmark study and the flagship for a number of studies that established the value of intensive control of blood glucose to prevent the complications of diabetes. However, treatment and prevention of diabetes mellitus anywhere in the world is usually accomplished by

1. Insulin therapy,
2. Drug therapy,
3. Diet therapy (Nutritional management).

1. Insulin therapy

Insulin therapy is the main treatment for type 1 diabetes. It may also be required by some people with type 2 diabetes. The aim of insulin therapy is to control the amount of insulin in the blood stream so that glucose levels are normal or near normal. Insulin injection is given at regular intervals especially after meals to maintain the blood glucose level. The insulin regimen is usually tailored to the nutritional plan and physical activity of the individual.

1. Drug therapy

A great deal of research towards the development of more effective ways of treating the disease has led to the development of orally active agents such as sulfonylurea, biguanides, tolbutamide and glyblenclamide. These oral agents are being extensively used and are primarily used for the treatment of noninsulin-dependent diabetes. These oral anti-diabetic drugs lower the blood sugar by increasing the amount of insulin secreted by the pancreas or by increasing the action of insulin in the body or by delaying the absorption of glucose. These drugs are called hypoglycemic drugs. They will not be effective unless they are combined with dietary restrictions. Biguanides like phenformin will enhance the

utilization of glucose by the tissues. The drugs like acarbose delay the absorption of carbohydrates from the gastrointestinal tract (Sharma et al. 2004).

2. Diet therapy or Nutritional management

Diet is important in management of both type 1 and type 2 diabetes. In type 2 diabetes, effective dietary management may be sufficient to control the disorder. The main aim is to maintain a normal blood sugar level. Nutritional management is a key component for the long-term health and quality of life for people with diabetes. Early physicians in pre-insulin era depended mainly on diet for diabetes management (Wood et al. 1972). The diet regimen usually consisted of low carbohydrate, high fat diet with boiled vegetables and without milk. The dietary measures also included the use of traditional medicines mainly derived from plants. A large number of plants all over the world have been tested for the blood glucose lowering effect in the laboratory experiments (Sridhar et al. 1987; Torangatti et al. 1995). Considerable interest is seen in the management of diabetes by natural anti-diabetic plant products.

Development and utilization of antidiabetic plants have attracted increasing interest. More than 400 plants with glucose-lowering potential are known. Hypoglycemic activity of polysaccharides from plants has been reported (Marles et al. 1995). Some botanical polysaccharides are considered as important bioactive components responsible for hypoglycemic property. Nutritional factors including antioxidants have great influence in the management of diabetes mellitus and its complications. An imbalance between oxidative stress and antioxidative defense mechanisms in diabetics can result in cell and tissue damage and accelerate diabetic complications. Hence, administration of appropriate antioxidants could prevent or retard diabetic complications to some extent. American Diabetic Association (ADA) has recommended that diet should be rich in low caloric carbohydrates. Dietary fibers are one such low caloric carbohydrate,

which has emerged as a major dietary component in the management of diabetes (Trowell, 1975). The studies carried out on the foods and medicine plants having beneficial effect on diabetes have shown that, these one or a combination of dietary fiber, antioxidants and or bioactive compounds are effective in delay the diabetic complications.

Dietary fiber

Dietary fibers are generally defined as the macromolecules present in the diet that are resistant to the digestion by human endogenous enzymes. These are essentially composed of plant cell wall components such as non-starchy polysaccharides and lignin. The term dietary fiber was first coined by Hipsley in 1953 to describe the unavailable carbohydrates in plant foods (Hipsley, 1953). Whereas, Trowell defined dietary fiber in the context of human medicine as the sum of lignin and polysaccharides that are not digested by endogenous secretions of the digestive tract of man (Trowell et al. 1976). However, the exact definition of the term has been controversial, as scientists have studied various aspects of the food supply and dietary fiber's impact upon health. Most recently, dietary fiber was defined with broad sense as the remnants of the edible part of plants and analogous carbohydrates that are resistant to digestion and absorption in the small intestine with complete or partial fermentation in the large intestine. It includes polysaccharides, resistant starch, lignin and associated plant substances. Dietary fiber exhibits one or more of either laxation (fecal bulking and softening; increased frequency; and/or regularity), blood cholesterol attenuation, and/or blood glucose attenuation. Dietary fibers found in the cell walls of all plant-based foods and do not constitute a defined chemical group. Instead it represents a combination of chemically heterogeneous substances such as cellulose, hemicelluloses, pectins, gums, mucilages, resistant starch and other

polysaccharides and lignin-a non-carbohydrate polymer of phenyl propane residues.

Classification

Dietary fibers can be divided into two main types based on their solubility. They are soluble dietary fiber (SDF) and insoluble dietary fiber (IDF). Most of the whole plant foods contain combination of these two types of fiber, each one of these exerts specific physiological effect.

Soluble dietary fiber

These due to their viscous nature influence the absorption by delaying gastric emptying, impairing mixing in the upper small intestine, changing the absorption site and delaying small bowel transit time. They also bind with cholesterol and prevent it from being reabsorbed into the blood stream. This lowers the amount of cholesterol in the blood, thereby reducing the risk of heart disease. Soluble fibers also form a gel in the intestine, which slows down the digestion and absorption of carbohydrates, especially glucose thereby helping to keep blood sugar levels steady. Foods rich in soluble fiber include fruits, vegetables, oats, barley, and pulses such as beans, lentils and peas.

Insoluble dietary fiber

These are the tough, fibrous parts of the plant. It helps to keep the digestive system in good working order by increasing the bulk and softning of the stool, which in turn assists the smooth passage of food through the body. IDF reduces intestinal transit time, thus reducing contact time for faecal mutagens to interact with the intestinal epithelium. As fermentative substrate, they modify the activity of digestive microflora and leads to modification or reduction in the production of mutagens. They also help to prevent bowel complaints like constipation and cancer. Foods rich in insoluble fiber include whole meal flour bread, whole grain

breakfast cereals, bran, brown rice, whole meal pasta, grains and some fruits and vegetables.

Health benefits of high fiber diet

Dietary fiber is known for a decade for their beneficial role against many diseases. The health benefits associated with high fiber foods are delayed nutrient absorption, increased bulk, lowering of blood lipids, prevention of colon cancer, barrier to digestion, mobility of intestinal contents, increased faecal transit time and fementability characteristics. This also helps to keep the digestive system healthy, preventing bowel problems such as constipation, biventricular disease and haemorrhoids (piles), as well as reducing the risk of bowel cancer.

Physiologically and metabolically, dietary fibers can affect protein digestibility and utilization, bioavailability of minerals and metabolism, faecal weight and their composition, intestinal transit time, microbial population (intestinal flora), digestive enzymes, SCFA production, intestinal luminal mutagens and carbohydrate metabolism. Their physiological property and important health benefits can be summarized as follows;

Table 2: Physiological properties of health benefits of dietary fiber

Property	Mechanism	Related conditions
Substrate for fermentation	Microbial growth stimulated Short chain fatty acids Changes in nitrogen, bile acid and xenobiotic metabolism	Bowel habit/constipation Diverticular disease Colo-rectal cancer
Physical effects in small bowel	Gel properties Secondary affects on insulin secretion and gut hormones	Glycaemic responses/diabetes Lipid absorption/coronary heart disease
Satiety and gastric emptying	Chewing of food Delay in gastric emptying	Short term appetite reduced

*Principally cell wall NSP and related polysaccharides.

Source: Cummings et al. 2004.

In contrast, DF is often considered as beneficial for human health due to its well-known effect of reducing blood cholesterol, affecting glycaemic response, delaying gastric emptying, diminishing nutrient absorption, effecting motility in the small bowel, and prolonging satiety after a meal (Blaak et al. 1995; Potty et al. 1996). Moreover, consumption of DF has also been shown to be correlated with the prevention of many health threatening intestinal diseases, especially cancer (Goodlad, 2001). Thus, inclusion of fiber in diets may also have positive non-nutritional effects.

Dietary fibers are also well known for their beneficial effects on bowel health (Meunier-Salaun et al. 1999). Nevertheless, the average daily intake of dietary fiber is far from sufficient in many developed countries. The recommended daily intake of total fiber for adults is set at 38 g for men and 25 g for women in the United States according to the Dietary Reference Intake for fiber (Institute of medicine of the national academies, 2002). Dietary fiber has been shown to play a role in the treatment of conditions such as gastrointestinal disease, constipation, hemorrhoids, heart disease, and diabetes.

Diabetes and dietary fiber

Dietary fiber is known for their beneficial role against diabetes for a long time. However, Trowell first identified the link between fiber and diabetes (Trowell, 1972; Trowell, 1973) and Jenkins *et al* published the first experimental evidence in man that fiber moderated blood glucose and insulin responses (Jenkins et al. 1976). Since then, recommendations for diet for diabetics have changed from a low carbohydrate high fat high protein diet to one moderately low in fat and high in non starchy polysaccharides (Dietary recommendations for diabetics for the 1980's 1982; Diabetes and Nutrition Study Group of the European Association for the Study of Diabetes 1988). This subject has been extensively researched and

evidence is summarized in recent reviews (Berger et al. 1992; Wolever et al. 1993). It is well known that a high intake of dietary fiber, particularly of the fermentable type, improves glycemic control, decreases hyper-insulinaemia, and lowers plasma lipid concentrations in patients with diabetes (Jenkins et al. 1978; Komal Mehta et al. 1992). High fiber diets have been found to be effective in controlling hyperglycemia and it has been introduced in the diet in two ways, first by incorporating purified form of fiber and secondly by overall enrichment of the diet. Studies are also carried out with purified form of vegetables. Dietary fibers are beneficial to diabetics either by acting as an insoluble matrix facilitating the slow absorption of glucose there by decreasing the blood glucose, or by short chain fatty acids released during dietary fiber fermentation.

Glycemic effect of dietary fiber

Glycemic index is a means of classifying carbohydrates in food, according to their effects on blood sugar levels. A food that has a high GI causes a sharp rise in blood glucose, whereas a low GI food causes a slower and more sustained increase in blood glucose. Large population studies suggest that low GI diets are associated with a reduced risk of chronic disease, especially type 2 diabetes and coronary heart disease (Frost et al. 1999). However, there is little evidence that a low GI diet is associated with a lower body weight or reduced risk of obesity (David, 2000). Low GI foods are associated with increases in fullness and decreases in hunger, which may help, reduce energy intake. However, claims that low GI diets can specifically help with weight loss or weight control are premature. High fiber diets, especially those with high carbohydrate content, probably exert their effect on glycemic control by insulin sensitivity. This is obvious from the reduced need for antidiabetic medication or insulin in subjects on these diets. However, reports on insulin clamp studies to assess insulin sensitivity

in these subjects is not consistent, being variously reported as unchanged or improved. However, various studies (Frost et al. 1999) report increased insulin binding to monocytes and adipocytes in subjects on high fiber diets. Dietary fiber is able to reduce the postprandial glycemic response by variety of mechanisms, including delay in gastric emptying, decrease of glucose diffusion across the unstirred water layer over the intestinal mucosa, modulation of G-I hormone secretion, and production of alternative energy substrates (e.g. SCFA) as a result of bacterial fermentation. (Anderson et al. 1987; Jenkins et al. 1977; Wolever, 1993)

Short chain fatty acids

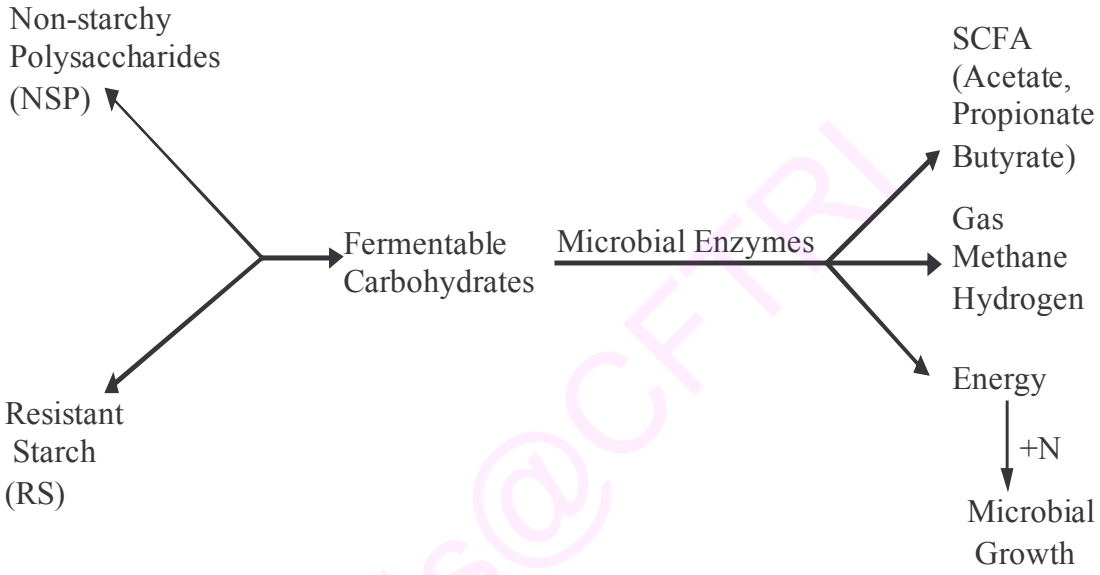
Nature and origin

The short chain fatty acids or volatile fatty acids are saturated fatty acids made of 1-6 carbon atoms produced by the fermentation of colonic microflora. Each molecule of short chain fatty acid present only one carboxylic organic acid function (-COO). Acetate, propionate, and butyrate, having 2, 3 and 4 carbon atoms respectively, constitutes to the main short chain fatty acids. Besides the exogenous source of fatty acids from the food supply, the host flora from a number of fermentable substances produces most of these short chain fatty acids endogenously. The major fermentable food substrate is the non-starchy polysaccharides, which are the main constituents of dietary fiber.

Fermentation of dietary fibers to short chain fatty acids

Although, role of fiber as a source of bacterial fermentation has been known for decades, fermentation of dietary fibers to short chain fatty acids and their biological activities and their metabolic products are gaining lot of attention these days. Plant fiber components and other nutrients that escape digestion in the

small intestine are broken down in the large bowel by anaerobic bacteria in a process known as fermentation. Caecal fermentation occurs by the action of anaerobic microbes. These microbes are dependent on dietary and endogenous sources for nutrition. The consequence of bacterial fermentation is summarized as follows.



Bacteria in the hindgut produce enzymes to digest fermentable substrates entering the large intestine. Anaerobic fermentation enables bacteria to use products of this process to supply their own energy needs for maintenance and growth, thus increasing their biomass. The primary products of fermentation in the hindgut are the short chain fatty acids (SCFA) - *n*-butyrate, propionate and acetate. The molar ratios of the different SCFA produced vary depending on the substrate.

Approximately 200 mM of short chain fatty acids are produced from the intake of 20 g of fiber, (acetate - 120 mM, propionate - 50 mM and butyrate - 30 mM). Qualitative and quantitative profiles of short chain fatty acids produced by different fibers depends both on their physical and chemical nature. For example Wheat bran, a source of insoluble dietary fiber produces acetate 52%, propionate

11.4%, butyrate 19.2%, whereas guar gum, a source of soluble dietary fiber produces 57% acetate, 27% propionate and 8% butyrate. The amount of short chain fatty acids observed in blood in fed and non-fed status is presented in Table 3.

Table 3: Concentration of short chain fatty acid in blood.

µmol/L	Portal	Sub-hepatic	Peripheral
Non fed status (Peters)			
Acetate	128.0 ± 70.8	-	67.0 ± 23.0
Propionate	34.4 ± 32.3	-	3.7 ± 1.2
Butyrate	17.6 ± 18.4	-	Traces
Nutritional status unknown (Cummings et al.)			
Acetate	257.8 ± 89.7	115.3 ± 63.0	70 ± 41.5
Propionate	88.3 ± 62.2	21.2 ± 21.9	5 ± 4.0
Butyrate	28.7 ± 17.6	11.7 ± 9.8	3.8 ± 3.8

Source: Bornet, F.R.G., Alanowitch, C and Slama, G., (1994), In: Gums and stabilizers for the food industry-7, G.O. Philips, P.A. Williams and D.J. Wedlock, (Eds), Oxford university press, New York. pp. 217.

Factors affecting the SCFA production/ fermentation

Generally, factors affecting the SCFA production are either related to the substrate availability for fermentation or the microflora and its activity, and the host. These can be summarized as follows.

1. SCFA production can be characterized by the rate, extent and the sites of fermentation (Jamroz et al. 2002; Marounck et al. 1996).

2. The physical properties of the fiber and the chemical structure of individual polysaccharides can influence the fermentation pattern of dietary fibers (Green et al. 1998; Roland et al. 1995) Accessibility to the fiber is one of the limiting parameters.

3. Soluble polysaccharides are generally more rapidly fermented than equivalent non-starchy polysaccharides. For insoluble fiber, the porosity of the fiber and the particle size may control the fermentation (Telung et al. 1987).

4. Structural features of the polysaccharides are a limiting factor. The nature of monomers and of the glycosidic linkages, the presence and distribution along the backbone of some of the functional groups or side chains can modulate their utilization by bacteria (Botham et al. 1998). In case of arabinoxylans from cereals, their extent of fermentation has been shown to depend on their degree of branching.

5. Colonic bacteria possess a broad spectrum of enzyme activities. However, colonic bacteria appear to be subjected to regulation. Their activities and in particular their polysaccharidase activities can be induced by exposure to substrate and there is evidence for repression of enzyme synthesis by products of the reaction. Thus, microflora can exhibit preferential utilization of certain substrates (Gibson et al. 1995; Nordgaard et al. 1995).

6. Transit time can affect fermentation, depending upon the amount and nature of the substrate available for fermentation (Goodlad et al. 1987; Husebye et al. 1994), the colon environment (pH, hydrogen pressure, etc), the quantity and activity of the microflora, which can have influence on the relative utilization of different carbohydrates. An accelerated transit time decreases the pH, increases the amount of substrates reaching the colon, the stool output and excretion of bacterial biomass.

Factors determining the ratio SCFA produced from bacterial fermentation are not fully understood. Acetic acid is always the major SCFA produced. Resistant starch is generally associated with large proportion of butyric acid, pectins with large amount of acetic acid while arabinogalactan, galactomannan, ispaghula and starch are associated with large proportion of propionic acid. The supplied substrates and the bacteria that ferment them may be involved in determining the SCFA profiles. The main point is that different fibers are fermented to different degrees, at very different rates, and to different ratio of SCFAs.

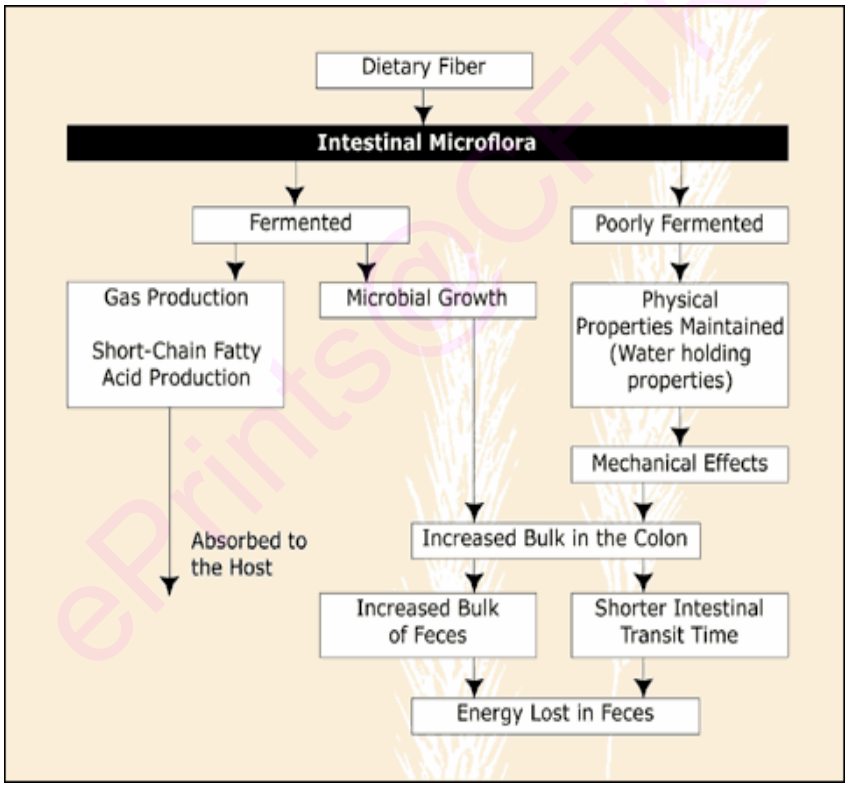


Fig. 11: Mechanism of action of dietary fiber and unabsorbed carbohydrates in increasing colonic and faecal weight and bulk

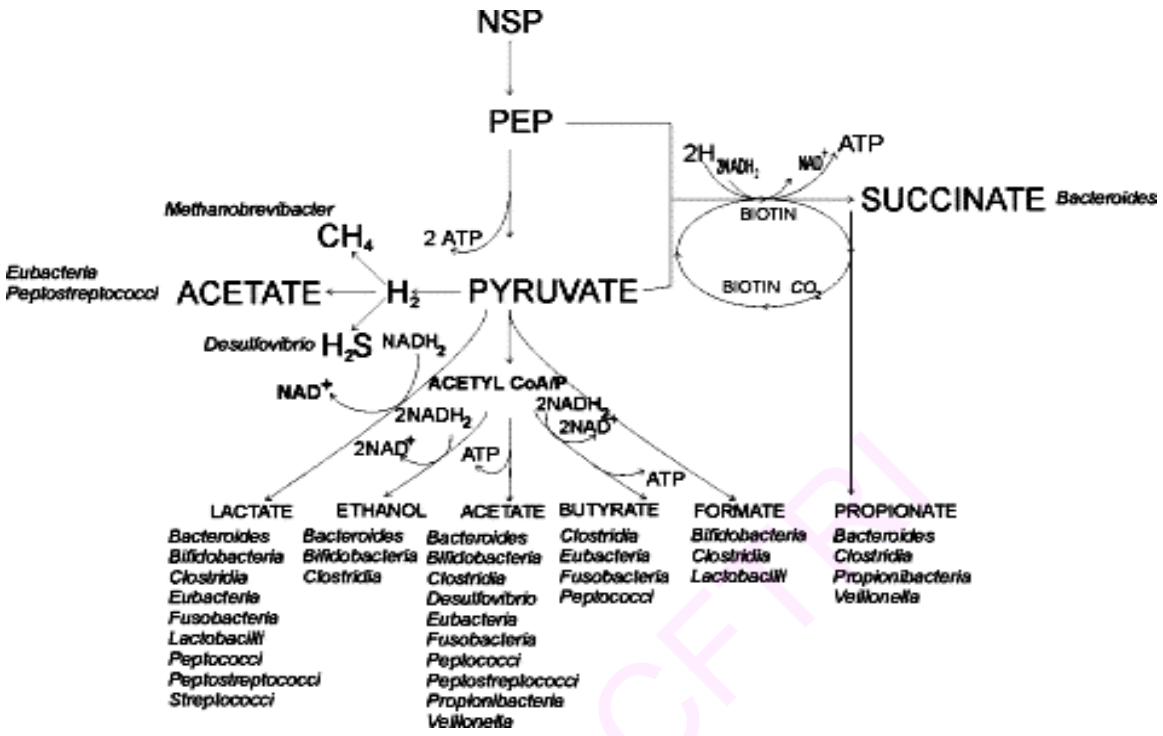


Fig. 12: Metabolic pathways of SCFA in colon.

Absorption of SCFA

Short chain fatty acids are absorbed in the monogastric animals including man via passive diffusion in a manner similar to that observed for rumen epithelium (Fleming et al. 1991). Alternatively, it has been proposed that SCFA may be absorbed via anionic exchange (Argenzio et al. 1977). Short chain fatty acids could be absorbed as un-dissociated acids (non-ionic diffusion), or sodium or potassium salts of short chain fatty acids (ionic diffusion. The absorption has been shown to be accompanied by luminal increase in HCO3 and decrease in CO2, and by increased G1 absorption of sodium, potassium and water. SCFA are most effectively transported at pHs lower than 7.0 and it has been proposed that in the human large intestine 60 % of SCFA are absorbed in the un-dissociated acid form (Ruppin et al. 1980).

Metabolism of SCFA

The SCFA are directly absorbed at the site of production and may be metabolised either locally in the gut, by the liver or by peripheral tissues. The SCFA absorbed may then be used for maintenance, growth and lipogenesis. The enzymatic activation of SCFA by formation of their respective acyl-CoA eg. acetyl-CoA, propionyl-CoA and butyryl-CoA are important factors regulating the rate of uptake of SCFA by different tissue (Roediger, 1982). Rat colonocytes have been shown to possess a butyryl-CoA synthetase, which is more active than the acetyl-CoA and propionyl-CoA synthetases (Roediger, 1982). Most of the butyrate is usually oxidised to CO2 and ketone bodies in pig (Imotso et al. 1978), rabbits, (Marty et al. 1984), rats and humans (Roediger, 1982) by the colonic mucosa during its transportation to the bloodstream. Some of the propionate is also metabolised by the gut. The remaining butyrate, propionate and acetate are transported to the liver via portal vein. The liver removes propionate and butyrate very efficiently and the uptake is close to 100% whereas acetate uptake is generally limited in the liver. Goodlad and Mathers, (1990) observed very low concentrations of propionate and butyrate in peripheral blood and it has been observed that acetate comprises 90-98 % of the SCFA present in both arterial and peripheral blood.

Metabolism of acetate

In ruminants the liver utilizes only a small proportion of the absorbed acetate and acetyl-CoA synthetase activity is low (Bergman, 1990). The acetate metabolism of monogastric animals varies from species to species. For example, lipogenesis occurs in humans and birds mainly in the liver whereas in ruminants and pigs lipogenesis occurs in adipose tissues (Bauman et al. 1975; Leaft, 1983; Pearce, 1977). In rodents, lipogenesis occurs in both tissues. These differences in

lipogenesis seem related to metabolism of acetate, since, acetyl-CoA can be easily incorporated into lipogenesis. In some studies it has been shown that hepatic acetate uptake is directly proportional to the concentration of acetate in the portal vein (Buckley et al. 1977; Remesy et al. 1980). In ruminants, acetate carbon is incorporated into fatty acids by both adipose tissue and the mammary gland more rapidly than is glucose carbon (Ballard et al. 1969; Ballmain et al. 1954; Vernon, 1981), whereas in rats glucose is the preferred substrate. Acetate can be a significant source of fuel for skeletal muscles (Snoswell et al. 1982). However, the quantitative importance and the metabolic fate of acetate in the simple stomach species such as humans and rats are not well understood.

Metabolism of propionate

Propionate is partially metabolized to CO_2 and there is no net gain of oxaloacetate and, therefore, in ruminants propionyl-CoA carboxylase activity has been shown. Feeding food rich in grains increased its activity and amount of propionate absorbed (Baird et al. 1975). Biotin and vitamin B12 have been shown to be essential for the metabolism of propionate (Elliot, 1980). However, the net contribution of propionate to glucose production in simple-stomached animals is still not clear.

Metabolism of butyrate

Butyrate is metabolized by rumen and gut epithelium and by liver. Large amounts of butyrate are taken up by the gut epithelial tissue and by the liver. It has been suggested that only trace amounts can enter the post-hepatic bloodstream. The metabolic pathways of butyrate in ruminants and non-ruminants seem to be similar. Peripherally, butyrate is utilized for the production of energy or used for lipogenesis and is removed for milk fat synthesis1 (Black et al. 1961). Butyrate is

readily oxidized by isolated rat hepatocytes (Roediger, 1982) and is an important source of energy for human colonocytes (Roediger WEW 1980). In liver, butyrate is converted to butyryl-CoA by an enzyme butyryl-CoA synthetase (Ash et al. 1973; Dougherty, 1984). Then it is rapidly converted to acetyl-CoA, longer chain fatty acids or to ketone bodies (Bergman et al. 1964; Katz et al. 1969).

Butyric acid and their derivatives

Among the SCFAs butyric acid has gained lot of attention. The role, played by butyric acid, a four-carbon fatty acid in particular on various physiological functions has received great attention (Bourquin et al. 1996; Cummings et al. 1991). Recent studies have revealed butyric acid to have a number of functions. Butyric acid plays a critical role in colonic homeostasis, because it stimulates pathways of growth arrest, differentiation, histone acetylation and apoptosis (Bordonaro et al. 1999). Butyric acid is shown to have potential role in decreasing the incidence of bowel cancer, which is associated with decreased fiber intake (Riggs et al. 1977; Calabresse et al. 1993). Butyric acid is known to modulate activities of many cellular enzymes including enzymes (Smith et al. 1998) involved in glycoconjugate metabolism such as sulphotransferases and sialyl transferase (Jacobsson et al. 1985; Shah et al. 1992). Analogues of butyric acid having different functional properties have been evaluated during cancer and some of them are undergoing clinical trials for tumor therapy. Tributyrin, an oral butyric acid analogue, induces apoptosis through the activation of caspase (Clark et al. 2000). In *in vitro* studies using cell culture system, sodium butyrate is shown to induce insulin gene expression (Karlsen et al. 1991). JTT-608 (4-trans 4- methyl cyclohexyl 4-0 oxybutyric acid), an analogue of butyric acid is shown to selectively improve glucose tolerance in both normal and diabetic rats (Ohta et al. 1999). Hence, it was our interest to study the effect of butyric acid on diabetic

status in experimentally induced diabetic rats and study fermentation of various dietary fibers *in vitro* and *in vivo*.

Table 4: Butyric acid derivatives and their functions

Analogs	Use
Arginine butyrate	Stimulates expression of human fetal globin gene *in vivo*
Butyramyde	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 antitumer 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
Sodium butyrate	Alleviates leukemia *in vivo*

Source: Smith et al. 1998.

Syzygium jambolana

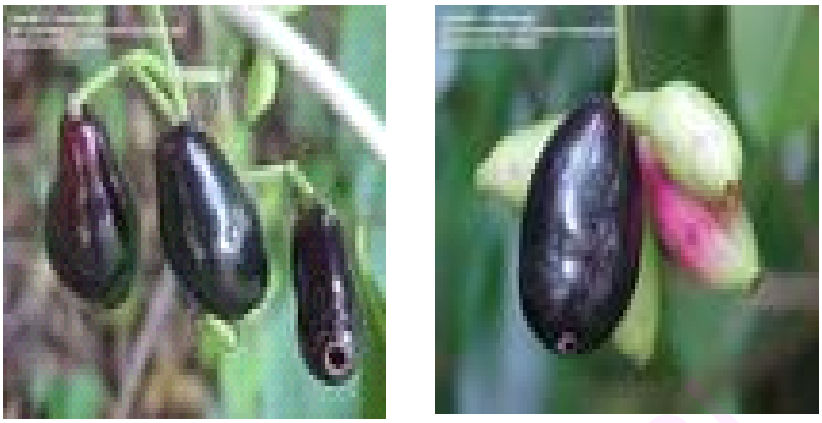


Fig. 13: *Syzygium jambolana* fruit

Syzygium jambolana, commonly called as jamun known for its ethnopharmacological potential in the management of diabetes and is used widely in Ayurvedic medicine. It has been attributed to posses several medicinal properties in the folklore system of medicine (Warrier et al. 1996). The leaves have been extensively used to treat diabetes, constipation (Bhandary et al. 1995; Rastogi et al. 1990), leucorrhoea, stomachalgia, fever, gastropathy, strangury, dermopathy (Korina et al. 1997) and to inhibit blood discharges in the faeces (Tanaka, 1994). Recently, the plant has been reported to poses acetyl oleanolic acid, triterpenoids, ellagic acid, isoquercitin, quercetin, kaempferol and myricetin (Martinez et al. 1981) in different concentrations. Most of these compounds have been reported to possess antioxidant and free radical scavenging activities (Jagetia et al. 2002).

The bark of the plant is astringent, sweet, refrigerant, carminative, diuretic, digestive, antihelminthic, febrifuge, constipating, stomachic and antibacterial. The fruits and seeds are used to treat diabetes, pharyngitis, spleenopathy, and urethrorrhea and ringworm infection. The fruits of wild Indian *Syzygium cumini* L. Skeels (Black Plum) are edible and are reported to contain vitamin C, gallic acid,

tannins, anthocyanins, includes cyanidin, petunidin, malvidin-glucoside and other components (Prince et al. 1998; Sharma et al. 2003).

The juice of unripe fruits is used for preparing vinegar that is considered to be a stomachic, carminative and diuretic. The ripe fruits are used for making preserves, squashes and jellies. The fruits are astringent. Wine is prepared from the ripe fruits. The leaf extract of *S. cumini* protects against radiation-induced DNA damage (Grover et al. 2000). Extract of seed, which is traditionally used in diabetes has hypoglycemic action and antioxidant property (Ravi et al. 2004), possibly due to tannin (Chopra et al. 1956). Both the aqueous and ethanolic extracts from the seeds reduced the glycaemia in diabetic animals (Sharma et al. 2003; Pandey et al. 2002; Prince et al. 1998). Seeds of *E. jambolana* contain an alkaloid - jambosine (Ravi et al. 2004), gallic acid, ellagic acid, corilagin and related tannin, quercetin. The 'Cogent db' capsules contain the powder of jamun seeds as one of the ingredients (Fig. 14). These are available in the market and are popularly used for the management of diabetes.

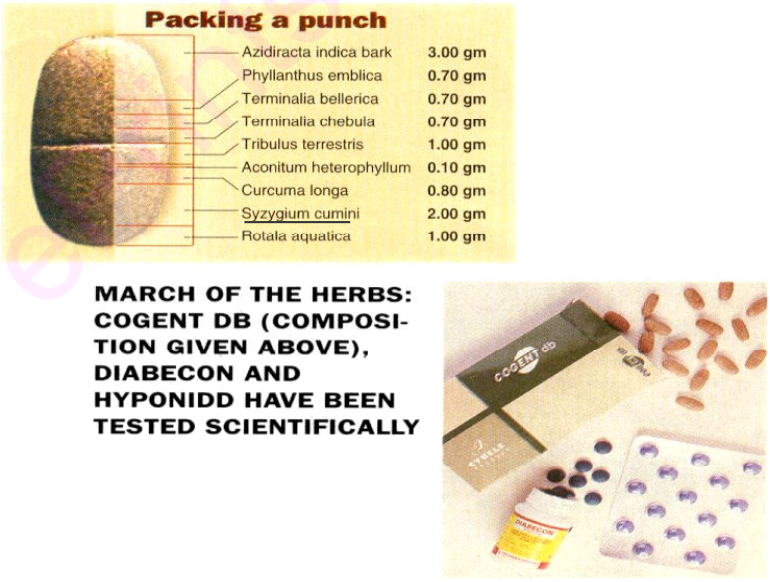


Fig. 14: Ayurvedic capsule - Cogent DB, and its constituents.

Source: *The week* - A biweekly magazine (1998). 147

Moringa oleifera

Fig. 15: *Moringa oleifera* fruit

Moringa oleifera Lam. commonly known as drumstick cultivated throughout India, mainly for its edible fruits. Parts of this plant like leaves, flowers, roots and fruits have been traditionally used for dietary purposes as vegetables (Qaiser, 1973; Siddhuraju et al. 2003) and are believed to possess some medicinal properties. The plant was reported to contain various amino acids, fatty acids, vitamins, and nutrients. Leaves of this plant are traditionally known for or reported to have various biological activities, including hypocholesterolemic (Ghasi et al. 2000) regulation of thyroid hormone status (Tahiliani et al. 2000), anti-diabetic effect (Makonnen et al. 1997), gastric ulcers (Pal SK etal. 1995) anti-tumor agent (Murakami et al. 1998) and hypotensive agent (Faizi et al. 1995), anti-tumor effect. The leaves, as well as the flowers, roots, fruits and seeds are extensively used for treating inflammation (Ezeamuzle et al. 1996) cardiovascular diseases (Limaye et al. 1995) liver disease (Rao et al. 1998) and renal disorders (Mazumder et al. 1999). It is generally known in the developing world as a vegetable, a medicinal plant and a source of vegetable oil (Oliveira et al. 1999). However, in Sudan it has been traditionally used in water purification. Numerous

recent studies have shown that *M. oleifera* seeds possess effective coagulation properties (Jahn, 1988; Ndabigengesere et al. 1998) and they are not toxic to humans or animals (Ghasi et al. 2000).

Moringa oleifera is incorporated in various marketed formulations, such as Rumalaya and Septilin (The Himalaya Drug Company, Bangalore, India), Orthoherb (Walter Bushnell Ltd., Mumbai, India), Kupid Fort (Pharma Products Pvt. Ltd., Thayavur, India) and Livospin (Herbals APS Pvt. Ltd., Patna, India), which are available for a variety of disorders. The leaves of *M. oleifera* were used by the Indians in their herbal medicine as a hypocholesterolaemic agent in obese patients. The scientific basis for their use in hypercholesterolaemia was examined by Ghasi et al 2000. They tested the crude extract of leaves of *M. oleifera* and showed that it possessed hypocholesterolaemic activity. This led them to conclude that there is a valid pharmacological basis for employing the leaves for this purpose. The fruit of *M. oleifera* contains proteins, fats, carbohydrates, minerals, fiber, Vitamin A, nicotinic acid, ascorbic acid.

Preliminary studies demand further inquiry so that their novel possibilities as a source of oral hypoglycemic agents could be investigated. There is scope for more extensive research in this field, especially to examine the long-term beneficial effect of dietary vegetables, to identify the active principle and to understand the mechanism of action, which is presently unclear. Since diet forms the mainstay in the management of diabetes mellitus, there is scope for exploiting the antidiabetic potency of vegetables to the maximum extent. Such plant food adjuncts possessing hypoglycemic activity appear to hold promise as potential anti-diabetic agents.

Fruit of *M. oleifera* is widely consumed by the people of India, Even though, much work has been done on beneficial effect of leaves and other parts of the plant, there are no reports on antidiabetic property of the fruit. The fruits are

good source of soluble dietary fiber, hence it was considered worthwhile to study the effect of fruit pulp on diabetic and diabetic nephropathy state with an emphasis on heparan sulphate.

Antioxidants and Bioactive polysaccharides

In normal physiological conditions, both oxidants and antioxidants are maintained in balance. During disease conditions these imbalances lead to oxidative damage to biomolecules and cells (Temple, 2000; Willett, 2002). Oxidative stress is now well established to play important role in many disorders including diabetes. Oxidative stress results from an imbalance between the generations of oxygen derived radicals and the organism's antioxidant potential (Halliwell et al. 1992). Various studies have shown that diabetes mellitus is associated with increased formation of free radicals and decrease in antioxidant potential. Due to these events, the balance normally present in cells between radical formation and protection against them is disturbed. This leads to oxidative damage of cell components such as proteins, lipids, and nucleic acids. In both insulin dependent (Type I) and non-insulin-dependent diabetes (Type II) there is increased oxidative stress (Maritim et a1. 2003). Conflicting results have been reported for the role of oxidative stress in diabetes. A correlation between impaired glycemic control and enhanced lipid peroxidation has been reported (Altamer et al. 1991; Velazquez et al. 1991).

Diet consisting of fruits and vegetables and spices has been shown to contain health beneficial components and are mainly attributed antioxidants. Among them phenolic compounds having antioxidant property are receiving much attention. These have been known for their stable radical intermediates, which can effectively prevent the oxidation at cellular and physiological level (Croft, 1998)

Antioxidants counter the action of free radicals by several mechanisms. These mechanisms include: Enzymes that degrade free radicals, proteins such as transferrin that can bind metals which stimulate the production of free radicals, and antioxidants such as vitamins C and E that act as free radical scavengers (Formica et al. 1995).

Recently, there has been a considerable interest in finding natural antioxidants from plant materials to replace synthetic ones. Data from scientific reports show that plants contain a large variety of substances that possess antioxidant activity (Ames et al. 1993). Phytochemicals with antioxidant effects include cinnamic acids, coumarins, diterpenes, flavonoids, lignans, monoterpenes, phenylpropanoids, tannins and triterpenes (Hertog et al. 1993). Natural antioxidants occur in all higher plants and in all parts of the plant. The occurrence of oxidative mechanisms in plants may explain why an abundance of antioxidant compounds have been identified in plant tissue (Maillard et al. 1996). Therefore, it seems that plants particularly those with high levels and strong antioxidant compounds have an important role in improvement of disorders including oxidative stress induced disorder such as diabetes mellitus. There are many investigations, which have reported the effects of different plants and their antioxidant ingredients on diabetes and its complications and have aroused interest (Galli et al. 2002).

Bioactive polysaccharides

Polysaccharides are gaining lot of importance in recent years because of their biological functions such as anti-tumor, anti-ulcer, immunological, anti-complimentary, anti-inflammatory, anti-coagulant, and immunomodulatary activities (Shin et al. 1997; Borchers et al. 1997; Masumorthy et al. 1995). Polysaccharides possessing such biological activities are found in seaweeds, fungi

and higher plants (Yamada, 1994; Whistler et al. 1976). Beta glucans are also receiving interest for the antitumorogenic activity. They are isolated from mushrooms and cereals and have been characterized (Takashi et al. 1995). Hypoglycemic activity of some of the polysaccharides from plants has been reported and considerable literature is available on selective polysaccharides of their hypoglycemic activity (Ling-Hua et al. 1993; Marles et al. 1995).

Though the antidiabetic property of *S. jambolana* has been done with different part of the plant and their isolated fractions, no work has been done on their effect on diabetic nephropathy status with particular emphasis on kidney heparan sulphate. Fruit of *M. oleifera* is widely consumed by the people of India, Even though much work has been done on beneficial effect of leave and other parts of the plant there are no report on antidiabetic property of the fruit, which is good source of soluble dietary fiber. Hence it was considered worthwhile to study its hypoglycemic activity. Although butyric acid plays an important role in modulation of different physiological conditions in many diseases, its beneficial effect on diabetes is not well understood.

Hence, the present investigation focuses on

1. Effect of butyric acid on modulation of diabetic status.
2. *In vitro* and *in vivo* fermentation of dietary fibers.
3. Effect of *S. jambolana* seed and *M. oleifera* pulp on diabetic nephropathy state with particular emphasis on heparan sulphate.
4. Carbohydrate profile of *S. jambolana* and the study of its antioxidant activity.

2. MATERIALS AND METHODS

2.1. Microbiological studies

2.1.1. Chemicals used

Nutrients used in the media preparation such as yeast extract, peptone, agar agar, were obtained from Hi Media, Mumbai, India. Acetic acid, propionic acid, butyric acid and valeric acids were procured from Sigma chemicals, USA. Glycine, arginine, sodium phosphate, potassium dihydrogen phosphate, magnesium sulphate, urea, ammonium sulphate and sodium nitrate were obtained from Ranbaxy laboratories Ltd, India. Acetone and ethanol were double distilled prior to their use.

2.1.2. Substrates used for fermentation

2.1.2a. Sugars: Glucose, galactose, rhamnose, xylose, arabinose, mannose, glucuronic acid (SRL, India).

2.1.2b. Polysaccharides: Citrus pectin, arabinoxylan, cornstarch.

2.1.2c. Dietary fiber: Wheat bran, guar gum.

2.1.2d. Fiber sources: Bitter gourd fruit (*Momordica charantia*), fenugreek seeds (*Trigonella foenum graceum*), spent turmeric rhizome (*Curcuma longa*), drumstick (*Moringa oleifera)*, *Syzygium jambolana* (*Eugenia jambolana*) and chitosan.

2.1.2e. Substrates for experiments

Wheat bran was obtained from Milling and Baking Technology Department, CFTRI, Mysore. Starch was removed by adopting the method described by Johansson et al. and was powdered. Fenugreek was purchased from local market and powdered in a dry grinder. Bitter gourd and drumstick fruits were purchased from local market, pulp of both fruits were scraped and dried at 40 ^{0}C and powdered. Turmeric-spent (produced after removal of curcumin), a by-

product of turmeric was obtained from turmeric industry. Cornstarch was purchased from local market and guar gum was obtained from Indian Gum Industry, Mumbai, India.

2.1.3. Anaerobic gas pack

Anaerobic gas pack from Himedia, Mumbai, India was used to create anaerobic environment in the anaerobic chamber for bacterial growth.

2.1.4. Media and their composition

Composition of various media used for experiments are shown in Tables 5-9. These media (50 mL) were prepared in 250 mL capacity Erlenmeyer flasks and autoclaved at 15 lbs and 121^{o}C for 20 min, cooled and used in the experiments. Experiments were carried out in duplicates.

Table 5: Anaerobic agar

Ingredients	**g/L**
Casein enzymatic hydrolysate	20.0
Dextrose	10.0
Sodium chloride	5.0
Sodium thioglycollate	2.0
Sodiumformaldehyde sulphoxylate	1.0
Methylene blue	0.002
Agar	20.0

Table 6: MacConkey agar

Ingredients	**g/L**
Peptic digest of animal tissue	20.0
Lactose	10.0
Sodium chloride	5.0
Bile salts II	1.5
Natural Red	0.05
Crystal Violet	0.001
Agar	15.0

Table 7: MRS agar

Ingredients	**g/L**
Proteose peptone	10.0
Beef extract	10.0
Yeast extract	5.0
Dextrose	20.0
Polysorbate 80	1.0
Ammonium citrate	2.0
Sodium acetate	5.0
Magnesium sulphate	0.1
Manganese sulphate	0.05
Dipotassium phosphate	2.0
Agar	12.00

Table 8: Composition of dissolving solution (pH 7.0)

Components	**mg/100 mL**
KH_2PO_4	90
$NaCl_2$	90
Na_2SO_3	90
$CaCl_2$ H_2O	2
$MgCl_2$	2
Na_2CO_3	400
Cystein HCl	50

Table 9: Nutrient agar

Ingradients	**g/L**
Peptic digest of animal tissue	5.0
Sodium chloride	5.0
Beef extract	1.5
Yeast extract	1.5
Agar	20.0
Peptic digest of animal tissue	5.0

2.1.5. Sterilization of glassware: Glasswares such as pipettes and petriplates used for the experiments were sterilized using dry heat in copper containers at 180^{o}C for 90 min.

2.1.6. *In vivo* study

2.1.6.1. Animals and group

Male albino rats (Wistar strain) of 90 days old weighing 100-110 ± 2g were obtained from animal House facility of CFTRI, Mysore. The studies had clearance from Institutional Animals Ethics Committee. The animals were grouped as shown below in to starch fed control (SFC), wheat bran fed (BFC) and guar gum fed (GFC) groups. Each group consisted of six animals. They were housed individually in stainless steal cages with screen bottom in a room maintained at 25 ± 2^{o}C and relative humidity of 45%. Food and water were given *ad libitum*. Composition of experimental diets is given in Table10-12. Food intake by animals was monitored. Body weights were measured once in a week and after 35 days the animals were sacrificed and the colon, intestine and other organs were collected in sterile containers.

2.1.6.2. Diet composition and feeding

Diets of defined compositions were prepared (Table 10) and the animals were maintained with these diets for 35 days.

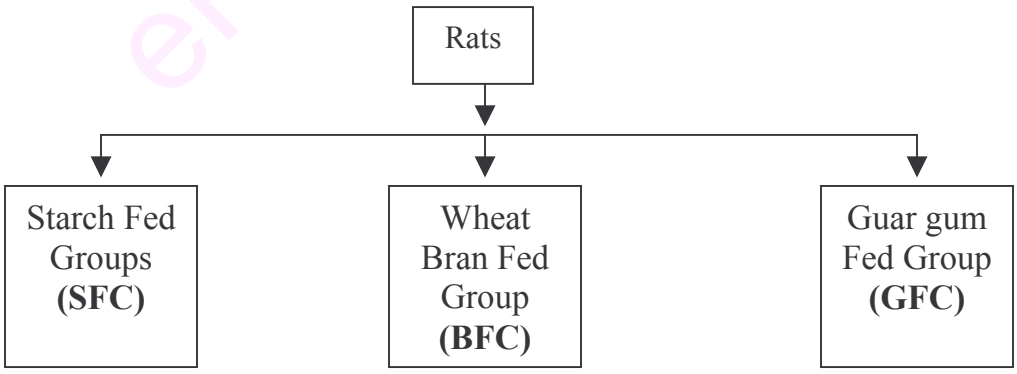


Table 10: Composition of experimental diets (Bieri et al. 1997)

Components	SFC	BFC	GFC
		g/kg diet	
Casein	200	200	200
AIN-76 vitamin mix*	10	10	10
AIN-76 mineral mix**	35	35	35
Choline chloride	2	2	2
Fat	100	100	100
Cornstarch	653	628	553
Wheat bran (10%)	-	100	-
Guar gum (5%)	-	-	50

*, ** Details are given under nutritional studies;
SFC: Starch fed control; BF: Wheat bran fed group;
GF: Guar gum fed group

2.1.6.3. Collection of samples

2.1.6.3a. Faeces and urine

During sampling animals were transferred to metabolic cages equipped with funnels and mesh at the bottom of the cage to separate urine and faeces. Urine was collected in bottle placed in the bottom of each funnel containing a layer of toluene. Both samples were stored under refrigeration for further analysis.

2.1.6.3b. Collection of caecal content

Immediately after the dissection, sides of the colon were tied with thread to maintain anaerobic environment and transferred to sterile beaker placed in anaerobic jar having anaerobic gas pack. Caecal contents were removed from colon and weighed under sterile condition. Known amount of the sample was taken for short chain fatty acid analysis.

2.1. 6. 3c. Isolation of bacteria from caecal content (AOAC, 1960)

Caecal bacteria were collected from albino rats after feeding the diet for 30 days. Rats were anaesthetized and their intestines were collected under sterile conditions. The caecal contents were transferred to sterile saline, serially diluted three times, and were plated on nutrient agar, MRS agar, and Mac Conkey agar plates. The plates were incubated at 37^{o}C for 24-48 h. Colonies with different morphologies were picked-up, streaked repeatedly on respective agar plates for purification. Isolated colonies from the above mentioned media were transferred on to the respective agar slants, incubated at 37^{o}C for 48 h and stored at 4^{o}C till further use. Sub-culturing of isolated cultures was carried out once in a month. Amongst various cultures isolated, 4 were selected based on preliminary experiments and maintained on nutrient agar medium at 4^{o}C for further studies.

2.1.6.3d. Collection of intestine

Intestine was collected in sterilized container. Length of the intestine was measured and content was flushed with saline to remove the undigested material.

2.1.6.4. Viable counts of bacteria from caecal/ faecal content

Fresh caecal/ faecal contents were collected in sterile beaker covered with aluminum foil and immediately transferred to anaerobic chamber containing

anaerobic gas pack. One gram of sample (faecal/ caecal) was taken in sterilized test tubes containing 9 mL dilution buffer, serially diluted and plated on to MRS, Nutrient agar, Maconckey agar and anaerobic agar. Plates were incubated at 37 ^{0}C under aerobic and anaerobic conditions for 24 and 48 h. Colonies were counted and bacterial load was calculated per g of sample.

2.1.6.5. Inoculum

2.1.6.5a. Faecal inoculum

Fresh faeces were collected from three groups separately in to sterilized beakers, immediately transferred to anaerobic chamber, pooled and homogenized with the media or dilution buffer. Sediment free supernatant was used as faecal mixed culture or inoculum (10%, v/v). The inoculum had 1 x 10^5 cells/mL.

2.1.6.5b. Caecal inoculum

Known amount of caecal content was diluted in sterile saline and used as inoculum (1 x 10^5 cells/mL) at 10% (v/v).

2.1.6.5c. Purified strains

Cells from 24 h old slant culture were inoculated into 500 mL Erlenmeyer flasks that contained nutrient medium. The culture was grown on a rotary shaker maintained at 200 rpm at the ambient temperature of 30^{o}C for 24 h. The cultures were used as inoculum at 10%, v/v (1 x 10^3 cells/mL) level for subsequent experiments.

2.1.7. *In vitro* fermentation

Various experiments were carried out to find out the effect of caecal and faecal mixed flora and purified cultures on fermentation of various substrates rich in dietary fibers to release SCFA. All the experiments were carried out in duplicates in 250 mL capacity Erlenmeyer flasks with 50 mL of nutrient medium

and 1% of dietary fiber rich substrates. Unless otherwise mentioned, the flasks were inoculated with 10% of faecal or caecal or purified inoculum (as mentioned above) and the flasks were incubated anaerobically at 37^{o}C for 48 h. At the end of fermentation the samples were extracted to estimate SCFA. Viable cell counts or optical densities (620 nm) were also analysed wherever needed. Different experiments carried out are as follows:

2.1.7a. Production of SCFA from different sugars and polysaccharides by faecal mixed flora

Various carbon sources such as glucose, rhamnose, galactose, fucose, arabinose, mannose, xylose, glucuronic acid, maltose and polysaccharides such as pectin, cornstarch and arabinoxylan were used at 1% (w/v) in nutrient medium. Substrates such as wheat bran, guar gum and cornstarch (1%) were used in the experiment. Flasks without the substrate but 1% glucose served as control.

2.1.7b. Effect of different caecal and faecal microflora (based on substrate used for growth of rats) on fermentation of substrate for butyric acid production

Caecal and faecal microflora obtained from rats fed with cornstarch, wheat bran and guar gum were used as inoculum sources to ferment cornstarch, wheat bran and guar gum as substrates (1%) in nutrient medium. Viable cell counts obtained under different feeding of rats were also analyzed.

2.1.7c. Effect of incubation period, substrate concentration and pH on butyric acid production by rat faecal mixed culture

Effect of fermentation period (24 h and 48 h) on butyric acid production was studied using rat faecal mixed flora on cornstarch (CS), wheat bran (WB) and

guar gum (GG) as substrates at 1% concentration. pH of the fermented broth was measured at the end of fermentation.

2.1.7d. Production of short chain fatty acids by various dietary fiber sources by rat faecal and caecal mixture

Dietary sources such as powdered fenugreek seeds, dried pulp powder of bitter gourd and drum stick and dried powder of jambolana (1%) in nutrient medium were fermented by mixed faecal and caecal microflora.

2.1.7e. Production of SCFA from different isolated polysaccharide fractions of jambolana by faecal mixed culture

From the previous experiment it was noted that black plum supported production of good quantity of butyric acid. Hence various polysaccharide fractions were isolated from the pulp, seed coat and seeds and they were used individually (1%) in nutrient medium to assess their effect on butyric acid production by mixed faecal microflora.

2.1.7f. Butyrogenic property of purified bacteria isolated from rat intestine from drumstick pulp

By preliminary experimentation it was found that out of various bacterial cultures isolated from rat intestine, cultures 3a, 3b, 6a and 6b had good SCFA production property. Hence these cultures were grown in the presence of drumstick pulp powder (1%) in the nutrient medium.

2.1.7g. Butyric acid production by isolated bacteria grown on guar gum (soluble dietary fiber) and wheat bran (insoluble dietary fiber)

Purified isolates (3a, 3b and 6b) isolated from rat intestine were also cultivated on wheat bran and guar gum (1%) containing nutrient medium to assess their ability to produce SCFA on these substrates.

2.1.7h. Butyric acid production from various dietary fiber sources (1%) incubated with *Bifidobacterium sp.* (isolate 6a)

Amongst the four purified cultures used in the above-mentioned experiment, culture no. 6b produced higher titers of butyric acid on various substrates. This was identified by morphological and biochemical methods as *Bifidobacterium sp.* This isolate was cultivated in the presence of cornstarch, wheat bran, drum stick and bitter gourd pulp, fenugreek seed powder, spent turmeric powder and chitosan.

2.1.8a. Effect of dietary fibers on the *in vivo* fermentation and production of SCFA by caecal and faecal microflora

Cornstarch, wheat bran and guar gum were fed to rats as mentioned earlier for a period of 35 days. Gain in body weight, urine and faecal output were measured regularly. Viable cell counts, SCFA concentration in caecal and faecal materials were analyzed.

2.1.8b. Changes in caecal weight and viable microbial population due to butyric acid supplementation to diabetic animals

Control animals and diabetes-induced animals were fed with cornstarch, wheat bran plus guar gum diets. Another group of animals were fed similarly but with additional supplementation of butyric acid (500 mg/kg body weight). At the

end of 35 days, microbial population in caecum, faecal matter, weight of caecum and caecal matter were analysed.

2.1.9. Analysis of fermented broth

2.1.9a. Estimation of biomass

Culture broth was centrifuged at 10,000 rpm for 20 min. Sedimented cells were washed with distilled water and biomass was dried at 70^{o}C to a constant weight.

2.1.9b. pH measurement: pH was measured by using pH meter (C.D. Instruments PVT Ltd India).

2.1.9c. Bacterial density: Taking Optical Density (OD) at 620 nm in a Shimadzu UV-160A spectrophotometer measured microbial density.

2.1.10. Short chain fatty acid extraction

2.1.10a. Fermented sample

Fermented samples were centrifuged at 6,000 rpm at 4^{o}C for 10 min and supernatant was used for SCFA extraction. Supernatant was acidified with 50% sulphuric acid and then short chain fatty acids were extracted twice with diethyl ether (Karppien et al. 2000) and concentrated with nitrogen gas (Silvi et al. 1999) and used for gas liquid chromatography analysis.

2.1.10b. Faecal and caecal matter

The samples (0.5 g) were taken in test tubes and acidified with 50 % H_2SO_4. The acidified solution was then extracted thrice with diethyl ether.

2.1.11. Gas liquid chromatography analysis (Mahadevamma et al. 2004)

PEG 20 M column was used and nitrogen (N_2) was the carrier gas with a flow rate of 40mL /min. Oven, detector and injector temperatures were 120, 220 and 230^{o}C, respectively. SCFAs were identified based on retention time of standards-acetic acid, propionic acid and butyric acid (Fig. 16). Relative percentage of individual acids was calculated by using the formula;

$$\text{Relative \% of Acid} = \frac{\text{Individual area}}{\text{Total area}} \times 100$$

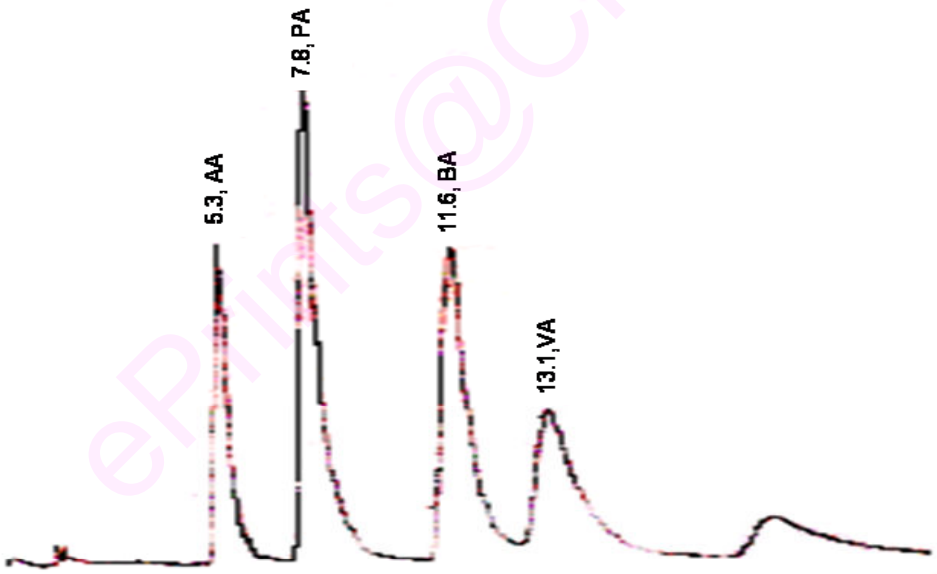


Fig. 16. GLC chromatogram of standard short chain fatty acids

AA: Acetic acid PA: Propionic acid

BA: Butyric acid VA: Valeric acid

2.1.12. Identification of isolated bacterium (*Bifidobacterium sp*.)

The strain was subjected to various morphological and biochemical tests as per standard microbiological procedures. Gram stain, cell morphology, spore formation, catalase test, production of ammonia from arginine, oxidase test, liquefaction of gelatin, indole production, resistance to bile salt, acid and gas production (at 2% concentration) from glucose, arabinose, cellobiose, fructose, lactose, galactose, dextrin, starch, raffinose, maltose, inulin, sorbitol, salicin, sucrose, trehalose, ribose, melibiose and xylose were tested.

2.2. Nutritional studies

2.2.1. General chemicals used

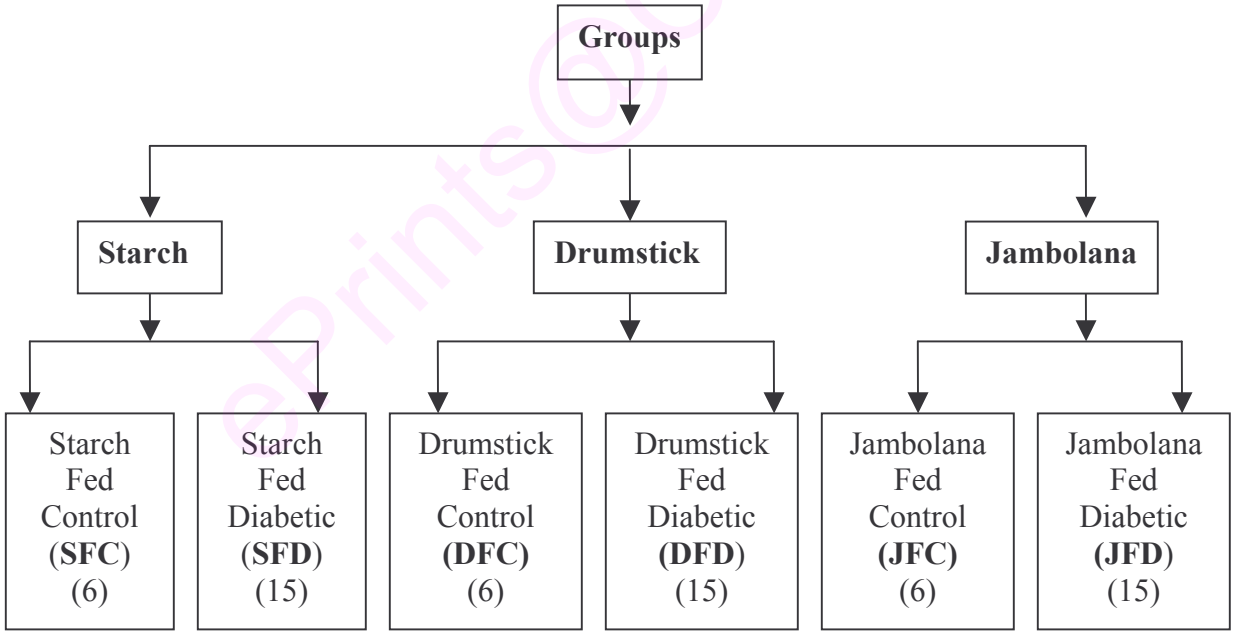
Glucose oxidase, p-nitrophenyl-N-Acetyl-glucosaminide, P-nitriphenyl-β-glucuronide, streptozotocin, toludine blue, alcian blue, carbozole, dimethyl aminobenzaldehyde, chondoitinase ABC, peroxidase, Dowex-2 (chloride form), blue dextran, Coomassie brilliant blue G- 250, carbodimide (1-cyclo-hexyl -2 (4-methymorpholine-ethyl p-toluene sulfonate), carbazole, catechin, dinitrosalicylic acid, deuterium oxide, iodomethane (methyl iodide), ruthenium red, sodium azide, sodium borohydride, Tris [(hydroxyl methyl) methyl amine (2-amino-2-(hydroxyemthyl) propane -1,3 diol)], 1,1-diphenyl-2 picryl hydrazyl (DPPH), 2-thiobarbituric acid (TBA), were from Sigma Chemical Company, St. Louis, USA.

Dimethyl methylene blue was procured from Aldrich, Milwaukee, USA. Folin and Ciocaliteu 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.

Trifluroacetic acid (spectroscopy grade) was procured from spectrochem, Mumbai, India. Vitamins and casein were obtained from Hi media, Mumbai, India. Cornstarch and groundnut oil (refined, postman Brand) was purchased from a local market, Mysore, India. All other chemicals and reagents used were of Analytical grade.

2.2.2. Animals and their classification

Male Wistar rats weighing between 110-120 g were taken from Animal house facility of CFTRI, Mysore. The study had the clearance of Institutional Animal Ethical Committee. The rats were divided in to three main groups as starch based, jamun based and drumstick based control and diabetic. The control group had six rats, while diabetic had fourteen rats. Animals were housed individually in stainless steel cages and were maintained for 45 days.



Abbreviations: SFC: Starch fed control, DFC: Drumstick fed control, JFC: Jambolana fed control, SFD: Starch fed diabetic, DFD: Drumstick fed diabetic, JFD: Jambolana fed diabetic, Numbers in the parenthesis indicates number of animals in that group.

2.2.3. Diet preparation and feeding:

Fresh jamun *(S. jambolana)* and drumstick (*M. oleifera*) were obtained from the local market. Pulp of the jamun fruits was manually separated from the seeds and the seeds were washed thoroughly, sun dried and powdered to 60 M size. Fresh drumsticks were cleaned manually, opened and the pulps along with seeds were dried in air drier maintained at 40^{o}C. Jamun seed powder was supplemented at 25 g/kg diet and drumstick pulp powder was given at 100 g/kg diet in AIN-76 diet (Table 11). The diet and water were given *ad libitum*. Rats were acclimatized for 1 week with respective diets before the induction of diabetes. After induction of diabetes rats were fed for 45 days before sacrificing.

Table 11: Composition of basal and experimental diet

Components	SFC/SFD	DFC/DFD	JFC/JFD
	g/kg diet		
Casein	200	200	200
AIN-76 vitamin mix*	10	10	10
AIN-76 mineral mix**	35	35	35
Choline chloride	2	2	2
Fat	100	100	100
Cornstarch	653	553	628
Drumstick pulp	-	100	-
Jamun seed	-	-	25

*AIN-76 Vitamin mixture: (g/kg mixture)

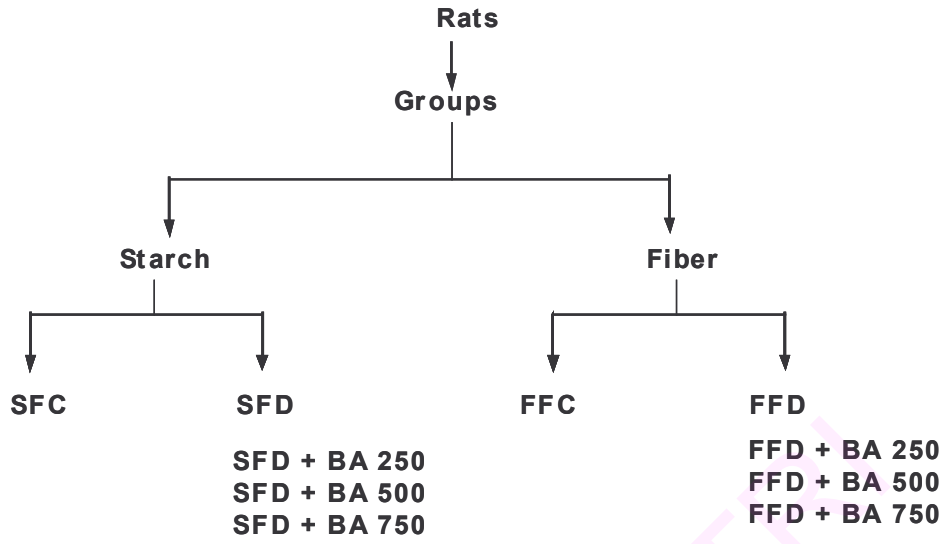
Thiamine hydrochloride 0.6; riboflavin 0.6; pyridoxine hydrochloride 0.7; nicotinic acid 3.0; D-calcium pantothenate 1.6; folic acid 0.2; D-biotin 0.02; cyanocobalmine (Vit. B_{12}) 0.001; retinyl acetate 0.4 (4,00,000 I.U); DL-α-tocopherol acetate 7.7 (5000 I.U); cholecalciferol 0.0025 (1,00,000 I.U);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

**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% Zn_2O) 1.6; cupric carbonate (53-55% Cu) 0.3; potassium iodate 0.01; sodium selenite 0.01; chromium potassium sulphate 0.55; sucrose (finely powdered) was added to make to 1000 g.

2.2.4. Classification of animals for butyric acid experiment

Male Wistar rats weighing between 110-120 g were divided into two main groups. The first group received starch-based diet without fiber and the second group received diet supplemented with fibers. Each group was subdivided into control and diabetic groups. The control group had 6 animals while the diabetic group had 14 animals. The experimental group received butyric acid at 250, 500 and 750 mg/kg body weight/day in drinking water. Composition of basal AIN-76 diet is shown in Table 12.



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

FFC: Fiber fed control, FFD: Fiber fed diabetic,

Butyric acid (BA) is supplemented at 250, 500, 750 mg/kg body weight.

2.2.5. Butyric acid feeding

Butyric acid feeding was started after a week of streptozotocin injection. Diabetic animals were further classed in to three groups and butyric acid was fed at 250, 500, and 750 mg/kg body weight for each group. Known amount of butyric acid (SRL, Analytical grade, Mumbai, India) was given in drinking water via feeding bottles. From the quantities of water-containing butyric acid consumed by the rats, the amount of butyric acid and water consumptions were calculated.

Table 12: Composition of basal and experimental diet (for butyric acid experiment)

Components	SFC/SFD	FFC/FFD
	g/kg diet	
Casein	200	200
AIN-76 vitamin mix	10	10
AIN-76 mineral mix	35	35
Choline chloride	2	02
Fat	100	100
Cornstarch	653	578
Wheat bran	-	50
Guar gum	-	25

2.2.6. Induction of diabetes (Hatch et al. 1995)

Streptozotocin dissolved in freshly prepared citrate buffer (0.1 M, pH 4.5) was injected intraperitonially at a concentration of 55 mg/kg body weight. Control rats were injected with citrate buffer only. The rats were given 5% glucose solution over night, following streptozotocin injection.

2.2.7. Collection of blood and urine

After the induction of diabetes, blood was drawn from retro-orbital plexus using heparinised capillary tubes in test tubes containing heparin (20 U/mL blood) for measuring fasting blood glucose. At the end of the experiment, blood was

drawn before sacrifice of animals. Urine was collected under a layer of toluene by keeping rats in metabolic cages.

2.2.8. 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) were excised, washed with cold saline, blotted dry, weighed and stored at -20^{o}C till further analysis.

2.2.9. Processing of kidney tissues (Allalouf et al. 1964)

All the tissues were cut into small pieces and stored in acetone in cold room for a month. At the end of every week acetone was changed with fresh acetone. After drying in acetone, the tissues were defatted by Soxhlet extraction with petroleum ether (40-60) for 6 h. Dried and defatted tissues were ground to a fine powder using pestle and mortar.

2.2.10. Isolation of glycosaminoglycans (GAGs) from kidney tissues (Scott, 1960)

Kidney tissue (1 g) was suspended in 20 mL of phosphate buffer (0.1 M, pH 6.5). Papain [10 mg papain in 1 mL phosphate buffer containing EDTA (0.005 M)] was first activated by keeping at 65^{o}C for 30 min in a water bath. The activated papain solution (1 mL) was added to the tissue suspension and digested for 44 h at 65^{o}C in an oven. Enzyme solution (1 mL) was again added at the end of 24 h. After digestion, it was centrifuged. 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 (Mikuni et al. 1979)

To 0.05 mL of glycosaminoglycan solution containing 10-15 µg of sulphated polysaccharide, 0.05 mL of 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 control without chondroitinase ABC was subjected to digestion as described above. After the digestion, an aliquot was 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 digested glycosaminoglycan gives the amount of chondroitin sulphate.

2.2.12. Agarose gel electrophoresis (Van de Lest et al. 1994)

Preparation of reagents

Solution A: Agarose (1 g, low EEO) was dissolved in 100 mL dH_2O by warming.

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

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

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

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

Agarose gel was casted on the 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 containing solution B. The samples were run for 5 h at 80 V using LKB Broma 2297 Macrodrive-5 power pack. After the run, the gel was stained in solution D overnight. It was then distained with solution E, till the gel was cleared of background colour.

2.2.13. Urine and plasma analysis

2.2.13a. Reducing sugar estimation (Miller, 1989)

Preparation of reagent

Dinitrosalicylic acid (DNS, 1 g) and sodium potassium tartarate (30 g) were dissolved in 100 mL of sodium hydroxide (0.4 M). To 1 mL of the urine sample containing approximately 100-1000 µg of reducing sugar, 1 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).

2.2.13b. Uronic acid estimation (Dische, 1947)

Sample (0.5 mL) was taken in test tubes and kept in ice cold for 10 min and then 3 mL of concentrated sulphuric acid was added slowly. Mixed thoroughly and kept for incubation for 20 min in boiling water bath. To the cooled sample, 0.1 mL carbazole (0.1%, prepared by dissolving carbazole in ethanol) was added and kept in dark for 2 h and the absorbance was recorded at 530 nm. Uronic acid was determined by refering to the standard graph prepared using D- glucuronic acid.

2.2.13c. Creatinine estimation (Folin, 1919)

To 250 µL of sample, 0.5 mL of distilled water, 3 mL of saturated picric acid reagent and 2 mL of sodium hydroxide were added. It was mixed well and allowed to stand at room temperature for 20 min and the absorbance of Blank (B), Standard (S) and Test (T) was measured against distilled water on a spectrophotometer at 520 nm.

Calculation

$$\text{Plasma creatinine in mg \%} = \frac{\text{A of (T)} - \text{A of (B)}}{\text{A of (S)} - \text{A of (B)}}$$

2.2.13d. Glucose estimation (Dahlqvist, 1964)

To 10 µL of plasma, 1 mL of glucose reagent was added and kept for 15 min at room temperature. Absorbance was read at 505 nm in a spectrophotometer.

$$\text{Glucose (mg/dL)} = \frac{\text{Absorbance of sample at 505 nm X 1000}}{\text{Absorbance of standard at 505 nm}}$$

2.2.14. Estimations in tissues

Estimations of total sugars, uronic acid, amino sugar and sulphates were carried out after hydrolyzing the samples. Proteins were estimated after solubilising the tissues in 0.1 N sodium hydroxide.

Trifluoroacetic acid hydrolysis

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

2.2.14a. Total sugar estimation (McKelvy and Lee, 1969)

Preparation of reagent

5% phenol (distilled) was prepared in brown bottle. Prior to estimation, to the dried and powdered kidney sample (20-30 mg), 2 ml of 2 N trifluroacetic acid was added and hydrolyzed at 100^{0} C for 8 h in sealed tubes in an oven.

To 0.5 mL of the sample containing 5-25 µg of sugars, 0.3 mL of 5% phenol was added. After mixing, 1.8 mL of conc. H_2SO_4 was blown on to the sample (solution). It was kept at room temperature for 10 min and the absorbance was read at 480 nm in a spectrophotometer. Standard graph was prepared using 0-25 µg of glucose.

2.2.14b. Amino sugar estimation (Ludowieg and Benmanan, 1967)

Preparation of reagents

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

Solution B: p-Dimethyl aminobenzaldehyde (1.6 g) was dissolved in a mixture containing 30 ml of conc. 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 1h in a water bath. After cooling the tubes to room temperature, 5 mL of ethanol and 0.5 mL of solution B were added. Contents of the tubes were thoroughly mixed and left at room temperature for 1 h. The absorbance was read at 535 nm in a spectrophotometer. Glucosamine hydrochloride (0-50 µg) was used to prepare a calibration curve.

2.2.14c. Sulphate estimation (Dodgson, 1961)

Formic acid hydrolysis:

Tissues (20-30 mg) were hydrolyzed with 60% formic acid prior to estimation. To the sample 3 mL of 60% formic acid was added and kept for hydrolysis 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 mL of water.

Preparation of reagents

Solution A: Trichloroacetic acid (4 g) in 100 mL distilled water.

Solution B: Bactograde gelatin (2 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 gelatinous 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 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.

2.2.14d. Protein estimation (Lowry et al. 1951)

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

Preparation of reagents

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

Solution B_1: Copper sulphate ($CuSO_4.5H_2O$, 1 g) in 100 mL of distilled water.

SolutionB_2: Sodium potassium tartarate (2 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 mL, ~2.0 N) diluted to 2.5 mL with distilled water before use.

To the tissue sample solution (1 mL), 5 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).

2.2.14e. Glycosaminoglycan estimation (Farndale et al. 1986)

Preparation of Dimethylmethylene blue reagent

Dimethylmethylene blue (8 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 0.1 mL of the sample solution containing 0-5 μg of sulphated polysaccharide, 2.5 mL of dimethylmethylene blue reagent was added and absorbance read at 525 nm in a spectrophotometer with in a minute. Standard graph was prepared using chondroitin sulphate (0-5 μg).

2.2.15. Intestinal maltase, sucrase and lactase activities (Dahlquist et al. 1968)

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 Elvehjem-Potter homogenizer for 10 min at 4°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) and lactose (56 mM), respectively, prepared in maleate buffer (0.1 M, pH 6.0) with the enzyme preparation after suitable dilutions. The activity was stopped by adding Tris-glucose oxidase reagent (1.2 mL). Control was done in the same way, but the enzyme preparation was added after stopping the reaction. The contents were incubated with Tris-glucose oxidase at 37°C in a water bath for 1h and the amount of glucose released was measured as described in section, Protein was estimated by Lowry's method as described in the section.

2.2.16. Renal disaccharidases

Enzyme preparation

Kidney tissue (100 mg) was taken in 1 mL cold saline (0.9 %) and homogenized in Potter-Ehlveym homogenizer for 10 min at 4°C for 10 min. It was then centrifuged at 2,000 rpm (Hermle, Z320K) at 4°C for 10 min. The supernatant was used as an enzyme source.

2.2.17. GAG metabolizing enzymes

2.2.17a. L-Glutamine fructose-6-phosphate aminotransferase (Pogell and Gryder, 1957)

Enzyme preparation

Kidney tissue (200 mg) was homogenized in potter-Ehlveym homogenizer in phosphate buffer (0.1M, pH 6.5) containing potassium chloride (0.154 M), EDTA (0.001 M) and glucose-6-phosphate (0.012 M). It was then centrifuged at 2,000 rpm for 10 min at 4°C. Supernatant was used as the 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, reaction was stopped by adding 1.0 mL of trichloro-acetic acid (0.4 M). The mixture was then centrifuged and the supernatant (1.5 mL) was evaporated to dryness in a rotary evaporator maintained at 35^{o}C. It was reconstituted with 0.025 mL water. Amino sugar was estimated as described in section 2.2.14b.

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

2.2.17b. β-N-Acetyl glucosaminidase (Kawai and Anno, 1971)

Preparation of enzyme solution

Kidney tissue (100 mg) was homogenized in 1 mL of acetate buffer (0.1 M, pH 4.5) and centrifuged at 2,000 rpm for 10 min at 4^{o}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. 0.1 M), 0.3 mL of acetate buffer (0.1 M, pH 4.5) and 10 μL of enzyme solution was added and incubated at 37^{o}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 section 2.2.14d.

Activity: It is expressed as μmoles of paranitro-phenol formed / h / mg or g protein.

2.2.17c. β-Glucuronidase (Kawai & Anno, 1971)

Preparation of enzyme solution

It was prepared in a similar way as that of β-N-acetyl glucosaminidase.

Assay: p-Nitrophenyl-β-glucuronide was used as the 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 (0.1 M, pH 4.5) were added and incubated at 37^{o}C for 1 h. 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

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

2.2.18. Glomerular filtration rate (GFR) (Yakozawa et al. 1996)

GFR is determined by measuring the amount of urinary and plasma creatinine. This was calculated using the formula;

$$\frac{\text{Urinary creatinine (mg/dL) X Urine volume (mL) X 1000 (g)}}{\text{Plasma creatinine (mg/dL) X Body weight (g) X 1440 (min)}} = \text{---- mL/min (GFR)}$$

2.2.19. Statistical analysis

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

Statistical analysis (Snedecor et al., 1976)

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' are 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.

Students 't' test was used for evaluating the results and the '*P*' value equal to less than 0.05 was considered to be significant.

2.3. Chemical and biological studies

2. 3.1. General methods

2.3.1a. Instruments

- Centrifugation of the samples was done either using Hermle (Z 320K) or Sigma (202C) centrifuge.
- Evaporation of the samples was carried out using Buchi rota vapor with a water bath temperature of 35-40^{0}C.

- Samples from column chromatographic analysis were collected by using LKB Broma 2211 fraction collector.
- Lyophylization was done using Vertis Freeze Mobile (12SL).
- Colorimetric and spectrometric determinations were carried out using Shimadzu double beam spectrometer (UV-160A).
- GC-15A Shimadzu.
- HPLC-LC 6A was Shimadzu equipped with UV and RI detectors and LC 10A equipped with RI, fluorescence and photodiode array detectors.

2.3.1b. Enzymes

Glucoamylase from *Aspergillus niger*, glucose oxidase from horse radish and pancreatin were from Sigma Chemical Company, St Louis, USA. Termamyl was obtained from Novo, Denmark and Milwaukee, USA. Papain was obtained from Flukam, Buchs, Switzerland.

2. 3.1c. Sugar standard

Rhamnose, fucose, xylose arabinose, glucose, galactose, mannose, maltose and inositol were from ICN Pharmaceutical Inc, Cleveland, USA.

2. 3.1d. Phenolic acid standards

Gallic acid, caffeic acid, coumaric acid, ferulic acid, genticic acid, proto-catechuic acid, syringic acid and vanillic acid, butylated hydroxyl anisole (BHA), butylated hydroxytoluene (BHT) were from Sigma Chemical Company, St, Louis, USA

2. 3.1e. GC and HPLC columns

3% OV-225 (1/8' x 6') on Chromosorb W (80-190 mesh) was from Pierce Chemical Company, Rockford, USA. All other chemicals and solvents used for HPLC and GLC were HPLC grade. Other analytical reagents were obtained from E- Merck, SRL or BDH Mumbai, India. Shimpak - C18 column (4.6 mm x 250 cm) was from Shimadzu Corporation, Tokyo, Japan.

2. 3.1f. Extractions

Extractions were carried out by using double distilled water, Dialysis against doubled distilled water was carried out using dialysis bags having a cutoff of 12,000 Da. Few drops of toluene was added to aqueous solutions to prevent microbial growth.

2. 3.1g. Purifications of solvents

Phenol, acetic anhydride and pyridine were purified by distillation in 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.

2.3.2. Preparation of sample for compositional study

Jambolana fruits were purchased from local market and washed thoroughly in tap water. Seeds were separated from pulp manually and once again seeds were washed with water to remove the adhered pulp and kept for drying in an oven at 40^{o}C.

2.3.3. Determination of starch (Hassid & Neufeld, 1964)

Sample (0.5-1 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°C for 2 h. The solution was filtered and made up to 100 mL and the liberated glucose was determined by Tris glucose oxidase (TGO) method. The glucose value was multiplied by factor 0.9 to get starch content.

2.3.4. Isolation of total dietary fiber (Aspinal et al. 1983)

The flour was first extracted with petroleum ether for 30 min to remove fat. The defatted sample (1 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°C for 1 h. Once again water 20 mL was added and the pH adjusted to 6.0 with 4M NaOH. To this, pancreatin (100 mg) was added and incubated at 40°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).

Insoluble dietary fiber

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

Soluble dietary fiber

The volume of the filtrate was adjusted to 100 mL and the soluble fibers were precipitated by adding 4 volumes of warm ethanol (60°C). The precipitate was filtered through celite, dried and weighed after drying at 105°C (D_2) followed by incineration at 550°C. Blank was prepared as above without sample.

Soluble and insoluble dietary fiber contents (%) were calculated by using following formula;

$$\% \text{ Soluble fiber} = \frac{D1 - (I1 - B1)}{W} \times 100$$

$$\% \text{ Insoluble fiber} = \frac{D2 - (I2 - B2)}{W} \times 100$$

2.3.5. Extraction of polysaccharides

Isolation of non - starch polysaccharides (Paramahans and Tharanathan, 1982)

The alcohol insoluble residue of the flours was suspended in water (1: 100, w/v). The suspension was kept in boiling water bath for 1 h to facilitate gelatinization. Termamyl (1.0 mL) was added at intervals to digest the starch. Starch was digested till it shows negative 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). Destarchified residue was further extracted with 0.5% ammonium oxalate (x3) in a boiling water bath for 2 h in order to extract pectin. The residue was further extracted twice with 10% alkali (1:10, w/v) under nitrogen atmosphere for 4 h. 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 3,500g for 15 min, and was designated as

hemicellulose A. To the supernatant, 3 volumes of ethanol were added to precipitate hemicellulose B. AIR was dried by solvent exchange (alcohol/ether).

2.3.6. Hydrolysis of polysaccharides

2.3.6a. Trifluoroacetic acid hydrolysis

Sample (10-15 mg) was taken in 1 mL of trifluroacetic 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, x3).

2.3.6b. Sulphuric acid hydrolysis (Selvendran et al. 1987)

Polysaccharide (10-15 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.

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

The Amberlite resins were washed with water until fines, color and other impurities are removed. Nylon cloth was used to successive washes by filtration, and 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 with water till the filtrate gave neutral pH.

2.3.8. 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.

2.3.8a. Aniline phthalate reagent (Partridge, 1949)

Pthalic acid (1.66 g) was dissolved in water saturated butanol (95 mL butanol + 5 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.

2.3.8b. Silver nitrate reagent (Trevelyan, 1950)

The air-dried chromatogram was dipped in silver nitrate solution (1 mL saturated $AgNO_3$ in water, diluted to 6 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.

2.3.9. Determination of total nitrogen by microkjeldal method (Hawk, 1965)

Sample (0.5-1 g) was digested with 20 mL conc. H_2SO_4 in the presence of catalyst (98 parts K_2S0_4 + 2 parts $CuS0_4$) 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 (40%, 10 mL) 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. Titer value of the blank was deducted from test values. Ammonium sulphate solution (1 M) was used as the standard.

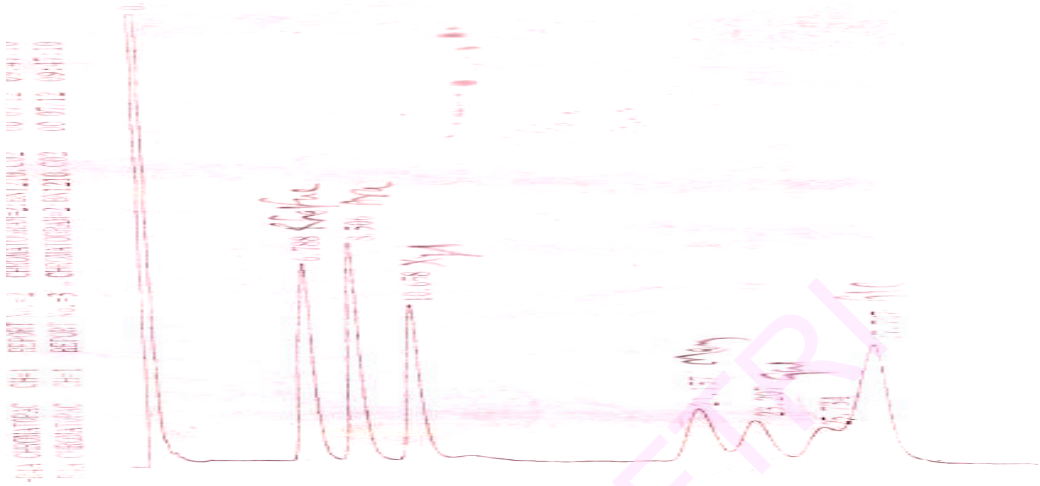


Fig. 17: Alditol acetate of standard sugars identified by GLC

1. Rha: Ramnose, 2. Ara: Arabinose, 3. Xyl: Xylose, 4. Man: Mannose, 5. Glc: Glucose, 6. Gal: Galactose.

2.3.10. Gas Liquid Chromatography

2.3.10a. Operating conditions for GLC

The alditol acetates were detected in gas liquid chromatography fitted with FID detector using OV-column at column temperature 170^{o}C, detector 250^{o}C and injector temperature of 250^{o}C. Nitrogen was the carrier gas.

2.3.10b. Preparation of alditol acetates (Sawardekar, 1967)

The neutralized and deionised sample was concentrated to about 0.5 mL and sodium carbonate was added to 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 over night.

Next day, excess borohydride was destroyed with acetic acid (2 M). The excess borate and other salts were removed by co-distillation 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^{o}C for 2 h after tightly stoppering the tubes. Excess reagent was removed by co-distillation with water (1 mL, x3) and toluene (1 mL, 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.

2.3.11. High performance liquid chromatography (HPLC) (McGinnis, 1980)

E-Linear (7.8 mm x 30 cm) connected in series with E-1000 (3.9 mm x 30 cm) column were used. Degassed triple distilled water was the mobile phase used. Flow rate was maintained at 0.6 mL/min. Oven temperature was 50^{0}C and RI setting was 8 x 10 RIU. Calibration curve was prepared using dextran standard (5 to 10 µL) of different molecular weights (T-40, T-70, T-150, T-500 and T-2000).

2.3.12. Biological activity

2.3.12a. Determination of total phenol content (Singleton, 1965)

The total phenolic content of the sample was determined calorimetrically using the Folin–Ciocalteu method. To the sample aliquots of 100 µL was added to 990 µL of water, 5 mL of 0.2 N Folin-Ciocalteu reagent and 4 mL of saturated sodium carbonate solution (100 g/L) and vortexed. The absorbance was measured at 765 nm with a Shimadzu UV – Visible spectrometer after incubation for 2 h at room temperature. The total phenolic content was expressed as gallic acid equivalent (GAE) in milligram per gram sample.

2.3.13. Antioxidant activity

2.3.13a. Scavenging effect of extracts on DPPH radical

The effect of free and bound phenolics on DPPH radical was estimated according to the method of Lih-Shiuh et al. (2001). An aliquot of 200 µL of different polysaccharide fractions (2- 20 µg, GAE) and standard antioxidants (2-20 µg) were mixed with 100 mM Tris- HCl buffer (800 ML, pH 7.4) and then added to 1 mL of 500 mM DPPH in ethanol (final concentration of 250 mM). 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;

$$\text{Scavenging effect (\%)} = \frac{\text{Absorbance of sample A}}{\text{Absorbance of control at 517 nm}} \times 100$$

2.3.13b. Measurement of reducing power (Yen and Chen, 1995)

The free and bound phenolic extract (2-20 mL) of the sample and standard BHA (2-20 mg) 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^{o}C for 29 min. Then an equal volume of 10% trichloroacetic acid was added to the mixture and then centrifuged at 1,600 g for 10 min. Upper layer of solution, distilled water and 0.1% $FeCl_3$, at a ratio of 1:1:2 were taken and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

2.3.14. Isolation of free phenolic acids (Ayum et al. 1999)

Samples (1 g each) were extracted with aqueous ethanol (70%, 50 mL, 1h, x4) and the supernatant was obtained by centrifugation. The combined supernatant was concentrated, and the pH was adjusted to 2-3 with 4 M HCl. Phenolic acids were extracted with ethyl acetate (50 mL, x5) and phase separation was followed and dried with anhydrous sodium sulphate. Sodium sulphate was removed by filtration and evaporated to dryness. The dried materials were 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, coumeric, ferulic acid, gentisic, galic proto-catecheuic, syringic and vanilic acids were used as standards and the phenolic acids in the samples were identified based on the retention time of the standards.

2.3.15. Bound phenolic acids (Nordkvist et al. 1998)

Samples (1 g each) were first extracted with ethanol (70%, 50 mL, 1h, x4) and then hexane (50 mL, 1h x4) to remove free phenolics, sugars and fat. The residues were extracted with 1M NaOH (100 mL, 2 h, x2) containing sodium borohydride (0.5%) under nitrogen. The clear supernatants obtained after centrifugation were pooled, acidified with 4M HCl till the pH became 1.5. Released phenolic acids were extracted, quantified and characterized, as above.

2.3.16. Reverse phase chromatography

C-18 (Shimpack) column was used to analyse 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/ mL) of which 5-10 µL was injected.

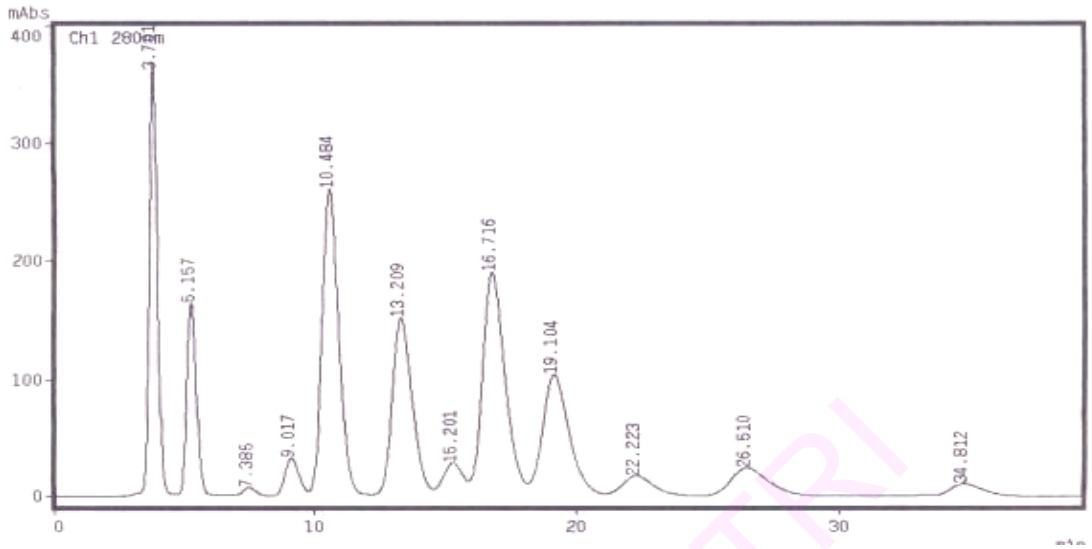


Fig 18: Standard graph of phenolic acids

Standards: 1. Protocatechuic acid, 2. Gallic acid, 3. Syringic acid, 4. Vannilic acid, 5. Para coumaric acid, 6. Caffieic acid, 7. Ferulic acid.

3. RESULTS AND DISCUSSION

3.1. Effect of butyric acid on experimentally induced diabetic rats

3.1.1. Introduction

Fermentation of dietary fibers and effect of fermented products on various physiological processes are gaining a lot of interest all over the world in recent years. Dietary fibers besides acting as insoluble matrix in slow absorption of nutrients are fermented by microbes present in the colon to short chain fatty acids (SCFA) such as acetate, propionate and butyrate (Cummings et al. 1986). The SCFA thus released are partially consumed by microflora for their growth and the remaining enters circulation and is shown to modulate many physiological events (Bourquin et al. 1996; Cummings et al. 1991). Butyrate among them is gaining lot of interest in recent years and is shown to modulate various physiological processes, including enzymes involved in glycoconjugate metabolism (Jacobsson et al. 1985; Shah et al. 1992). It has also been shown to modify nuclear architecture, induce cell death apoptosis in colon cancer and potential to decrease the incidence of bowel cancer (Riggs et al. 1977; Calabress et al., 1993). Many analogs of butyric acid are undergoing clinical trials for tumor therapy. One of the butyric acid derivatives JTT- 608 (4- trans-4-methyl cyclohexyl-4-O-oxobutyric acid) is shown to reduce glucose level in diabetic rats (Ohta et al. 1999). Sodium butyrate is also shown to induce insulin gene expression in *in vitro* study (Karlsent et al. 1991).

Although butyric acid plays an important role in modulation of different physiological conditions in many diseases, its beneficial effect on diabetes is not well understood. Hence, beneficial effect of butyric acid over and above moderate levels of dietary fiber on diabetic status needs to be evaluated.

3.1.2 Results

Male Wistar rats (OUTB-Wistar IND cftri) weighing between 110-120 g were maintained on diets containing starch in the control group, wheat bran (5%) and guar gum (2.5%) in the fiber-fed groups as shown in Table 12. Diabetes was induced using streptozotocin at a dosage of 55 mg/kg/body weight. Butyric acid was given to rats orally in drinking water. Initially, various concentrations of butyric acid (1, 5, 10, 50, 100, 250, 500, 750 and 1000 mg/kg body weight/day) were given to diabetic rats and their status was assessed. Of these concentrations, butyric acid feeding at concentration of 250, 500 and 750 mg/kg body weight/day showed promising results and hence was taken for further studies. The rats were monitored for dietary intake, water intake, butyric acid intake, urine output and urine sugar. The rats were sacrificed under ether anesthesia when mortality was about to set in, which was 45 days after the streptozotocin injection. Mortality started initially in the starch-fed diabetic group. A night before sacrificing, the rats were fasted and blood was collected to measure fasting blood sugar.

Effect of butyric acid on diet intake and gain in body weight in control and diabetic rats

Diet intake was greater in diabetic rats compared to control rats (Table 13). The starch-fed control (SFC) animals consumed about 13.0 ± 0.49 g/day whereas fiber-fed control (FFC) animals consumed 13.2 ± 1.35 g/day and was comparable. The higher diet consumption observed in diabetic group was apparently due to the hyperphagic condition developed during diabetes. Fiber-fed diabetic rats compared to starch fed diabetic rats consumed greater amounts of diet. Butyric acid feeding at 500 mg/kg body weight/day to starch-fed diabetic rats resulted in a lower diet intake (13.9 ± 2.30 g/day). Diabetic rats fed with fiber and butyric acid at 500 mg/kg body weight/day showed a significant reduction in the amount of

diet intake (13.8 ± 0.21 g/day) when compared to starch-fed diabetic animals (SFD). In accordance with diet intake, the control rats showed a steady increase in body weight. The gain in body weight in fiber-fed control rats (116.0 ± 7.1 g) was little less compared to starch-fed control rats (125.0 ± 5.4 g). There was a marginal gain in body weight in FFD rats (13.0 ± 1.1 g), whereas SFD rats lost weight (-7.0 ± 10.8 g) in spite of higher diet consumption. Butyric acid feeding at 500 mg/kg body weight/day to diabetic animals showed a slight improvement in gain in body weight (15%). The FFD group of rats showed an improvement over the SFD group. Butyric acid feeding to the fiber fed diabetic group showed significant improvement in gain in body weight and was best at 500 mg/kg body weight/ day level.

Butyric acid consumption and effect of butyric acid on water intake in control and diabetic rats

Butyric acid consumption was measured daily. The average consumption of butyric acid was around 250 (230-280), 500 (480-530) and 750 (720-780) mg/kg body weight/day level in the three groups reported SFD/FFD-250, SFD/FFD-500 and SFD/FFD-750, respectively. Water intake in different groups was measured daily (Table 14). The SFC and FFC groups did not show much alteration in water intake over a period of 5 weeks (30 mL). The SFD group showed a significant increase in water intake (120 ± 1.3 mL/24 h) compared to control rats. The FFD rats consumed less water (110.9 ± 0.7 mL/24 h) compared to the SFD. Feeding of butyric acid (500 mg/kg body weight/ day level) to SFD resulted in a lower consumption of water (92.0 ± 1.2 mL/24 h). On the other hand, butyric acid feeding (500 mg/kg body weight/ day level) along with fiber showed a significant reduction in water intake (77.1 ± 1.2 mL/24 h).

Table 13: Effect of butyric acid on diet intake and gain in body weight in control and diabetic rats

Groups	Diet intake (g/day)	Initial body weight (g)	Final body weight (g)	Gain in body weight (g)
SFC	13.0 ± 0.49	116 ± 4.7	241 ± 3.8	125.0 ± 5.4
SFD	15.4 ± 0.90[a]	114 ± 3.3	107 ± 13.5[a]	-7.0 ± 10.8[a]
SFD-250	14.6 ± 1.50	111 ± 2.7	121 ± 2.8	10.0 ± 2.1
SFD-500	13.9 ± 2.30	109 ± 6.4	126 ± 0.7	17.0 ± 0.6
SFD-750	14.8 ± 2.20	117 ± 3.7	122 ± 3.9	5.0 ± 2.8
FFC	13.2 ± 1.35	115 ± 3.4	231 ± 9.7	116.0 ± 7.1
FFD	17.5 ± 0.28[b]	119 ± 3.8	132 ± 1.9[b]	13.0 ± 1.1
FFD-250	17.0 ± 2.00	120 ± 4.8	144 ± 7.0	24.0 ± 5.7
FFD-500	13.8 ± 0.21[b]	115 ± 4.3	155 ± 8.4[b]	40.0 ± 6.7[b]
FFD-750	14.4 ± 1.91	120 ± 5.6	146 ± 5.6	26.0 ± 4.8

SFC - Starch Fed Control, SFD - Starch Fed Diabetic,

FFC - Fiber Fed Control, FFD - Fiber Fed Diabetic,

Groups with the numbers (eg. SFD–250) represent mg of butyric acid fed/day/kg body weight,

Values are mean ± SEM of 6 rats in control and 14 rats in diabetic groups,

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

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

Table 14: Butyric acid consumption and effect of butyric acid on water intake in control and diabetic rats

Groups	Butyric acid consumption (mg/kg body weight)	Water intake (mL/24 h)
SFC	--	30 ± 4.1
SFD	--	120 ± 1.3
SFD-250	253.5 ± 13.4	100 ± 3.0
SFD-500	492.6 ± 6.8	92 ± 1.2
SFD-750	723.1 ± 18.6	97 ± 2.8
FFC	--	31 ± 1.5
FFD	--	111 ± 0.7
FFD-250	243.1± 4.7	85 ± 1.5
FFD-500	486.0 ± 12.9	77 ± 1.2
FFD-750	756.3 ± 8.3	78 ± 0.9

Footnote: As in Table 13.

Effect of butyric acid on urine volume, urine sugar and fasting blood sugar in control and diabetic rats

The excretion of urine was around 20 mL/24 h in control animals (Fig. 19). The urine output in the SFD group increased significantly with the progression of diabetic status to about 117 mL/24 h. Fiber feeding to diabetic rats showed a significant reduction in the volume of urine output (96 mL/24 h). Butyric acid feeding at 500 mg/kg body weight/ day to the SFD group showed around a 10% improvement (108 mL/24 h) compared to the diabetic group. The FFD group supplemented with butyric acid (500 mg/kg body weight/day level) excreted around 84 mL/24 h and was the most effective group. There was an increased excretion of reducing sugar during diabetes (Fig. 20). Control rats excreted around 20 mg/24 h, while the diabetic animals excreted up to 4 g/24 h initially, which increased significantly over a period of 5 weeks to around 7.2 g/24 h. The excretion of sugar in diabetic rats was significantly higher compared to control animals. Butyric acid feeding at 500 mg/kg body weight/day to the starch-fed diabetic group prevented the increase in excretion of urinary sugar (5.7 g/24 h) by 20%. The sugar excretion in the fiber-fed diabetic group did not show a significant increase over a period of time and was around 4.8 g/24 h at the end of the fifth week. This indicates that fiber in the diet minimized the increase in sugar levels and did not permit the diabetic status/diabetic nephropathy status to deteriorate further. Butyric acid feeding at 500 mg/kg body weight/day along with fiber showed a marked reduction (3.6 g/24 h) in sugar excretion (50%) compared to the starch-fed diabetic group (7.2 g/24 h). Concentration of fasting blood glucose in various groups is given in Table 15. The control rats had fasting blood glucose level around 105 mg/dL. The fasting blood glucose level in the SFD group rats significantly increased to around 270 mg/dL. Butyric acid feeding at 500-mg/kg-body weight/day to the SFD group brought down the fasting blood glucose level

(211 mg/dL) by 21%. The percentage of reduction was significant compared to the starch-fed diabetic rats. Fiber feeding improved the fasting blood glucose level to 212 mg/dL. The increase of fasting Blood glucose levels in the FFD group were not as great as with the urinary sugar. Butyric acid feeding at 500 mg/kg body weight/day along with fiber showed a further reduction in fasting blood glucose level (180 mg/dL) to about 33%. This was significant compared to the starch-fed diabetic animals. The results prove that butyric acid in the diet has beneficial effects in ameliorating diabetic conditions.

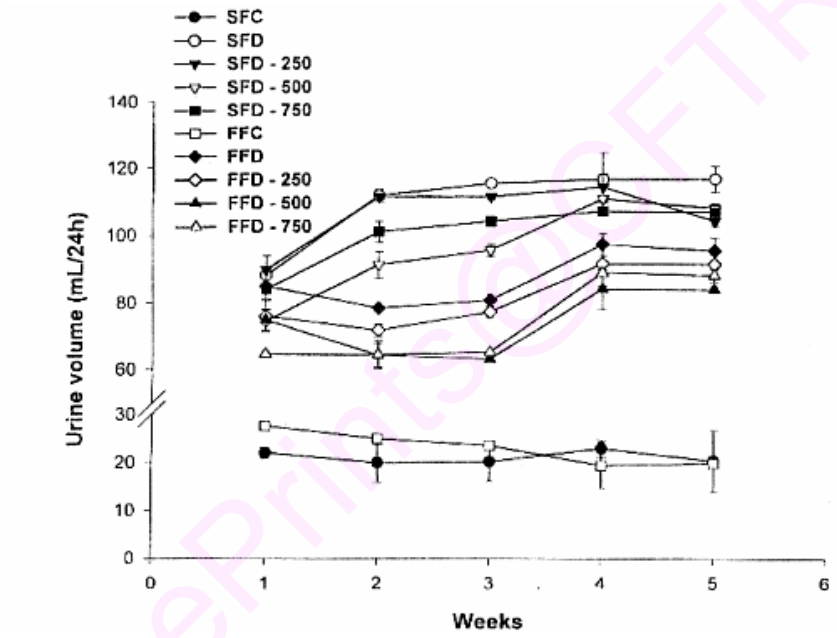


Fig. 19: Effect of butyric acid on urine volume in control and diabetic rats.

Abbreviations as given in footnote to Table 13.

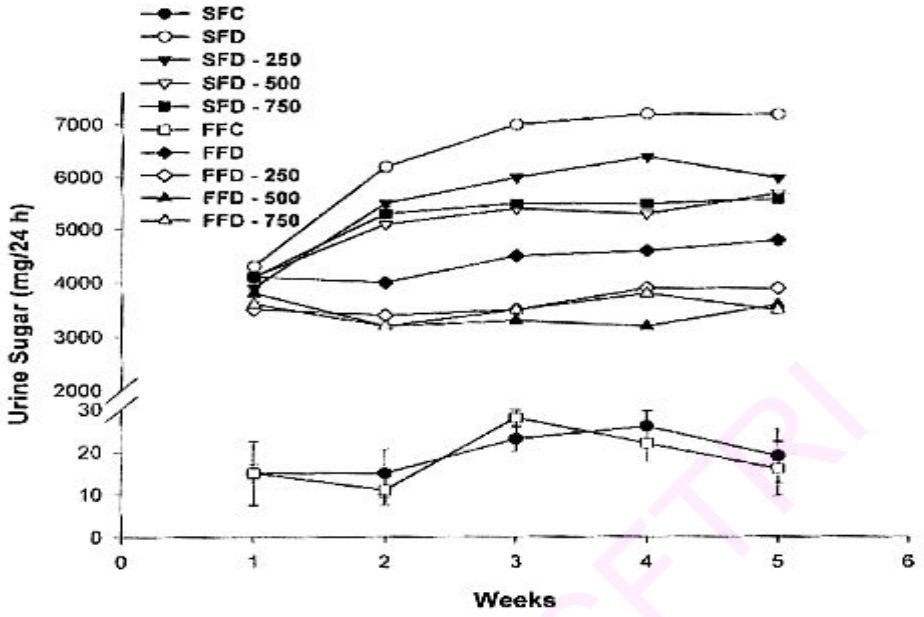


Fig. 20: Effect of butyric acid on urine sugar in control and diabetic rats

Abbreviations as given in footnote to Table 13.

Table 15: Effect of butyric acid on fasting blood glucose in control and diabetic rats

Groups	Fasting blood sugar (mg/dL)		
	Initial *	Fourth week	Fifth week
SFC	101.8 ± 11.1	105.4 ± 3.5	105.4 ± 1.2
SFD	217.5 ± 38.9[a]	258.3 ± 17.1[a]	269.5 ± 3.6[a]
SFD-250	212.3 ± 32.1	246.2 ± 20.2	248.5 ± 2.1
SFD-500	212.2 ± 18.4	216.3 ± 17.0	211.6 ± 1.7
SFD-750	214.0 ± 11.2	227.2 ± 4.2	217.9 ± 1.9
FFC	86.8 ± 11.0	106.0 ± 7.3	107.0 ± 0.4
FFD	208.4 ± 30.8	207.8 ± 14.6[b]	212.2 ± 4.7[b]
FFD-250	204.9 ± 24.7	205.0 ± 4.8	194.6 ± 0.4
FFD-500	201.2 ± 14.5	178.6 ± 13.7[b]	180.0 ± 1.3[b]
FFD-750	203.0 ± 18.7	187.3 ± 20.7	187.8 ± 2.3

* One week after the induction of diabetes

Footnote: As in Table 13.

3.1.3. Discussion

Diet and dietary fibers play a significant role in the management of various diseases including diabetes (Cummings et al. 1985; Spiller et al. 1986). The fermentation of dietary fibers to short chain fatty acids and butyric acid, in particular, has beneficial role on various physiological functions (Bourquin et al. 1996; Cummings et al. 1991). The beneficial effect of dietary fibers on the

diabetic status is well documented (Nandini et al. 2000). The results of the present study have shown that butyric acid feeding can further ameliorate the diabetic status, in terms of urine sugar, fasting blood sugar and various other parameters. The diabetic animals lost body weight in spite of a high diet consumption compared to control animals. The loss in weight was nearly 6%, which improved significantly by 15% with butyric acid feeding over a period of time. Fiber in the diet and butyric acid feeding showed a significant improvement (50%) on gain in body weight. The levels of urine sugar, an important criterion of the diabetic status showed a progressive increase every week compared to control rats. Excessive urine output (polyuria) and increased intake of water (polydipsia) were seen in the diabetic condition compared to control rats. Fiber in the diet alone showed a 10% reduction in urine output. Feeding of butyric acid at 500 mg/kg body weight/day to fiber-fed diabetic rats showed nearly 28% reduction. This was significant in terms of management of the diabetic status. The elevated levels of various metabolic parameters such as reducing sugar in urine and fasting blood sugar were significantly decreased in the presence of fiber and butyric acid. Butyric acid feeding at 500 mg/kg body weight/day levels was most effective in controlling the diabetic status. The results showed that the diabetic rats fed with moderate levels of dietary fiber (FFD) fared comparably better than starch-fed diabetic animals that received 500-750 mg butyric acid/ kg body weight/day (SFD 500-750). However, butyric acid feeding to the fiber-fed diabetic group (FFD-500) showed maximum beneficial effect. The benefits observed in FFD-500 group are attributed to combined effects of slow absorption of glucose resulting in better glucose levels in the blood as a consequence of insoluble matrix formed by dietary fiber in the intestine (Nuttall et al. 1993; Mujumdar et al. 1995; Plaami, 1997) and slow release of butyric acid over a period of time by the fermentation of dietary fiber which *in situ* acts as reservoir of butyric acid (Cummings et al. 1986; Bourquin et

al. 1992; Smith et al. 1998) and supplementation of butyric acid. The results clearly demonstrated that butyric acid has beneficial role on diabetes and ameliorated urine sugar, urine volume and blood sugar levels besides various other parameters. These regimens may also be beneficial in preventing diabetic complications at the cellular and molecular levels.

3.1.4. Summary and conclusion

Dietary fiber is beneficial to diabetics either by slow absorption of glucose there by decreasing blood glucose or by their fermented product including butyric acid. In the present study, attempts were made to evaluate antidiabetic property of butyric acid in STZ-induced diabetic rats and results can be summarized as follows.

1. Diabetic symptoms such as polyphagia, polydipsia, and polyuria and body weight gain were better controlled by fiber-fed group. Further amelioration was observed in feeding butyric acid at 500 mg/kg body weight along with fiber.
2. Excretion of reducing sugar in urine and fasting blood glucose was best controlled by feeding butyric acid at 500 mg/kg body weight along with fiber.

Conclusion

Diet that includes both fiber and butyric acid at 500 mg/kg body weight is effective in ameliorating diabetic status to higher extent than fiber alone. However, the exact mechanism by which butyric acid controls the fasting blood glucose needs to be evaluated.

3.2. *In vivo* and *in vitro* fermentation of dietary fibers to short chain fatty acids

3.2.1 Introduction

Dietary fibers (DF) are unabsorbable carbohydrates of food that encompasses diverse macromolecules that exhibit a large range of physicochemical properties (Smith et al. 1998). Their beneficial role against many diseases such as colorectal cancer, bowel syndrome and diabetes are reported (Guillon et al. 2000; Topping and Clifton, 2001). DF are often considered as beneficial for human health due to their effect on reducing blood cholesterol, affecting glycemic response, delaying gastric emptying, diminishing nutrient absorption, effecting motility in the small bowel, and prolonging satiety after a meal (Csordas, 1996; Hunt and Groff, 1990). Besides many of their important physiological effects, fermentation of DF to short chain fatty acid (SCFA) such as acetate, propionate, butyrate and their beneficial role in maintaining normal physiological functions is gaining importance (Stark and Madar, 1993). One of the SCFA's - butyric acid is receiving much attention in recent years. Many analogs of butyric acid are being tested clinically for tumor therapy. One of the butyric acid derivatives, JTT-608 (4-trans-4-methyl cyclohexyl-4-O-oxobutyric acid) is known to selectively reduce glucose level in diabetic rats. In the previous section we have provided evidence to show hypoglycemic effect of butyric acid in STZ induced diabetic rats.

Optimum concentration of SCFA is essential to maintain the healthy gut and butyric acid is a main energy source for colonic mucosa (Bourquin et al. 1996). Substrates producing high molar ratio of butyric acid are important for healthy colon. Though the amount of total SCFA produced can be used as a measure of fermentability, molar proportion/ relative percent of individual acids provides additional information about the quality of the substrate. Therefore,

substrates producing higher molar ratio of butyrate could be desirable for inclusion in diets for diabetics. SCFA profile depends on the type of dietary fiber and microbial source. Bacteria are known to degrade DF in colon to produce SCFAs and importance was given to the production of butyric acid from these substrates. Experiments in this chapter deals with butyric acid production by rat faecal and caecal mixed cultures under *in vitro* and *in vivo* conditions from various dietary fiber rich substrates.

3.2.2. Results

3.2.2.1. Effect of dietary fibers on the *in vitro* fermentation and production of SCFA by caecal and faecal microflora

Various experiments were carried out to find out the effect of mixed bacterial cultures on production of SCFA from dietary fibers and the results are as follows:

3.2.2.1.1. Production of short chain fatty acids from different sugars and polysaccharides incubated with faecal mixed culture

Anaerobic fermentability of various carbohydrate sources by faecal mixed cultures is presented in Table 16. Sugars such as glucose, rhamnose, galactose, fucose, arabinose, mannose and xylose were used as substrates for fermentation. These carbohydrates supported the production of acetic acid as major short chain fatty acid. Propionic acid was produced in the presence of rhamnose (48%) and fucose 23.8%). Poor butyrogenic property was observed in mixed culture fermentation of glucose (22%) and fucose (22%). Fermentation of glucuronic acid and maltose produced more acetic acid whereas arabinoxylan showed better butyrogenic property, which was around 44% and guar gum showed higher propionic acid production (51.5%).

Table 16: Production of short chain fatty acids from various sugars and polysaccharides incubated anaerobically with faecal mixed culture.

Substrate	Relative µmole %		
	Acetic acid	Propionic acid	Butyric acid
Glucose	62.0	16.0	22.0
Rhamnose	42.3	48.2	9.5
Galactose	67.6	21.4	11.0
Fucose	54.2	23.8	22.0
Arabinose	87.3	7.5	5.2
Mannose	69.5	18.5	12.0
Xylose	76.6	19.0	4.4
Glucuronic acid	62.0	19.0	19.0
Maltose	71.3	19.5	9.2
Pectin	70.7	15.8	13.5
Cornstarch	59.5	20.3	20.2
Guar gum	26.5	51.5	22.0
Arabinoxylan	18.6	37.4	44.0

3.2.2.1.2. Effect of different caecal and faecal microflora (based on substrate used for growth of rats) on fermentation of substrate for butyric acid production

Mixed culture obtained from caecal and faecal sample of rats fed with cornstarch, guar gum and wheat bran were used for *in vitro* fermentation of these substrates. Data in Table 17 indicates that variations were found in caecal and faecal viable cell counts amongst the substrates. Wheat bran and guar gum had higher number of microorganisms compared to cornstarch-fed animals. Inoculum from cornstarch fed group produced more butyric acid on wheat bran (33.6 and 40.8%) compared to other substrates (Table 18). Better propiogenic property was observed when guar gum was fermented with caecal inocula of cornstarch fed rats. Butyric acid production was found to be higher in all three substrates when all substrates were incubated with faecal, caecal inocula of wheat bran fed rats. However, faecal inocula of wheat bran fed rats were comparatively better than caecal inocula in butyric acid production. Butyric acid produced by faecal inocula with cornstarch, wheat bran and guar gum were 52.3, 70 and 60.41%, respectively. Use of guar gum-fed rat faecal inocula for fermentation resulted in lower butyric acid with cornstarch and wheat bran than caecal inocula of same rats. Similar amounts (29%) of butyric acid were produced on cornstarch as substrate by both the inocula. Among cornstarch based, wheat bran based and guar gum based caecal and faecal inocula, wheat bran based inocula were found to be better suited for butyric acid productions.

3.2.2.1.3. Effect of incubation period, substrate concentration and pH on butyric acid production by rat faecal mixed culture

Effect of fermentation period (24 h and 48 h) on butyric acid production was studied using rat faecal mixed flora on cornstarch (CS), wheat bran (WB) and

guar gum (GG) at 1% substrate concentration (Figs 21-23). SCFA was estimated after the fermentation (Fig 21). Acetic acid production (24 h) from all the three substrate were between 70-83% of total SCFA, whereas their molar percent decreased to 41-53%, under prolonged incubation of 48 h. There was about 30% reduction in acetic acid production with increased incubation period from 24 to 48 h. Propionic acid production increased from 10-25% at 24 h to 18-30% after 48 h. There was significant enhancement in butyric acid production from 24 h to 48 h by all the three substrates studied. Substrate concentration also influenced fermentation with faecal inocula. Fig. 22 shows that the fermentation of CS, WB and GG after 48 h incubation produced increased acetic acid concentration from 1% to 5%. Production of propionic acid decreased with increased substrate concentration. Levels of butyric acid varied marginally in the presence of different concentrations of cornstarch. Low concentration of guar gum (1%) was feasible for butyric acid production compared to higher concentrations.

Table 17: Effect of type of dietary fiber on aerobic and anaerobic viable counts (cfu/g) in caecal and faecal samples.

Diet fed to animal groups		Viable cell counts /g	
		Nutrient agar	Anaerobic agar
Starch	A	2.0×10^5	11.0×10^5
	B	1.5×10^1	2.0×10^1
Wheat bran	A	3.2×10^5	15.2×10^5
	B	2.5×10^4	2.2×10^2
Guar gum	A	8.0×10^5	14.4×10^5
	B	5.0×10^4	2.0×10^2

A: Caecal sample; B: Faecal sample.

Table 18: *In vitro* fermentability of starch, wheat bran (starch free), and guar gum by caecal and faecal mixed culture obtained from rats fed with respective dietary sources.

Microbial source	Fermented substrate	Relative µmole %		
		Acetic acid	Propionic acid	Butyric acid
Faecal and caecal mixed culture obtained from starch-fed rats				
Faecal mixed culture	**Starch**	55.8	23.6	20.6
	Wheat bran	43.5	22.9	33.6
	Guar gum	49.9	28.2	27.9
Caecal mixed culture	**Starch**	54.6	26.9	18.5
	Wheat bran	36.2	23.0	40.8
	Guar gum	29.6	44.8	25.6
Faecal and caecal mixed culture obtained from wheat bran-fed rats				
Faecal mixed culture	**Starch**	15.4	23.2	52.4
	Wheat bran	6.7	24.2	70.1
	Guar gum	11.5	28.1	60.4
Caecal mixed culture	**Starch**	21.4	26.8	51.8
	Wheat bran	15.5	25.7	58.8
	Guar gum	28.1	17.2	54.7
Faecal and caecal mixed culture obtained from guar gum-fed rats				
Faecal mixed culture	**Starch**	45.1	15.4	29.5
	Wheat bran	59.2	25.2	15.6
	Guar gum	51.7	28.9	19.4
Caecal mixed culture	**Starch**	38.8	32.1	29.1
	Wheat bran	26.0	37.2	36.8
	Guar gum	29.7	36.8	33.5

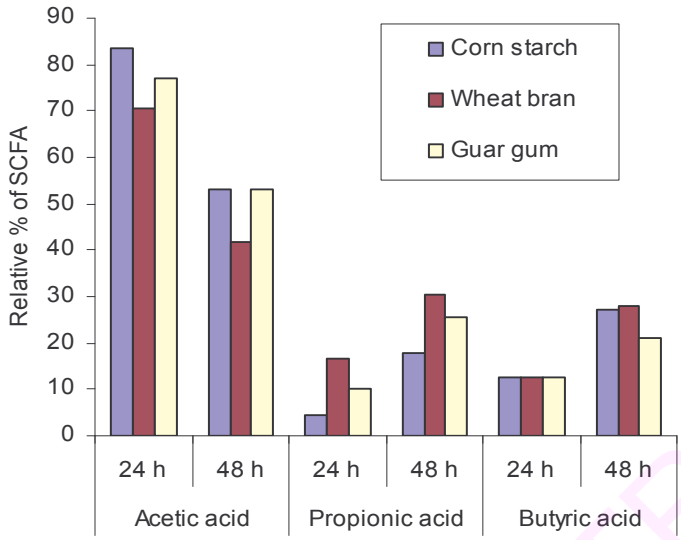


Fig. 21: Effect of incubation period on relative % of short chain fatty acid production by rat faecal mixed flora from various substrates

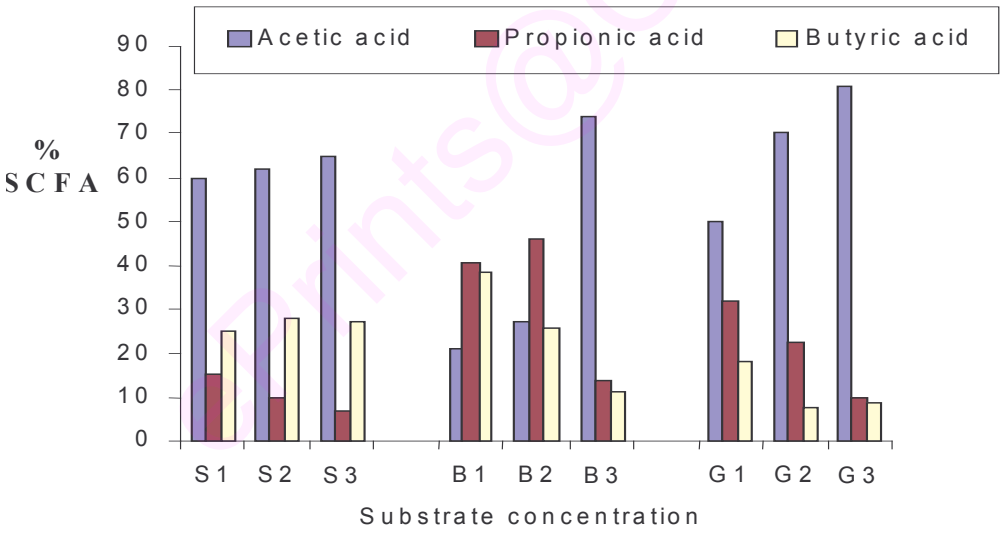


Fig. 22: Effect of substrate concentration on relative % of short chain fatty acid production from faecal mixed flora of rats

S1, B1 and G1: Corn starch, Wheat bran and Guar gum at 1%, respectively,

S2, B2 and G2: Corn starch, Wheat bran and Guar gum at 2%, respectively,

S3, B3 and G3: Corn starch, Wheat bran and Guar gum at 5%, respectively,

Anaerobic incubation was carried out for 48 h at 30^0 C.

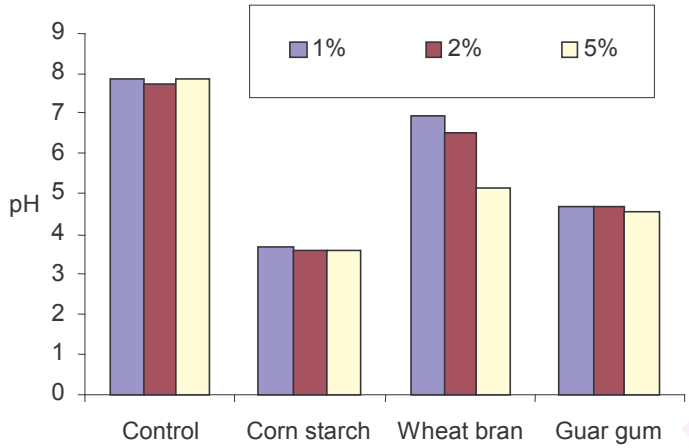


Fig 23: Effect of substrate concentration on pH after 48 h of microbial growth

Short chain fatty acid (SCFA's) production is correlated with lowering of pH. Lower pH after incubation indicated higher fermentability of substrates and higher SCFA production. Hence, pH of fermented broth was measured after 48 h incubation (Fig. 23). Control without substrate was also measured and the pH was between 7.8-8.2. Corn starch, a highly fermentable substrate showed lower pH (3.6-3.7). Guar gum showed pH value between 4.6-4.7, which was next to corn starch in fermentability. Wheat bran at 1% concentration showed pH 5.6 and was further increased to 6.5 with 2.5% and 7.1 with 5% substrate concentration.

3.2.2.1.4. Production of short chain fatty acids by various dietary fiber sources by rat faecal and caecal mixture

Short chain fatty acid production varies with substrate and microbial source (faecal/ caecal/ intestinal). Hence, fermentablity of various dietary fiber sources was studied by using both rat caecal and faecal mixed cultures and the short chain fatty acids production was measured (Table 19).

Table 19: SCFA production from different dietary fibers by faecal and caecal mixed microflora.

Substrate	Inoculum	Relative µmole %		
		Acetic acid	Propionic acid	Butyric acid
Fenugreek	Caecal mixed culture	62.7	12.5	24.8
Bitter gourd		63.8	09.4	26.8
Drumstick		64.3	10.5	25.2
Fenugreek	Faecal mixed culture	75.4	14.0	10.6
Bitter gourd		66.5	15.5	12.0
Drumstick		61.2	23.8	15.0
Jambolana		54.6	23.8	21.6

Substrates that are rich in dietary fiber sources such as fenugreek, bitter gourd and drumstick were fermented and different proportions of butyric acid were observed under anaerobic incubation with caecal and faecal mixed cultures. Acetic acid was the major SCFA produced from all the substrates with both the faecal and caecal inocula. Propionic acid production was lower in all the substrate incubated with faecal mixed culture, whereas butyric acid production was more and it was around 24% for fenugreek, 26% for bitter gourd and 25% for drumstick as substrates.

When all the above-mentioned substrates were incubated with faecal mixed culture SCFA pattern obtained was quiet different from the above experiment. Acetic acid production was higher in all the substrates, whereas butyric acid production decreased and propionic acid production enhanced during fermentation of various substrates. Butyric acid produced on fenugreek and bitter gourd and

drumstick were 10, 12 and 15% of total SCFA, respectively. Jambolana supported maximum concentration of butyric acid production.

3.2.2.1.5. Production of short chain fatty acids from different isolated polysaccharide fractions of jambolana by faecal mixed culture

As higher butyric acid production was found in pulp of jambolana, experiments were conducted to find out the effect of various polysaccharides fractions isolated from the pulp, seed and seed coats of the fruit (soluble in water, ammonium oxalate and alkali soluble (hemicellulose fractions) on SCFA production. Data in Table 20 shows that the short chain fatty acid production depends on the nature of polysaccharide and its composition. In the present study water soluble, pectic and hemicelluloses isolated from *S. jambolana* (jambolana) were subjected to anaerobic incubation with rat faecal inocula for 48 h. Water-soluble polysaccharide fractions of pulp, seed and seed coat produced higher molar ratios of acetic acid than propionic and butyric acid. Pectic polysaccharide fraction of pulp and seed coat produced 33.8% and 19.7% of propionic acid respectively. However, among WSP and pectic polysaccharide fractions of pulp, seed and seed coat, pectic polysaccharide of pulp showed better butyric acid production (24%). Butyric acid production was significantly better in hemicellulose of seed coat (50%), However, arabinoxylan (60%) isolated from wheat was found to exhibit better butyrogenic property than other polysaccharide fractions.

Table 20: Cultivation of faecal mixed microflora on different fractions of isolated polysaccharides from black plum for SCFA production

Substrate	Relative µmole %		
	Acetic acid	Propionic acid	Butyric acid
P* - WSP[a]	81.0	13.5	5.5
P - ASP[b]	42.0	33.8	24.2
P - HC[c]	40.6	24.2	35.2
S** - WSP	91.0	5.7	3.4
S - ASP	80.4	7.6	12.0
S - HC	63.5	8.4	28.1
C*** - WSP	85.8	10.5	3.7
C - ASP	75.8	19.7	4.5
C - HC	47.8	1.8	50.3
Wheat arabinoxylan	18.3	21.6	60.0

*P: Pulp, **S: Seed, ***C: Seed Coat, [a]WSP: Watersoluble polysaccharides, [b]ASP: Ammonium oxalate soluble polysaccharide (Pectic polysaccharides), [c]HC: Hemicellulose.

3.2.2.1.6. Butyrogenic property of purified bacteria isolated from rat intestine and cultivated on drumstick pulp

Butyric acid production by four purified isolated cultures on drumstick pulp is given in Table 21. When isolates were incubated anaerobically with drumstick pulp (1%) for 48 h, short chain fatty acid production varied among them. Isolate

no 3b produced lesser molar percent of butyric acid (15% substrate) compared to 6a, which produced about 21.4%. Isolate no 6b produced maximum level of butyric acid (67%) with drumstick pulp compared to isolate no 3a (34%).

3.2.2.1.7. Butyric acid production by isolated bacteria grown on guar gum (soluble dietary fiber) and wheat bran (insoluble dietary fiber)

Butyric acid production from isolate no. 6a, 6b and 3a grown on wheat bran and guar gum are presented in Table 22. Among three isolates grown on wheat bran, isolate no. 3a showed poor butyric acid production (13.8%), whereas isolate no 6a and 6b showed better butyrogenic property which was around 21% and 42%, respectively. Among isolates grown on soluble dietary fiber (guar gum), isolate no. 6a and 6b showed a poor butyric acid production with 14.3 and 10.5%, respectively, where as isolate no. 3a showed an enhanced butyric acid production (29%).

Acetic acid production was higher in isolate no 6a grown on guar gum (79%), where as higher propionic acid (45%) production was observed with isolate no 3a indicating that it had good propiogenic property. Isolates no. 6a, 6b and 3a cultivated on guar gum produced biomass of 0.07, 0.06 and 0.02 mg/gram, respectively. However, isolate no. 6b grown on wheat bran showed better biomass (0.98%) production in spite of lower fermentability. However, among all six different combinations isolate no. 6b grown on wheat bran showed maximum butyric acid production along with higher biomass.

Table 21: Butyric acid production (%) by isolated organism grown on drumstick pulp

Isolate number	Relative µmole %		
	Acetic acid	Propionic acid	Butyric acid
6a	63.4	15.2	21.4
6b	15.2	17.4	67.4
3a	52.4	13.6	34.0
3b	19.0	65.4	15.6

Table 22: Butyric acid and biomass production by isolated bacteria grown on guar gum (soluble dietary fiber) and wheat bran fiber (insoluble dietary fiber)

Isolate No.	Substrate	Relative µmole %			Biomass
		Acetic acid	Propionic acid	Butyric acid	mg/g substrate
6a	**Wheat bran**	37.3	41.6	21.1	0.021
6b		16.1	41.4	42.5	0.090
3a		47.1	38.1	13.8	0.010
6a	**Guar gum**	79.3	6.4	14.3	0.070
6b		45.9	33.6	10.5	0.059
3a		25.7	45.2	29.1	0.026

3.2.2.2. Identification of the bacterial isolate

Bacterial culture 6b which showed maximum butyric acid concentration from dietary fiber was characterized by morphological and biochemical methods. The bacterium was gram +ve and had varied shapes (curved, clubbed), branched and also had V shaped cells (Fig. 24). It was non-motile, anaerobic, and negative for catalase, oxidase and nitrate reduction. It was 0.5-1.3 µm breadth and 1.5-8 µm in length. It was non-sporulating, lacked indole production and was resistant to bile salt up to 2%. It produced acids from glucose, fructose, galactose, sucrose, inulin, dextrin, starch, maltose, raffinose and trehalose. The strain fermented arabinose. Optimum growth occurred at 37^{o}C. It was tentatively identified as *Bifidobacterium sp* (Fig 24).

Fig. 24: Gram stained cells of *Bifido bacterium sp.* (Isolate no. 6b)

3.2.2.2.1. Butyric acid production from various dietary fiber sources (1%) incubated with *Bifidobacterium sp.* (isolate 6b)

Butyrogenic property of isolate no 6b with different dietary fiber sources are given in Table 23. Dietary fiber sources studied were fenugreek, bitter gourd, spent turmeric, drum stick, guar gum, wheat bran and chitosan. They were (1%) fermented anaerobically with isolate no 6b for 48h. Short chain fatty acid production pattern varied and butyrogenic property was poor (12.4%) when cornstarch was as used as substrate. Higher acetic acid production was observed

with cornstarch (62.1%), bitter gourd (65.42%) and fenugreek (44.7%). Both bitter gourd (9%) and fenugreek (8.5%) proved to be poor substrate for propionic acid production. Higher butyric acid production was observed with bitter guard (25.6%), fenugreek (48%), chitosan (52.5%) and drumstick (59.8%). Wheat bran an insoluble dietary fiber source once again proved to be the best substrate for butyric acid production (77%). Spent turmeric, which is an industrial waste obtained after removal of curcumin is a rich source of insoluble dietary fiber (70%). This supported highest production of butyric acid (72%) compared to other sources used in the experiment.

3.2.2.3 Effect of dietary fibers on the *in vivo* fermentation and production of SCFA by caecal and faecal microflora

Influence of dietary fiber on food consumption and body weight are presented in Table 24. Starch-fed group and guar gum-fed group consumed an average of 13-14 g and 12-13 g of diet/day, respectively. Food consumption was significantly higher in wheat bran-fed group, which was around 15 g/day. Supplementation of wheat bran to feed showed a significant increase in the body weight (212 g) compared to control group (192 g). Food Consumption was less in the group provided with guar gum compared to starch fed group.

Table 23: Butyric acid production from various dietary fiber sources incubated with *Bifidobacterium sp*.

Substrate	Relative µmole %		
	Acetic acid	Propionic acid	Butyric acid
Cornstarch	62.1	25.5	12.4
Wheat bran	03.9	19.0	77.0
Guar gum	02.0	69.6	28.4
Drumstick	14.4	25.8	59.8
Bitter gourd	65.4	9.0	25.6
Fenugreek	44.7	8.5	46.8
Spent turmeric	05.0	22.7	72.3
Chitosan	25.4	22.1	52.5

3.2.2.3.1. Effect of type of dietary fiber on faecal out put, urine excretion, caecal weight and caecal wall weight

Rats fed with wheat bran showed a significant increase in faecal output compared to guar gum and starch fed rats. Whereas faecal output in guar gum fed group was slightly higher than starch-fed group (Table 25).

Urine excretion also differed between the three groups, however guar gum fed groups excreted more urine (27 mL/5 days) compared to other group. Both caecal content and caecal wall weight differed marginally among the three groups, which were not statistically significant. Caecal content values of CS, WB and GG were 3.83, 3.33 and 3.36 g/100g, respectively.

Table 24: Effect of type of dietary fiber on food intake and body weight gain

Substrate	Faecal out put (g/3 days)	Urine out put (ml/3days)	Colon weight (g/100g body weight)	Caeacal weight (g/100 g body weight)
Starch fed group	1.65 ± 0.075	19.25 ± 3.57	3.83 ± 0.156	2.15 ± 0.152
Wheat bran fed group	4.55 ± 0.35	13.0 ± 4.08	3.33 ± 0.191	1.99 ± 0.096
Guar gum fed group	2.38 ± 0.21	27.5 ± 8.25	3.36 ± 0.208	1.86 ± 0.095

Table 25: Effect of type of dietary fiber on various parameters (faecal weight, urine volume, colon and caecal weight)

Dietary fibers fed to animal groups	Food intake (g/day)	Body weight (Initial, g)	Body weight (Final, g)	Gain in weight (g)
Starch fed group	13.67 ± 0.68	107.62 ± 1.79	192.5 ± 16.47	84.9
Wheat bran fed group	14.85 ± 1.12	107.75 ± 1.81	212.67 ± 12.41	104.9
Guar gum fed group	12.25 ± 0.96	107.75 ± 1.89	181.83 ± 18.29	74.1

3.2.2.3.2. Effect of type of dietary fiber on caecal microbial population in different groups

Bacterial counts in the caecal mass from rats fed on starch, wheat bran and guar gum diet are presented in Table 26. Significant differences were observed in the total anaerobic counts among the groups. Rats fed with wheat bran and guar gum feed supplements harbored higher bacterial population in the caecal mass compared to cornstarch fed control group.

Table 26: Effect of type of dietary fiber on caecal microbial population in different groups

Diet fed to animal groups	Viable cell counts /g		
	Nutrient agar	MRS	Anaerobic agar
Starch	1.83×10^5	0.18×10^5	12.6×10^5
Wheat bran	3.05×10^5	10.3×10^5	16.2×10^5
Guar gum	10.5×10^5	6.22×10^5	15.4×10^5

3.2.2.3.3. Effect of type of dietary fiber on caecal and faecal SCFA

Molar proportion of acetic acid, propionic acid and butyric acid in caecal and faecal content of starch, guar gum and wheat bran fed group is presented in Table 27. Starch-fed group showed marginal variations in proportions of all three acids in both caecal content and faecal content. However, higher butyric acid concentration was observed in faecal (32%) content than caecal (28%) content. Faecal content of guar gum fed-group showed higher acetic acid concentration (56%) and higher propionic acid concentration was observed in caecal content of the guar gum-fed group (47.9%). Wheat bran-fed group showed a better butyric acid concentration in the both faecal and caecal content compared to other groups.

Table 27: Effect of type of dietary fiber on faecal and caecal short chain fatty acids production

Animal groups	Relative µmole %					
	Acetic acid		Propionic acid		Butyric acid	
	Faecal	Caecal	Faecal	Caecal	Faecal	Caecal
Starch	29.2	32.9	38.4	38.5	32.4	28.6
Wheat bran	37.7	28.8	28.3	29.6	34.0	41.6
Guar gum	56.1	30.4	35.1	47.9	7.8	21.7

3.2.2.3.4. Changes in caecal weight and viable microbial population due to butyric acid supplementation to diabetic animals

Supplementations of butyric acid (500 mg/kg body weight) have beneficial effect on the diabetes-induced animals. This group of animal was used to evaluate the caecal weight, caecal content and viable cell counts in the caecal material (Table 28).

Data in Table 28 shows that butyric acid feeding resulted in increased caecal weight and contents in cornstarch as well as wheat bran plus guar gum-fed group of animals. Viable cell counts of anaerobic microflora and lactics (on MRS agar) showed enhancements under butyric acid feeding.

Table 28: Effect of butyric acid supplementation to diet of diabetic animals, on the caecal content and viable cell counts

Animal group	Caecal weight (g)	Caecal content (g)	Viable cell count (cfu/g caecal content)			
			A	B	C	D
1	3.4	1.8	$5.0x10^{5}$	$3.6x10^{7}$	$8.0x10^{7}$	$1.5x10^{10}$
2	4.7	3.2	$3.0x10^{6}$	$4.5x10^{6}$	$1.4x10^{10}$	$2x10^{10}$
3	8.1	5.8	$3.6x10^{6}$	$1.1x10^{8}$	$9.5x10^{10}$	$4.6x10^{10}$
1a	2.5	1.4	$1.0x10^{6}$	$1.7x10^{7}$	$1.5x10^{9}$	$8.6x10^{10}$
2a	3.6	2.5	$5.5x10^{6}$	$1.0x10^{6}$	$7.7x10^{9}$	$6.4x10^{6}$
3a	14.0	11.3	$1.0x10^{6}$	$8.8x10^{7}$	$7.4x10^{9}$	$4.5x10^{10}$

Diets and treatment given to animal groups:

1, Starch (control); 2, Starch (diabetic); 3, Starch (diabetic) with butyric acid supplementation.

1a, Wheat bran and guar gum (control); 2a, Wheat bran and guar gum (diabetic); 3a, Wheat bran and guar gum (diabetic) with butyric acid supplementation.

Media used for bacterial counts:

A, MacConkey agar; B, Nutrient agar; C, Anaerobic agar; D, MRS agar.

3.2.3. Discussion

a) *In vitro* fermentation of dietary substrates

Increased intake of dietary fiber and their sources are recommended for people suffering from chronic constipation, bowel dysfunction and diabetes in particular (Csordas, 1996). These are well established to be beneficial to diabetics as they act as insoluble matrix or indirectly as short chain fatty acids such as acetate, propionate and butyrate. Butyric acid derivatives have been shown to have hypoglycemic property (Fuchs et al. 1999; Ohta et al. 1999). During the last

decade many studies have been conducted to investigate the factors responsible for production of butyric acid and to understand the physiological importance of butyiric acid concentration (Della et al. 2001; Kanauchi et al. 1999; Wolin and Miller, 1983; Zoetendal et al. 1998). Therefore, the effect of various dietary fibers and their sources on the *in vitro* production of butyric acid by rat faecal/caecal microflora was studied in the present investigation and some of the intestinal bacteria were also evaluated for their butyrogenic property with different dietary fiber sources.

The ecosystem of the human gastrointestinal tract is highly complex and consists of more than 500 species of bacteria in a constant state of change (Weaver et al. 1992; Jensen, 2001). This situation is influenced by various factors including diet, stress, age, intake of medicines etc (Hold et al. 2002; Macfarlane and Cummings, 1991; Knudsen and Hansen, 1991). It is now known that intestinal microflora play a role of prime importance for health. For this reason there is a growing interest in manipulating the composition of intestinal flora in order to achieve a more beneficial intestinal bacterial community. The anaerobic microbial communities of the mammalian large intestine and rumen produce the short chain fatty acids (SCFA) namely acetate, propionate and butyrate as main non-gaseous fermentation end products (Scheppach et al. 2001; Mortensen et al. 1996). Our data indicated that mixed caecal bacteria present in rats can utilize various dietary fibers such as powdered fenugreek seeds, pulp of bitter gourd and drumstick, wheat bran (destarched), guar gum and jambolana seed powder under *in vitro* conditions to produce SCFA. Concentrations of butyric acid produced on these substrates varied. This would be due to the nature of dietary fibers used for bacterial growth, composition of polysaccharide and water-soluble and insoluble components present in them. Studies on dietary fiber and their sources are of great interest as it appears that the source and type of these indigestible substances

determine the physiological effect that they will exert on the body as measured by molar ratios of specific SCFAs (Fredstrom et al. 1994). This may be due to the presence of soluble and insoluble dietary fiber. In the present investigation, dietary fiber sources, such as, fenugreek, bitter gourd and drumstick showed butyrogenic property with caecal inoculum, whereas they were propiogenic with faecal inoculum indicating that butyrate producers are rich in caecal inoculum. Results clearly indicate that the faecal and caecal microbial population vary and depends on the type of dietary fiber used as feed supplements.

Bacterial population in the faeces is representative of those in the intestine (Gary et al. 1986). Short chain fatty acid production depends not only on microbes but also on source and their constituent sugar. Different microbes may utilize different sugars to varied extents to produce SCFA. Utilization of sugars such as glucose, rhamnose, galactose, fucose, arabinose, mannose and xylose by mixed culture varied. However, fucose and glucose supported good microbial growth along with good amount of butyric acid production (Table 16). According to earlier study, glucose was the main contributing substrate for acetate production (Christine et al. 1997). One interesting finding observed in an earlier study in humans was that *in vitro* fermentation of rhamnose produced significantly more propionate (Mortensen et al. 1988). In our study also higher propionic acid production by rhamnose compared to other sugars was observed. Guar gum showed propiogenic and arabinoxylan showed butyrogenic properties. Earlier reports have shown that arabinoxylan produce more butyric acid (Bach Knudsen et al. 1993b). The intestinal microflora and its response to changes in carbohydrate type display wide variation among individuals (Hijova and Chmelarva, 2007) and the bacterial community present in the faeces mimics this variation. Therefore, faecal specimens can be used to assess the ability of intestinal microflora to metabolize carbohydrates. A homogenate of rat faeces pooled from three

individual rats were used as inocula in a batch culture system since this approach has been shown to produce reproducible fiber fermentation profiles and digestibility that correlates with metabolizable energy from fibers using animal models. In the present study faecal and caecal mixed flora from rats fed with different dietary fiber sources were used for *in vitro* study. Caecal mixed microflora produced higher concentrations of butyric acid compared to faecal mixed microflora which indicates that anaerobic bacteria which would be present in the caecal material than in faecal material that is exposed to atmosphere over a period of time may be mainly responsible for the synthesis of butyric acid. Several bacteria such as *Clostridium sp, Lactobacillus sp, Bifidobacterium sp, Eubacterium lentum, Bacterroides sp, Fusobacterium sp, coliforms* etc. are known to be present in the intestine of animals (Finegold et al. 1983; Moore and Moore, 1995). Cultivation of mixed flora on different fractions of polysaccharides obtained from pulp, seed coat and seed powder of jambolana showed that hemicelluloses present in the seed coat support synthesis of higher concentration of butyric acid (Table 20). Similarly. arabinoxylan fraction isolated from wheat bran also yielded higher concentration of butyric acid (Table 16). Total SCFA production from the microbial fermentation of complex oligosaccharides has been indicated by several investigators to be linearly substrate dependent rather than dependent on the individual (Cummings and Englyst, 1987; Wurm, 1996; Casterline et al. 1997; Alam et al. 1998). Our results also indicated significant differences when different polysaccharides were fermented with faecal mixed culture. Among water soluble, pectic, hemicellulose A and hemicellulose B of pulp seed and seed coat, water soluble polysaccharides of all the three produced acetic acid as a major SCFA. In general pectic substances and gums are easily metabolized, whereas the fermentability of hemicelluloses mainly depends on their solubility. The susceptibility of cellulose to bacteria is primarily determined by its

crystallinity. Lignin, however is not fermented. Previous experiments indicated that fermentation of citrus pectin produced higher proportion of acetic acid (Berggren et al. 1993; Brighenti et al. 1989). In our study, even though pectic polysaccharides supported acetic acid, pectic polysaccharide from pulp produced fair amount of butyric acid. Interestingly, hemicelluloses of all the three produce good amount of butyric acid, however among them seed coat hemicelluloses proved to possess better butyrogenic property. Difference in butyrogenic property among polysaccharide could be due to nature and composition of polysaccharide. Hemicellulose of the pulp, seed and seed coat were rich in glucose and xylose indicating the presence of either β-glucan, xylan or xyloglucan type of polysaccharide.

Utilization of substrates for fermentation is dependant on type bacteria involved, the solubility and fermentability of substrates used. Due to differences in solubility it is known that wheat bran is less fermentable than guar gum (Mc Intyre et al. 1993). However in the present experiments fermentability of wheat bran and spent turmeric, which are the sources of insoluble dietary fiber, produced good amount of butyric acid with mixed bacterial flora. Feeding of required dietary fiber would enhance the necessary bacterial population for butyric acid fermentation. Inoculum of bacteria obtained after feeding of wheat bran showed better butyrogenic properties compared to guar gum or cornstarch fed rats (Table 27). This could be because of feeding wheat bran for a month may stimulate the butyrogenic bacteria in their gut. Such bacteria could be responsible for butyric acid production with wheat bran, guar gum and cornstarch *in vitro*. However, both faecal and caecal culture with wheat bran produce higher butyric acid indicating that both faecal and caecal inocula could be rich in butyric acid producers.

It is shown that increasing incubation time leads to increase in the short chain fatty acids (Wisker et al. 1998; Finegold et al. 1983). Data from our

experiment clearly shows that increasing incubation time from 24 h to 48 h increases butyric acid production in all the three substrates. Increased substrate concentration however resulted in decreased butyric acid production. Several butyrate-producing species are shown to require SCFA for growth (Finegold et al. 1983; Moore and Moore, 1995) and this would lead to better utilization of soluble and insoluble dietary fibers.

Literature reports on cultivable human intestinal microflora have shown that the colon harbors significant populations of genera, such as *Clostridium, Eubacterium* and *Fusobacterium* that include butyrate-producing species. Bifidobacterial species are common members of the human gut microflora, comprising up to 3% of the total faecal microflora. *Bifidobacteria* are considered to be beneficial bacteria, and they are used in the preparation of probiotic products. The gastrointestinal microflora is influenced by diet, age and environmental conditions and by the host genotype. The number of food and other dietary products containing live *Bifidobacteria* has increased significantly in recent years, due in part to the beneficial effects. In the present study, amongst various purified isolates obtained from intestine of rats fed with wheat bran, *Bifidiobacterium sp* was isolated as potent bacterium that produced higher concentrations of butyric acid.

b) *In vivo* fermentation of dietary substrates

Physiological effects of dietary fibers are affected by their physical nature (Compheret al. 1999; McIntyre 1993; Edwards and Eastwood, 1992) and their solubility influences the fermentability of such substrates. Studies on SCFA production by soluble and insoluble dietary fibers are well documented. Here attempts were made to study the effect of type of dietary fiber on faecal and caecal butyric acid production in *in vivo*. Food intake was influenced by diet and it was

observed that wheat bran fed group consumed more diet and this may be due to high caloric dilution (Plaami, 1997). Less consumption of guar gum fed group could be because of nonpallatable nature of guar gum. Authors in earlier studies showed that rats consumed more of wheat bran containing diet than that of guar gum (Nandini et al. 2000). Body weight is found to be directly influenced by diet consumption and wheat bran based diet group showed significant increases in body weight compared to other groups. Others have also reported that wheat bran based diet improves body weight in control and diabetic animals (Nandini et al. 2003).

Faecal weight and urine exertion are greatly influenced by diet type and it is directly correlated with amount of diet consumption. Animals fed with wheat bran excreted less urine compared to other groups. This may be because of less consumption of water during feeding. Higher urine volume excretion in guar gum fed group could be because of more water consumption, as the diet is nonpalatable in nature. Faecal weight was more in wheat bran than starch and guar gum. This is because of faecal bulk due to insoluble dietary fiber (wheat bran). Starch fed group showed little difference in proportions of all three acids in both caecal content and faecal content. However, higher butyric acid concentration was observed in faecal (34%) content than caecal (28%) content. Faecal content of guar gum fed group showed higher acetic acid proportion (51%) and higher propionic acid production was observed in caecal content of the guar gum fed group (47.9%). Wheat bran fed group showed a better butyric acid proportion in both faecal and caecal content compared to other groups. Overall the experiments indicated that the guar gum has propiogenic and wheat bran has butyrogenic property as evidenced by *in vivo* and *in vitro* experiments.

3.2.4 Summary and conclusions

1. Substrates at 1% concentration and 48 h incubation were found to produce butyric acid at higher level and lower pH indicated higher SCFA concentration.
2. Rhamnose was found to be propiogenic in nature amongst various carbohydrates used for mixed microflora fermentaion, where as hemicellulose was found to posess butyrogenic property among various polysaccharides studied *in vitro*.
3. Dietary fiber source may influence the faecal and caecal bacterial composition as evidenced by butyric acid production and varied with microbial source (faecal and caecal).
4. Bacterial isolate that produced maximum quantity of butyric acid was identified as *Bifidobacterium* species.
5. Feeding insoluble fiber (wheat bran) for long duration may enhance the butyrate producers and degrade the slowly fermented fibers.
6. Type of dietary fiber influences the food and water consumption, body weight and faecal out put.

From the above data it can be concluded that the butyrogenic property of substrate depends on substrate type, composition, concentration, incubation time, microbial source and feeding period.

3.3. Effect of *Moringa oleifera* (drumstick) pulp and *Syzygium jambolana* (jambolana) seed on diabetic and diabetic nephropathy state with particular emphasis on heparan sulphate

3.3.1. Introduction

Diabetes is a disease of great concern to many all over the world (Zimmet, et al. 2001) and is known for its complications, which includes diabetic nephropathy, neuropathy and retinopathy (Brownlee, 2001). During diabetic nephropathy, kidney basement membrane becomes thicker making glomeruli more porous to the macromolecules (proteinurea). Analysis of glomerular basement membrane during diabetes have indicated changes in its constituents with an increase in the major component, type IV collagen and decrease in heparan sulfate and laminin (Rohrbach et al. 1982). Decrease in heparan sulfate content during diabetes results in high glomerular filtration rate (GFR), which is a characteristic feature of diabetic nephropathy and is well documented.

Diet plays a major role in the management of diabetes. Besides drugs, complimentary medicines are receiving increased attention all over the world. These include dietary fibers and medicinal plant therapies (Oubré et al. 1997; Atta-ur-rahman et al. 1989) Foods rich in dietary fibers have been advocated for better diabetes management because of their proven beneficial effects (Cummingset al. 1985). Fruits and vegetables are rich sources of dietary fiber (Plaami et al. 1997). Drumstick is one of the commonly consumed vegetables rich in dietary fiber and hence is of interest to study its hypoglycemic effect. Another plant material, jambolana is a potential source in the management of diabetes that is widely used in Ayurvedic medicine.

Hence, the present study aims at understanding the effect of *M. oleifera* pulp and *S. jambolana* seeds on diabetes and its role in minimizing the diabetic nephropathy status with particular emphasis on heparan sulfate in kidney.

3.3.2 Results

In the present investigation, male Wistar rats weighing between 110-120 g were used for the study. The rats were maintained on diet containing starch in starch fed group and the experimental group received drumstick pulp powder at the concentration of 10% (w/w). The jambolana seeds were powdered and used at 2.5% (w/w) level (Table 11). Animals were maintained with their respective food for one week for food acclimatization before the induction of diabetes. Diabetes was induced by injecting streptozotocin at 55 mg/kg body weight (Hatch et al. 1995) and diabetic status was examined by measuring the fasting blood glucose (Hugget and Nixon, 1957) in the blood, drawn from retro-orbital plexus. The rats were fasted overnight to obtain fasting blood glucose. Animals were grouped and fed with starch-based diet for starch-fed control (SFC) and starch-fed diabetic (SFD), drumstick based diet for drumstick-fed control (JFC) and jambolana-fed diabetic (JFD) and jambolana based diet for jambolana-fed control (DFC) and jambolana-fed diabetic (DFD) groups. The rats were given diet and water *ad libitum*. The rats were monitored for diet intake, water intake, and gain in body weight, urine output, urine sugar, fasting blood glucose, kidney weight and glomerular filtration rate. Urine was collected by keeping rats in metabolic cages. The rats were sacrificed when the mortality was setting in.

Effect of drumstick pulp and jambolana seed on food and water intake in control and diabetic rats

Effect of jambolana seed powder and drumstick pulp was studied on food and water intake in control and diabetic rats, which are presented in Table 29. Fresh diet was given daily and the consumption of diet was recorded. Starch fed control (SFC) rats consumed on an average around 14 g of diet/day. However, consumption of diet was more in starch fed diabetic group (SFD) compared to

control group (SFC), which was statistically significant. Diet consumption was higher in both drumstick-fed control and diabetic groups compared to other groups. DFD rats consumed around 18 g/day, whereas consumption of diet was a little less in jambolana fed groups. The JFC group consumed around 13 g/day and the JFD consumed around 15 g/day.

Water consumption in control groups (SFC, DFC and JFC) was almost similar (around 24 mL/day), whereas, consumption of water increased significantly in starch fed diabetic (SFD) group (94 mL/day) (Table 29). However, increased consumption of water during diabetes is controlled by both drumstick and jambolana in the diet, which reached statistically significant proportions when compared to starch fed diabetic groups.

Effect of drumstick pulp and jambolana seed on body weight in control and diabetic rats

Effect of drumstick pulp and jambolana seed on body weight is presented in Table 30. The control rats (SFC) showed an increase in body weight compared to diabetic rats (SFD) towards the end of the experimental period. The animals in SFD group lost weight, which was ameliorated by the presence of drumstick pulp and jambolana seed in the diet.

Table 29: Effect of drumstick pulp and jambolana seed powder on food and water intake

Groups	Food intake (g/24 h)	Water intake (mL/24 h)
SFC	14.4 ± 0.62	24.0 ± 2.33
SFD	17.1 ± 0.92[a]	94.1 ± 3.63[a]
DFC	16.3 ± 0.62	24.0 ± 7.23
DFD	17.8 ± 0.89	76.0 ± 7.23[b]
JFC	13.4 ± 0.47	23.4 ± 1.43
JFD	14.8 ± 0.72 [b]	74.0 ± 7.03[b]

SFC: Starch fed control, SFD: Starch fed diabetic, JFC: Jambolana seed fed control, JFD: Jambolana seed fed diabetic, DFC: Drumstick fed control, DFD: Drumstick fed diabetic. Values are mean ± SEM of 6 rats in control and 14 rats in diabetic groups,

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

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

Effect of drumstick pulp powder and jambolana seed powder on urine volume and urine sugar in control and diabetic rats

Urine volume and urine sugar levels form important features of diabetic status. These parameters were followed weekly (Figs. 25 and 26). Control rats in all groups (SFC, DFC and JFC) excreted about 10-15 mL urine/day. During diabetes (SFD), rats excreted around 80-100 mL/day (Fig. 25). However, DFD group excreted around 60-65 mL/day and was significantly less than the SFD

group. The JFD group showed comparatively less amount of urine excretion among the diabetic groups and was around 50-60 mL/day.

Urine sugar was monitored on weekly basis after induction of diabetes (Fig 26). The control rats showed very less reducing sugar in urine (10-20 mg/day), whereas SFD showed high amounts of sugar levels (7-8 g/24h). The animals in JFD and DFD groups showed lesser levels of urine sugar compared to SFD group, which were around 5-6 g and 4-5 g/24h.

Table 30: Effect of drumstick pulp and jambolana seed powder on body weight of control and diabetic rats

Groups	Initial body weight (g)	Final body weight (g)
SFC	129.0 ± 0.05	250.3 ± 5.17
SFD	130.0 ± 0.14	128.3 ± 7.48[a]
DFC	121.6 ± 0.07	240.3 ± 1.47
DFD	121.3 ± 2.66	154.7 ± 9.98 [b]
JFC	127.3 ± 5.18	260.0 ± 5.64
JFD	126.1 ± 0.88	150.1 ± 13.10[b]

Footnote: As in Table 29.

Effect of drumstick pulp and jambolana seed on fasting blood glucose in control and diabetic rats

Fasting blood glucose was measured in the blood collected while sacrificing the rats. The rats were fasted overnight to collect fasting blood and amount of glucose was estimated in plasma (Fig. 27). The control rats of all the groups showed fasting blood sugar of around 130 mg/dL. Among the diabetic groups, SFD group showed higher amounts of blood glucose (around 335 mg/dL), which was statistically significant when compared to SFC. The rats in DFD and JFD groups showed an improvement to the tune of 17% (~ 280 mg/dL) and 23% (~ 280 mg/dL), respectively when compared to SFD.

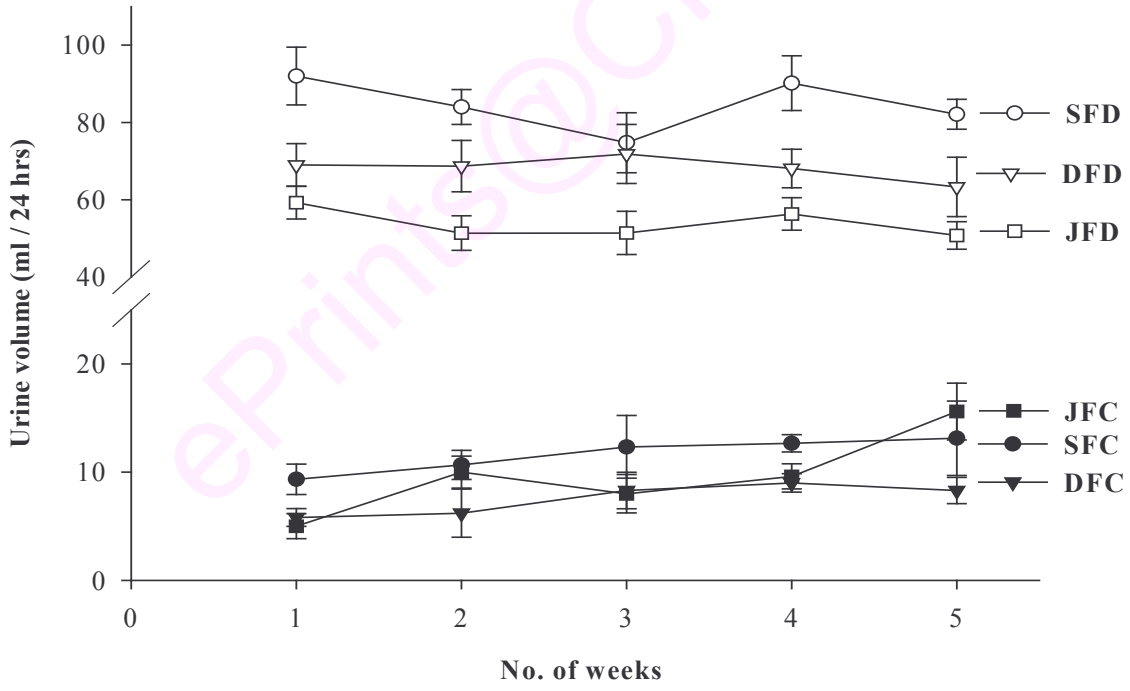


Fig. 25: Effect of drumstick pulp powder and jambolana seed powder on urine volume in control and diabetic rats.

Abbreviations as in Footnote to Table 29.

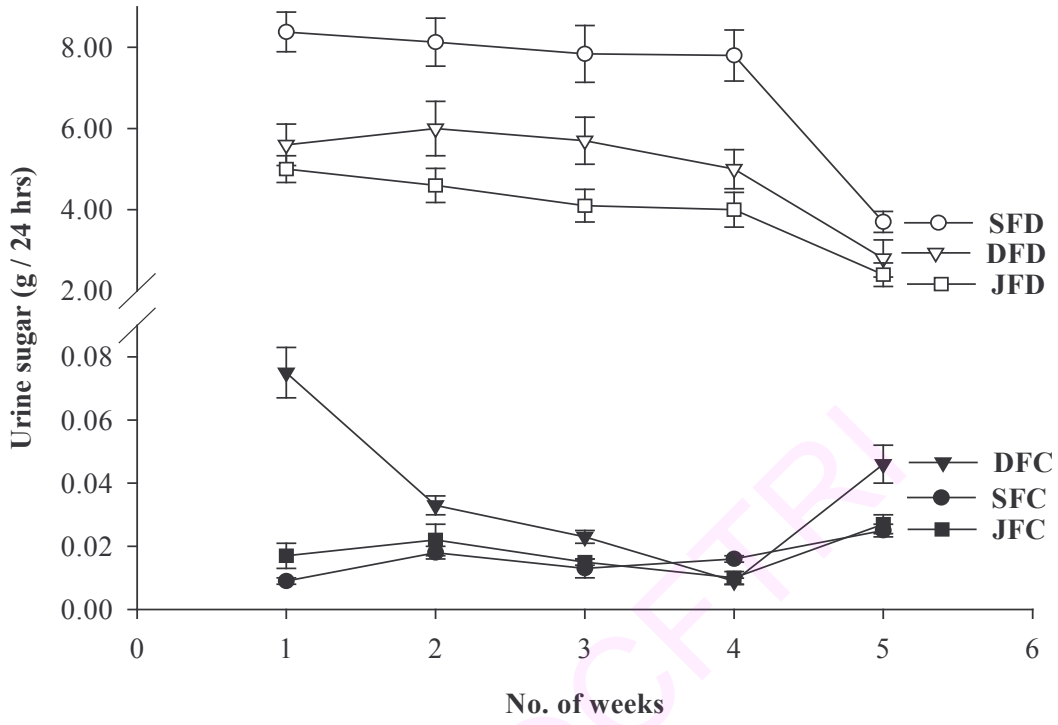


Fig. 26: Effect of drumstick pulp powder and jambolana seed powder on urine sugar volume in control and diabetic rats.

Abbreviations as in Footnote to Table 29.

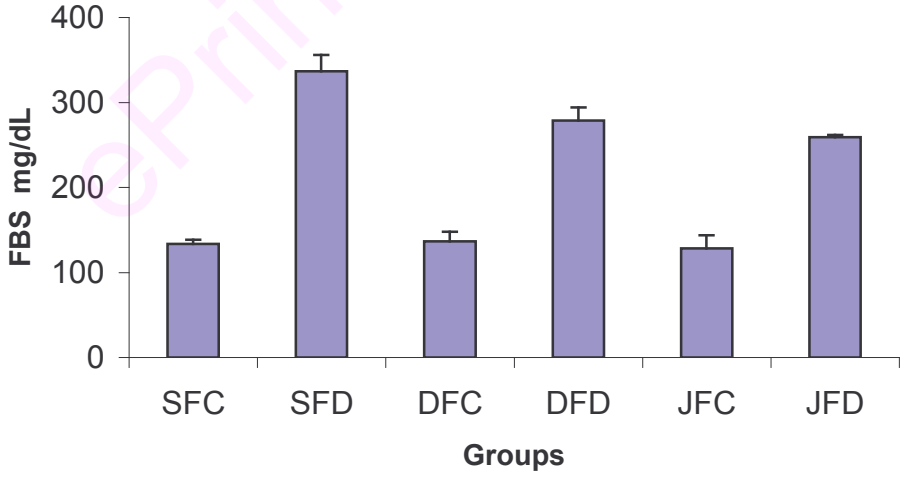


Fig. 27: Effect of drumstick pulp and jambolana on fasting blood glucose (FBS) in control and diabetic rats.

Abbreviations as in footnote to Table 29**.**

Effects of drumstick pulp and jambolana seed on intestinal disaccharidases in control and diabetic rats

Effect of drumstick pulp and jambolana seed on activities of intestinal maltase, sucrase and lactase are given in Table 31. Maltase and sucrase activities increased in diabetic rats (SFD), which was ameliorated to various extents by the presence of drumstick pulp and jambolana seed powder in the diet. Among the two, jambolana seed powder was more effective in bringing about the decrease of increased maltase activities. Jambolana seed in diet decreased the increased maltase and sucrase activities to the tune of 40% and 35%, respectively. Drumstick pulp on the other hand was not effective in bringing down sucrase activity. Lactase also showed an increase in activity during diabetes. However, unlike maltase and sucrase, presence of drumstick seed and jambolana powder was not so much effective in ameliorating it.

Table 31: Effect of drumstick pulp and Jambolana seed on intestinal disaccharidases in control and diabetic rats

Groups	µmoles of product formed/ g/ h		
	Maltase	Sucrase	Lactase
SFC	422.0 ± 5.51	82.4 ± 7.49	20.4 ± 2.71
SFD	678.8 ± 10.29[a]	129.0 ± 14.67[a]	26.9 ± 2.53[a]
DFC	435.6 ± 8.69	89.3 ± 10.55	22.1 ± 2.80
DFD	555.4 ± 19.38[b]	119.7 ± 4.55	25.8 ± 3.10
JFC	404.6 ± 8.22	91.9 ± 3.92	23.9 ± 0.88
JFD	508.5 ± 21.63[b]	100.5 ± 6.77[b]	25.3 ± 3.85

Footnote as in Table 29.

Effect of drumstick pulp and Jambolana seed powder on renal disaccharidases in control and diabetic rats

Effect of drumstick and jambolana on activities of renal maltase, sucrase and lactase are given in Table 32. In kidney, all the three enzymes, maltase, sucrase and lactase showed considerable variations in their activities. Unlike intestinal disaccharidases, the enzyme activities here showed a decrease during diabetes. Maltase activity was highest among them. Marginal variation was found in maltase activities in control groups (SFC, DFC and JFC). During diabetes (SFD) a significant decrease in maltase activity (57.7%) was observed when compared to SFC. Presence of drumstick and jambolana in the diet influenced the activities of maltase. Drumstick feeding controls the decreased maltase activity by 33.6%, whereas, jambolana by 34%. Sucrase activity also decreased significantly during diabetes (SFD), which was ameliorated by feeding jambolana but not drumstick. There was a significant decrease in lactase activity in SFD and both drumstick and jambolana was ineffective in controlling their decreased activities.

Effect of drumstick and Jambolana on organ weights in control and diabetic rats

Diabetes affects the weight of various organs (Table 33). The various organs examined were liver, spleen, lung, brain, stomach, colon, heart, testes, brain and intestine. It was observed that, there was significant increase in weight of some organs such as liver, lung, brain, testes, intestine and colon studied during diabetes. Decrease in weight in spleen and increase in weight of liver and kidney has been reported earlier. However, only jambolana in diet prevents the increased weight of some of these organs (lung, brain, testes and colon) significantly compared to control (SFC). But drumstick didn't show any beneficial effect on weights of these organs.

Table 32: Effect of drumstick and Jambolana pulp on renal disaccharidases in control and diabetic rats.

Groups	µmoles of product formed/g/h		
	Maltase	Sucrase	Lactase
SFC	173.4±10.27	2.9 ± 0.65	2.8 ± 0.25
SFD	73.6 ± 5.75[a]	1.9 ± 0.26[a]	1.7 ± 0.16a
DFC	189.8 ± 11.12	2.7 ± 0.32	2.6 ± 0.23
DFD	132.2 ± 8.29[b]	2.0 ± 0.28	1.9 ± 0.10
JFC	144.5 ± 17.01	2.6 ± 0.21	2.5 ± 0.24
JFD	132.9 ± 10.27[b]	2.1 ± 0.35[b]	2.0 ± 0.09

Footnote as in Table 29.

Effect of drumstick pulp and Jambolana seed on kidney weight and glomerular filtration rate in control and diabetic rats

Kidney damage is one of the serious consequences of the complications of diabetes. Kidney weight was examined in different groups of rats and the data is presented in Table 34. Diabetic animals (SFD) showed an increase in relative weight of kidney compared to control groups (SFC) significantly. Control rat kidney weighed around 0.6-0.7g, whereas, diabetic animals weighed around 1.6-1.7g. This increase in the weight of the kidney was ameliorated to various extents by both drumstick and jambolana fed diabetic groups. Comparatively, jambolana (JFD) was found better in controlling kidney weights by 20% and was statistically significant when compared to SFD.

Glomerular filtration rate (GFR) is one of the important markers of kidney function. The glomerular filtration rate (mL/min) increased during diabetes and

increased to about 16 mL/min in SFD (Table 34). The filtration rate was ameliorated to 9.9 mL/min in DFD and was much better in JFD group (8.6 mL/min). These results clearly demonstrated that kidney functions are improved by feeding drumstick and jambolana seed powder to diabetic rats.

Effect of drumstick and jambolana on enzymes of glycoconjugate metabolism in kidney

L-Glutamine-fructose-6-phosphate aminotranseferase (GFAT) is a biosynthetic enzyme involved in amino sugar synthesis. There was a significant increase in GFAT activity during diabetes (SFD) compared to control rats (SFC) (Table 35). Incorporation of jambolana and drumstick in diet was effective in controlling the increased GFAT activity by 19% and 32%, respectively.

N-acetyl-glucosaminidase and β-glucuronidase, which is degradative enzyme activities were also studied (Table 35). Degradative enzymes like N-acetyl-β-glucosaminidase and β-glucuronidase act on glycoproteins and GAGs. The activity of NAG was found to be significantly increased during diabetes (SFD). Jambolana in the diet was effective in preventing the increased activity, which was not the case with drumstick. β-glucuronidase activity was elevated during diabetes, which was statistically significant compared to control (SFC). However, both jambolana and drumstick supplementation in the diet resulted in the decrease of β-glucuronidase activity in both control and diebetic animals, which was statistically significant in JFD and DFD, when compared to SFD.

Table 33: Effect of drumstick pulp and jambolana seed on organ weights in control and diabetic rats

Group	Liver	Spleen	Lungs	Brain	Testes	Heart	Intestine	Stomach	Colon	Pancreas
	g/100g									
SFC	3.68 ± 0.103	0.25 ± 0.018	0.51 ± 0.017	0.7 ± 0.033	1.072 ± 0.113	0.354 ± 0.007	2.68 ± 0.21	0.88 ± 0.136	1.27 ± 0.152	0.093 ± 0.009
SFD	5.037 ± 0.140[a]	0.28 ± 0.02	0.79 ± 0.052[a]	1.24 ± 056[a]	1.53 ± 0.108[a]	0.44 ± 0.02	6.41 ± 0.329[a]	0.98 ± 0.066	7.28 ± 0.667[a]	0.104 ± 0.015
DFC	3.73 ± 0.080	0.25 ± 0.013	0.47 ± 0.017	0.73 ± 0.095	1.21 ± 0.023	0.339 ± 0.013	2.86 ± 0.103	0.78 ± 0.046	1.52 ± 0.24	0.067 ± 0.016
DFD	4.89 ± 0.320[NS]	0.29 ± 0.012	0.66 ± 0.033[NS]	1.042 ± 0.048[NS]	1.52 ± 0.066[NS]	0.426 ± 0.018	5.96 ± 0.257[NS]	1.27 ± 0.161	6.63 ± 0.83[NS]	0.106 ± 0.009
JFC	3.25 ± 0.081	0.26 ± 0.018	0.55 ± 0.062	0.66 ± 0.019	1.17 ± 0.018	0.341 ± 0.011	2.773 ± 0.19	0.73 ± 0.069	1.45 ± 0.183	0.077 ± 0.003
JFD	5.032 ± 0.190	0.28 ± 0.020	0.73 ± 0.063[b]	1.18 ± 0.076[b]	1.304 ± 0.082[b]	0.43 ± 0.026	6.73 ± 0.461	1.03 ± 0.09	7.58 ± 1.165[b]	0.093 ± 0.01

Footnote as in Table 29

Table 34: Effect of drumstick pulp powder and jambolana seed powder on kidney weight and glomerular filtration rate in control and diabetic rats

Groups	Kidney weight (g/100 g body wt)	Glomerular filtration rate (mL/ min)
SFC	0.671 ± 0.01	2.390 ± 0.51
SFD	1.259 ± 0.06 [a]	16.215 ± 2.36 [a]
DFC	0.672 ± 0.02	1.799 ± 2.25
DFD	1.141 ± 0.03	9.880 ± 1.26 [b]
JFC	0.638 ± 0.01	2.450 ± 0.28
JFD	1.080 ± 0.04 [b]	8.600 ± 1.52 [b]

Footnote as in Table 29

Effect of drumstick and jambolana on carbohydrate composition of kidney tissues of control and diabetic rats.

Kidney composition such as total sugars, uronic acids, aminosugars, sulfates and protein were estimated (Table 36). The above estimations were carried out before pooling of kidneys for GAG extraction. There was a significant increase in total sugars, uronic acids, amino sugar and decrease in protein and sulfate content in SFD groups compared to SFC. In both drumstick and jambolana fed diabetic animals the increase in total sugars was prevented by 29% and 35% respectively.

Table 35: Effect of drumstick and Jambolana pulp on kidney glycosaminoglycans metabolizing enzymes

Groups	L- Glutamine fructose-6-phosphate aminotransferase (GFAT)	N-Acetyl β-glucosaminidase (NAG)	β-Glucuronidase
	μmoles of product formed/ g of protein/ min		
SFC	47.7 ± 3.99	1238.0 ± 87.73	3.1 ± 0.19
SFD	73.2 ± 6.38[a]	1998.3 ± 119.28[a]	4.0 ± 0.32[a]
DFC	49.9 ± 4.09	1308.3 ± 66.54	3.1 ± 0.19
DFD	68.9 ± 5.94[b]	1837.3 ± 115.90[b]	2.8 ± 0.22[b]
JFC	53.5 ± 5.75	1214.0 ± 105.75	3.0 ± 0.19
JFD	59.3 ± 4.55[b]	1538.9 ± 117.14[b]	2.1 ± 0.16[b]

Footnote as in Table 29.

Uronic acid content increased during diabetes (SFD). Incorporation of jambolana and drumstick in the diet effectively prevented by the increase by 27 and 13%, respectively. Amino sugar content during diabetes (SFD) was significantly increased compared to control (SFC). In DFD and JFD, the increases in amino sugars were narrowed down to various extents, which were significant when compared to SFD. Content of proteins decreased during diabetes in kidney in SFD and ameliorated by both drumstick and jambolana. Sulfate groups have received lot of attention in recent past. During diabetes decrease in sulfate content is reported in GBM (Cohen and Surma, 1981). In our study significant change was observed in diabetic group compared to control.

Effect of drumstick and jambolana on carbohydrate composition of kidney glycosaminoglycan

Isolated GAG from kidney was used for their compositional study and data is presented in Table 37. After estimations were carried out, the kidney tissue of the respective groups was pooled to isolate GAGs because of being present in small quantities. Uronic acid, amino sugar, sulfates and total sugars were estimated in the isolated GAGs. Uronic acid is one of the major components of GAGs like heparan sulfate and chondroitin sulfate. Its estimation gives a picture of the profile of GAGs in the kidney tissues. Uronic acid content in all the control groups was almost the same. During diabetes, there was a decrease in uronic acid content in SFD and there was amelioration in the decreased uronic acid content in drumstick and jambolana fed groups. Total sugar content decreased during diabetes (SFD) significantly when compared to SFC. With drumstick and jambolana in the diet the decrease was 20 and 30%, respectively, when compared to SFC.

Amino sugar content decreased during diabetes by 20%, which was prevented to some extent by the presence of drumstick and jambolana in the diet. Jambolana was much better than drumstick in preventing the decrease in amino sugar content. It was interesting to observe a decrease in the content of sulfate in kidney during diabetes (SFD) compared to control (SFC). Significant amelioration in the content of sulfate was observed by the feeding of drumstick pulp and jambolana seed.

Effect of drumstick pulp and jambolana seed powder on total sulfated glycosaminoglycan, heparan sulfate and chondroitin sulfate

The content of sulfated GAG was estimated in the isolated GAG sample. Control groups SFC, DFC and JFC showed almost similar amounts of GAG in the

kidney. There was a significant decrease in the content of GAG in diabetic groups (SFD) compared to control (SFC). Incorporation of drumstick and jambolana in the diet ameliorated the decreased GAG to various extents. Jambolana was found to be more effective than drumstick in ameliorating the decreased GAG content.

GAGs were fractionated based on the specificity to chondroitinase ABC. The undigested fraction represents the heparan sulfate based on the estimation with DMMB. The content of heparan sulfate decreased in the starch fed diabetic group (SFD) compared to its control (SFC) by 56%. Both drumstick and jambolan were effective in ameliorating the decrease in heparan sulfate content in the kidney to various extents (Fig. 28). The content of chondroitin sulfate decreased in the SFD groups compared to control (SFC) and it was observed that both drumstick and jambolana are effective in preventing their decreased metabolism. However, jambolana was found to be more effective than drumstick. Similar pattern was observed with respect to chondroitin sulfate. Changes in total GAGs, heparan sulfate and chondroitin sulfate in diabetes and their modulation by drumstick and jambolana is given in Fig. 28.

Table 36: Effect of drumstick pulp and jambolana seed carbohydrate composition of kidney tissues of control and diabetic rats.

Groups	*Total sugar*	*Uronic acid*	*Aminosugar*	*Sulphate*	*Protein*
	mg/g tissue				
SFC	27.4 ± 1.612	7.43 ± 0.569	1.83± 0.314	6.98± 0.401	891.8 ± 30.883
*SFD**	32.2 ± 2.199[a]	10.06 ± 0.691[a]	2.98± 0.317[a]	6.30 ± 0.354[a]	780.4 ± 54.822[a]
DFC	28.4 ± 1.942	8.02 ± 0.669	1.98± 0.199	6.73± 0.339	870.5 ± 38.338
DFD	30.8 ± 2.756	9.25 ± 0.926	2.2± 0.170[b]	6.80 ± 0.517	808.6 ± 35.114[b]
JFC	26.8 ± 2.513	7.85 ± 0.782	1.73± 0.182	6.85± 0.672	886.3 ± 18.543
JFD	28.0 ± 3.110[b]	8.90 ± 0.738[b]	2.1± 0.078[b]	6.20 ± 0.329	820.2 ± 18.543[b]

Footnote as in Table 29.

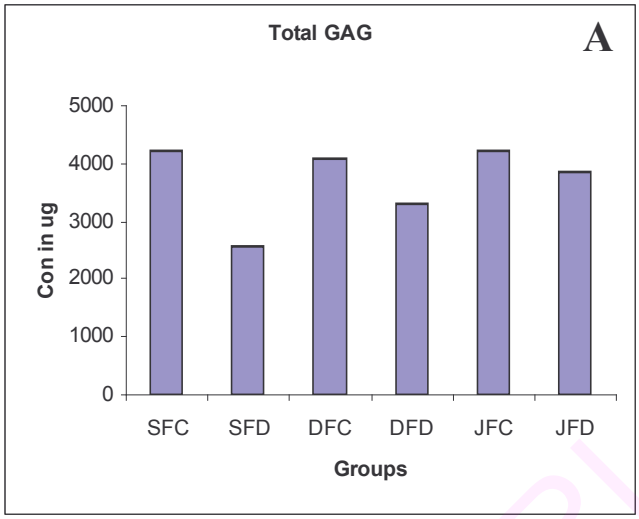
Table 37: Effect of drumstick and Jambolana seed on carbohydrate composition of kidney glycosaminoglycans

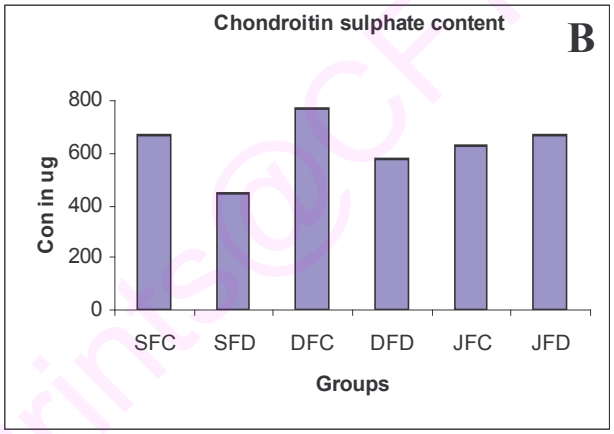
Groups	Amino sugar	Uronic acids	Sulphate content	Total sugar
	μg/g dry kidney tissue			
SFC	1062	1452	2893	8988
SFD	720	825	1582	7450
DFC	987	1503	2989	9020
DFD	790	920	1750	5682
JFC	1022	1587	2782	8789
JFD	810	1010	1890	6890

Footnote as in Table 29.

Agarose electrophoresis

Separation of GAG was done by agarose gel electrophoresis. Equal amount of kidney samples were loaded. Electrophoresis resulted in the separation of GAGs. Heparan sulfate was the major band present. The other band was that of chondroitin sulfate B. There was no band corresponding the chondroitin sulfate C. Identification of GAGs on the electropherogram was based on relative mobility against standard chondroitin sulfate C. The isolated GAGs from kidney consisted of major amounts of heparan sulfate and minor amounts of chondroitin sulfate B. Decrease in the content of heparan sulfate during diabetes and its amelioration by drumstick and jambolana was observed.





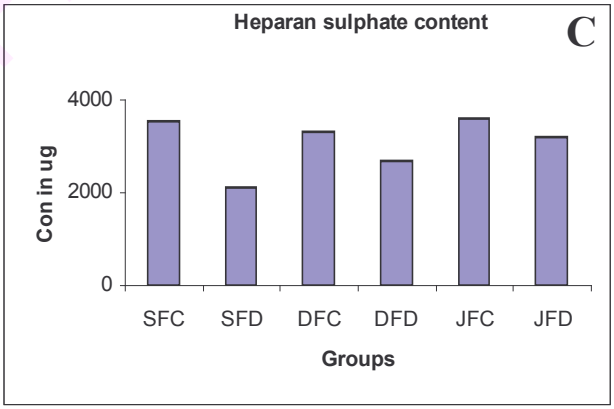


Fig. 28: Effects of fiber on total GAG (A), Chondroitin sulfate (B) and Heparan sulfate (C)

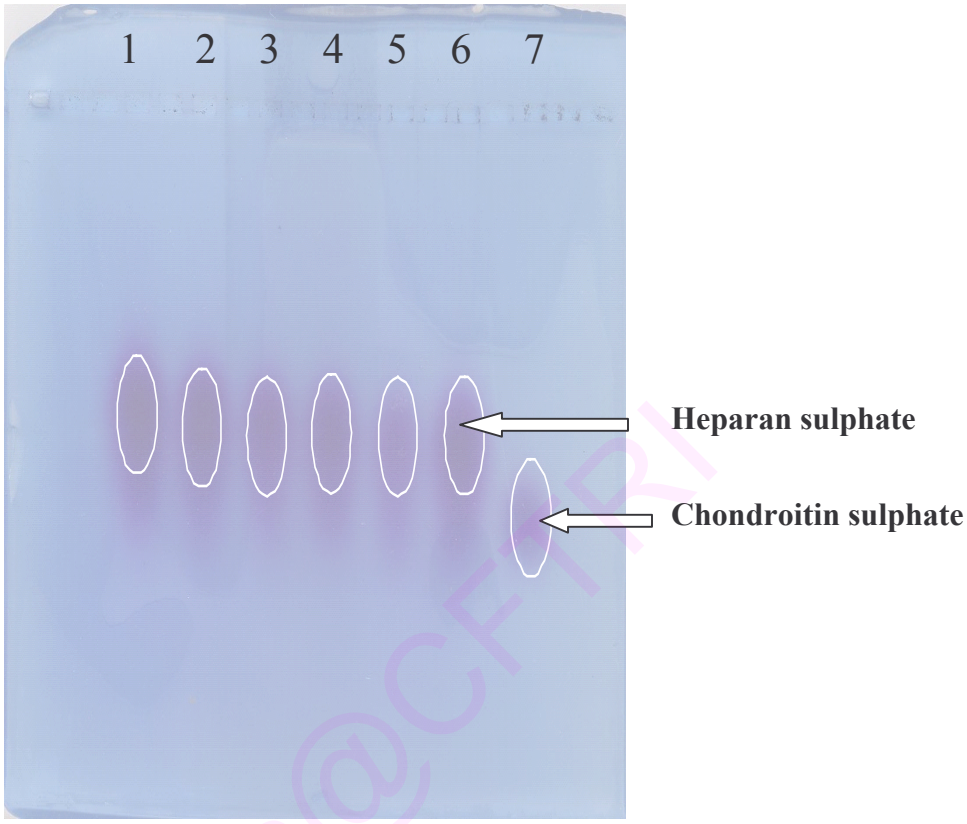


Fig. 29: Agarose gel electropheresis of glycosaminoglycans (GAG) isolated from control, diabetic and treated rat kidneys

Lane 1: SFC –Starch fed control

Lane 2: SFD - Starch fed diabetic

Lane 3: DFC – Drumstic fed control

Lane 4: DFD – Drumstic fed diabetic

Lane 5: JFC- jambolana fed control

Lane 6: JFD- Jambolana fed diabetic

3.3.3. Discussion

Diabetes mellitus, one of the major metabolic disorders, is marked by sustained hyperglycaemia and has deleterious effects on various organs. Nephropathy is one such manifestations of diabetes, which affect the kidney. During diabetic nephropathy, the glomerular basement membrane is known to become thicker with a reduction in the contents of heparan sulfate and laminin (Shimomura et al.1987; Kanwar et al.1983; Brown et al.1981) and an increase in type IV collagen (Rohrbach et al.1982) and reduced sulfation (Cohen et al.1981). In recent years the role of mesangial cell expansion during diabetic nephropathy state is receiving much attention (Kritchevsky et al. 1995). Apart from insulin and anti-diabetic drugs, fibers and their sources have become important nutritional components in the management of diabetes. Effect of fibers in the management of diabetes is well documented (Kritchevsky et al. 1995; Trowell, 1975; Khatter et al. 1990). Dietary fibers (DF) play an important role in facilitating slow absorption of glucose. We have shown that feeding wheat bran and guar gum brings about significant hypoglycemic activity and ameliorates the diabetic nephropathy (Felix and Zahola, 1993). In recent years fermentation of DF to short chain fatty acids (SCFA) such as acetate, propionate and butyrate has gained a lot of interest. Butyrate in particular is shown to modulate activities of many cellular enzymes including enzymes involved in glycoconjugate metabolism such as sialyl transferase (Kritchevsky et al. 1995) sulfotransferase (Shah et al. 1992; Gallaher et al.1992; Nandini et al. 2000) etc. In this study we have investigated antidiabetic effect of butyric acid in STZ induced diabetic rats.

Though dietary fiber rich sources are recommended to diabetics, their beneficial effect on diabetic nephropathy, especially in relation to their effect on glycosaminoglycans such as heparan sulfate, which plays an important role in the process of filtration, is not known. In this investigation we have studied the effect

of DF rich sources such as *M. oleifera* pulp and jambolana seeds on sulfated glycosaminoglycans.

In the present study, dietary fiber rich source drumstick and jambolana were administered to diabetic rats and maintained for five weeks. During this period diabetic status was assessed by diet consumption, body weight, urine volume, urine sugar and fasting blood glucose. Diabetic nephropathic status was evaluated by GFR and and relative kidney weight. Assays of various functional enzymes involved in glycoconjugate metabolism, kidney composition and GAG composition was also done to get a complete picture. The present study showed that both drumstick pulp and jambolana seed are beneficial in ameliorating diabetic nephropathy status to various extents. Present investigation possibly helps to understand the beneficial role of both drumstick pulp and jambolana seed in bringing down the nephropathy status with respect to heparan sulfate content.

Diabetes is a metabolic disorder with some characteristic symptoms such as poly uria, glucosuria, polydipsia and polyphagia, during which animals consume more food and water and excrete more urine and sugar (ADA, 1997; 2001) In the present study, higher consumption of diet in diabetic groups was statistically significant when compared to control groups (SFC) and was due to hyperphagic condition developed during diabetes. Nandini et al. (2000) reported that STZ induced diabetic rats consumed higher amount of diet compared to control (Chethankumar et al. 2002). Incorporation of drumstick in diet showed higher consumption in both diabetes and control rats due to the presence of dietary fiber in this diet. The symptoms observed in SFD were significantly altered by incorporating the fibers as reported by others also (Chethankumar et al. 2002). Foods rich in dietary fiber are known to be consumed in higher amounts, which may be due to better palatability and caloric dilution (Shetty et al. 2005a).

Jambolana fed diabetic group (JFD) showed less consumption and this could be because of the palatability of the diet and was reduced by jambolana seed powder.

Higher food intake was due to hyperphagia and was observed on other fiber fed diabetic groups also but there was comparatively better gain in body weight indicating amelioration in diabetic condition.

There was a decrease in body weight despite higher food intake which is one of the characteristic conditions of DM. Improvement in body weight was observed by both drumstick (8%) and jambolana fed groups (10%) when compared to SFC. Authors reported similar observation in diabetic rats where they fed 2% Fenugreek (Vats et al. 2003; Shetty et al. 2005b). Both polyuria and glycosuria observed on weekly basis in diabetic animals (SFD) was effectively controlled by drumstick pulp and jambolana seed in the diet.

Hyperglycemia is a characteristic feature of diabetes, where fasting blood glucose goes beyond the normal values 120-130 mg/dL and was reported by others (Shetty et al. 2005b; Olsen et al. 1971). Animals in SFD group showed an average FBS of 336 mg/dL, which is characteristic of hyperglycemia. Fiber sources (drumstick and jambolana powder) in the diet prevented the increase to various extents. Both drumstick and jambolana decreased the blood sugar either by forming as insoluble matrix thereby slowing the absorption of food making it less accessible or through SCFA.

Diabetes frequently results in severe metabolic imbalances and pathological changes in many tissues. In the small intestine this causes significant changes in the morphology and functions of the mucosa (Arvanitakis et al. 1973; Plotkin et al. 1964). Among a variety of biological functions in the small intestine, many investigators focus on the recovery of carbohydrate and protein metabolism including their digestion and transport. The small intestine plays an important role to digest and absorb the nutritive substances. Disaccharidases are a group of

enzymes present in intestinal mucosa, and their activity is a useful parameter of intestinal function. In normal mucosa an increased level of activity was observed between lactase, sucrase and maltase (Auricchio et al. 1963; Dahlqvist et al. 1963; Vinnik et al. 1965). During diabetes an increased absorption of hexoses by small intestine was observed in humans [Murakami and Ikeda, 1998]. The disaccharidases present in the brush border of the small intestine play an important role in hydrolyzing the disaccharides to monosaccharides, there by facilitating their absorption (Semenza, 1986). This could be due to increased activity of disaccharidases and their activities are also known to be contributory factor for severity of diabetes.

In our present study, there was a significant increase in activities of intestinal maltase, sucrase and lactase (Table 31). Drumstick prevented the increased intestinal maltase activity by 18% and jambolana by 25%. In case of intestinal sucrase, only jambolana ameliorates significantly (22%) but not drumstick, which prevents by 7%. Increased activities of intestinal lactase during diabetes were not ameliorated controlled either by drumstick or jambolana. Earlier studies, in our lab have also shown effect of dietary fiber and their source in ameliorating these enzymes (Nandini et al. 2000). The decrease in disaccharidase activities in diabetes in the presence of fiber has been reported by previous workers (Felix and Zahola, 1993; Taylor et al. 1972).

There was a significant decrease in activities of renal maltase, sucrase and lactase and showed considerable variation in both control and diabetic groups (Table 32). The presence of disaccharidase activities in subcellular fractions of rat kidney cortex has been reported (Taylor et al.1972). The decrease in maltase and lactase activities in kidney during diabetes has been reported previously (Yukisumi et al. 1989; Juretic et al. 1984; Sharma and Shivkami, 1984). In our present study, there was a decrease (about 57%) in activity of maltase, which was

alleviated by drumstick and jambolana. Decreased activity of renal sucrase was observed during diabetes. However, only jambolana in diet showed a significant amelioration (27%). Renal lactase activity was significantly decreased during diabetes (SFD) compared to control (SFC), which was ameliorated by 30% with drumstick and 31% with jambolana.

The distribution of lactase activity is probably associated with lysosomes. The activity may be due to the hydrolysis of lactose by the β-galactosidase found in rat kidney lysosome (Price and Dance, 1967). The presence of disaccharidase activities in subcellular fractions of the rat kidney cortex probably reflects an involvement in the digestion or transport of sugars across the membranes. A decrease in renal disaccharidase activity was observed during diabetes (Sharma and Shivakami, 1984; Juretic et al.1984). One of the reasons for the alleviation of altered activities of intestinal and renal disaccharidases may be due to alleviation in diabetic status as indicated by urine sugar, urine volume, FBS in treated groups, thus making diabetic animals more tolerant to hyperglycemia.

Vegetables are among the numerous plant adjuncts examined for the management of the diabetes mellitus. A few vegetables that are commonly consumed in India have been claimed to possess antidiabetic potency. In recent years, there has been a renewed interest to screen such plant food materials, for a possible beneficial use. Considerable amount of work has been carried out in this regard. Vegetables and their products are usually valued for their nutrient content but they are also regarded as rich sources of nonglycemic carbohydrates, collectively called as dietary fiber. Vegetables are rich source of dietary fiber, which is well known for their beneficial physiological effect such as improved bowel function and carbohydrate and lipid metabolism. Studies have reported that dietary fiber of leafy vegetable was effective for counteracting obesity, diabetes, hyperlipidemia, colon disease and constipation. *M. oleifera,* commonly called

drumstick, is a widely used vegetable and is a rich source of dietary fiber. Previously studies have been carried out to elucidate their antioxidant (Kumar and Pari, 2003) hypocholesterolomic (Ghasi et al. 2000) and antitumor (Murakami et al. 1998) effects. However, most of the work has been done on leaves, bark and seeds of the plant. But there are no reports on the hypoglycemic property of the fruit pulp and their effect on kidney function. In our present study, from all the above data drumstick pulp is proved to be hypoglycemic property with respect to urine sugar and FBS and intestinal and renal disaccharidases. However, dietary fiber or any other bioactive compounds may attribute their hypoglycemic property.

Indigenous medications prepared from extracts of plants have been used for controlling diabetes long before the discovery of insulin. *S. jambolana*, one of the ancient plants is widely studied by many authors for their immunomodulatory activities, antioxidants (Ravi et al. 2004a), anti-inflamatory (Chaudhuri et al. 1990), antifertility (Rajasekaran et al. 1998), anti- HIV antibacterial (Shafi et al. 2002; Shaikh et al. 1994) and gastroprotective activities (Mukherjee et al. 1998; Ramirez et al. 2003). *Syzygium cumini* (also shown as *Eugenia jambolana*) is plant with a putative antihyperglycaemic effect. Different components of the plant, such as seeds, bark, fruit and tea prepared from their leaves have been used to treat diabetes in many countries Bramachari, 1961; Kohliand Singh, 1985; Almeida and Agra, 1986; Rahman and Zaman, 2001; Haddad et al. 2001). Although earlier reports stated that administration of powdered seeds of *E. jambolana* do not produce appreciable difference in blood sugar levels in rabbits but Brahmchari et al., showed that its ethanolic extract has hypoglycemic activities in albino rabbits (Brahmachari and Augusti, 1961). Indira and Mohan Ram (1992) reported hypoglycemia and reduced glucosuria on oral administration of alcoholic extracts of dried seeds of *E. jambolana* (Achrekar et al. 1991). Antidiabetic activities of seeds is reported with ethanol (Ajit Kar et al. 2003; Sharma et al. 2003; Prince et

al. 2004; Ravi et al. 2004) and water (Prince et al.1998; Daisy et al. 2004; Jasmine and Daisy, 2004) extracted sample and only little work has been done on effect of seed powder on diabetic status. In our present study, jambolana seed at 2.5% was found to be better than drumstick in controlling diabetic status with all the parameters to various extents.

These studies indicate that both the plant sources are good in controlling the diabetic status to various extents**.** The mechanism of action of drumstick and jambolana seeds may be similar or different. The drumstick pulp as well as jambolana seeds are rich in dietary fibers and contain mainly soluble dietary fiber. Authors have reported that jambolana seed is having more soluble dietary fiber.

Dietary fibers being not digestible by the enzymes present in gastrointestinal tract act as insoluble matrix and facilitate slow absorption of glucose, thus making blood glucose levels not to raise (Oubre et al. 1997). The dietary fibers are further fermented by the microflora present in the colon to short chain fatty acids (Bourquin et al. 1992). Our studies show that butyric acid- a short chain fatty acid formed as a product of fermentation of dietary fibers ameliorates diabetic status (Chethankumar et al. 2002). Thus the beneficial effects that are observed by drumstick and jambolana are combined effect of dietary fiber and fermented products of dietary fiber - the short chain fatty acids, including butyric acid. It is also likely that beneficial effect may be due to some of the bioactive components present in jambolana (jambosine), phenolic compounds such as gallic acid having may have beneficial effect on diabetic status. Ravi et al., have observed that *E. jambolana* may bring about its hypoglycemic action through stimulation of surviving cells of islets of Langerhans by releasing more insulin (Achrekar et al. 1991; Ravi et al. 2004). Studies have reported on increase in glucose-6-phosphate content in liver thus by bringing about an overall increase in glucose influx thus bringing about and hence have an over all effect in increasing

glucose utilization (Grover et al. 2000). Bansal et al. (1981) has reported the hypoglycemic activity by increased insulin content through increased activity of cathepsin. Glycemic control is correlated with diabetic complications. Diabetic control and complications trial have proved that diabetic complications are controlled by glycemic control. The degree of renal enlargement was correlated with the degree of glycemic control. Since both plant materials showed a considerable effect on glycemic control we have further extended our studies to determine their effect on diabetic nephropathy status with particular emphasis on heparan sulfate content. There are no reports on drumstick pulp and their effect on diabetic complications or nephropathy status. However, Chirvan-Nia et al., (1972) have reported regression of cataract and hyperglycemia in diabetic rats with the feeding of the extract of *E. jambolana*. Rathi et al. (2000) have reported that lyophilized aqueous extract of *E. jambolana* seeds prevented the development of cataract in alloxan induced diabetic rats.

The increased kidney size is shown to occur during early stages of diabetes (Anderson and Geil, 1994). The relative weight of the kidney nearly doubled during diabetes. Renal enlargement is observed in diabetes of 4-weeks duration (Christiansen et al. 1981). It is reported by Seyer-Hansen (1976) that renal enlargement in rats begins within a day of induction of diabetes and continues for at least 6 weeks. The increase in weight of kidney during diabetes has also been reported by others. In this investigation, significant increase in kidney weight was observed in diabetic animals (SFD) compared to control (SFC). Supplementation of drumstick and jambolana in the diet prevents the kidney weigh to various extents. However, jambolana effectively controlled the kidney weight gain by 14% compared to SFD, whereas in DFD animals the effect was only marginal. Earlier works have showed that dietary fiber decreases the relative kidney weight during diabetes.

Glomerular filtration rate (GFR) is measured in terms of creatinine clearance. The daily excretion of creatinine is constant and unaffected by a wide variety of circumstances. Creatinine is not reabsorbed by the tubules. Hence, the rate of excretion is used as an index of GFR (Berkow, 1982). GFR gives the indication of the amount of blood passing through the kidney per minute during the test period. Lower values of GFR might indicate reduced flow of blood through renal vasculature and it occurs during end stage renal disease.

GFR was increased significantly in diabetic rats. In earlier stages, hyperfiltration of the kidney with typical 20-40% increase in kidney size and GFR was observed by Christiansen et al. (1981). STZ induced diabetic animals tend to show renal hypertrophy (Rash, 1980; Neilsen et al. 1999). Renal enlargement was correlated with the degree of glycemic control (Seyer-Hansen, 1976). Increased GFR was observed in diabetic rats (SFD) compared to control rats. Presence of fiber sources (drumstick pulp and jambolana seed) in diet showed lesser GFR indicating improved kidney function. It indicates the beneficial effect of these plant materials in delaying the development of diabetic nephropathy. Reversal of increase in GFR and renal hypertophy by blood glucose normalization has been observed by Stachouse et al. (1990). Jambolana, which is more effective in controlling the blood glucose level than drumstick helps in bringing down GFR to certain extent probably by blood glucose normalization. Long-term control of plasma glucose level is known to reduce kidney filtration in human diabetic subjects (Feld- Rasmussen et al. 1991).

Kidney plays an important role in maintaining normal physiological condition by filtering toxic materials from blood. However, during diabetes due to hyper functioning of kidney, filtration process gets affected which results in excretion of macromolecules in urine. Urinary excretion of glycosaminoglycans indicates an alteration in their synthesis and degradation. Hence, we measured

some of the important enzymes involved in synthesis and degradation of glycosaminoglycans in kidney and analyzed the carbohydrate content.

The increase in total sugars in diabetic kidney has been shown to be due to non-enzymatic glycation of tissues proteins. Increased uronic acid could be due to increased synthesis of small GAG or degradation of proteoglycan (Kasinath, 1995). Results clearly show that both drumstick and jambolana prevented the increase in total sugars and uronic acids to various extents. UDP-sugars, which are building block of glycoprotein, GAG, Glycolipids (Spiro, 1985) may contribute for increased amino sugar content during diabetes. However, jambolana significantly controls their increment. Authors have reported that there was decrease in sulphate content during diabetes (Cohen and Surma, 1981). There were no significant changes observed in sulphate content in this investigation. Decrease in protein content during diabetes is reported which may be due to increase in gluconeogenesis (Teng, 1954) or due to decrease in soluble protein during diabetes (Guptha et al. 1999). Both drumstick and jambolan affects marginally on these parameter.

GFAT is involved in the synthesis of glucosamine-6-phosphate from UDP-sugar by a series of enzymatic reactions. UDP sugars are important constituents of proteoglycans/ glycoproteins, which are constituents of glomerular basement membrane. Renal section from patients with diabetic nephropathy has shown significant increase in GFAT expression in glomerular epithelial cells. These changes in the expression of GFAT may be one of the reasons for the thickening of glomerular basement membrane (Nerlich et al. 1998).

During diabetes, increased activity of GFAT was observed and was significant compared to control (SFC). This elevation was brought down by both drumstick and jambolana based diet. However, jambolana (19%) was effective in controlling the elevation of GFAT compare to drumstick (6 %). Renal sections

from diabetic patients with diabetic nephropathy have shown significant increase in GFAT expression in some glomerular and epithelial cells. Since UDP sugars are important component of glycoproteins, increased synthesis of glucosamine points to the fact that there is glycoprotein accumulation. This may be one of the reasons for the thickening of glomerular basement membrane, which occurs during diabetic nephropathy. It was observed by Spiro (1984), that there is an increase in UDP glucose and UDP-galactose during diabetes.

NAG activity has been shown to be elevated in diabetic mice (Serrano et al. 1985). While others have shown a decrease in diabetic rats (Fushimi and Tarui 1976; Rao, 1981). β-Glucuronidase activity was shown to be unchanged or significantly elevated during diabetes depending on the diabetogenic agent used to induce diabetes (Bose and Sudha, 1984). The discrepancies in the reports are due to various factors like diabetogenic agents used to induce diabetes, duration of diabetes and the strain of the animals employed in experiments. NAG and B-glucuronidase activities are shown to be elevated in liver and aorta of athermanous rats (Vijaykumar and Kurup, 1995) and diabetic hypertensive rabbits (Kumari and Devi, 1993). Activities of NAG increased by about 60% during diabetes (SFD). There was an improvement in the drumstick fed diabetic groups (8%) and further improvement was observed with jambolana (23%). Increased activity of β-glucuronidase during diabetes was controlled effectively by both drumstick (22%) and jambolana (47%).

Glycosaminoglycans are carbohydrate moieties of proteoglycan, which are constituents of both glomerular basement membrane and mesangial matrix (Farqshar, 1964). GBM is organized as a network of fibrils or chords forming a small mashed sieve and it is believed to function as the major filtration barrier between the blood and urine. Composition of GBM is different in most of the basement membranes and it has a highly organized architecture. GAGs are located

as anionic sites in the lamina rarae of glomerular basement membrane and the mesangial matrix, where they serve as a charge selective barrier in the filtration of macromolecules. These anionic sites prevent clogging of the basement membranes by circulating plasma macromolecules (Kanvar, 1979). The main GAG constituent in GBM is heparan sulfate, which is an unbranched chain of repeating disaccharide units of amino sugar and uronic acid (glucuronic or iduronic acid). Other GAGs present in small portions constitute chondoitin sulfate, dermatan sulfate and hyaluronic acid. In our investigation it is observed that heparan sulfate is the major GAG presents in all the groups, which amounted to approximately 90% of the total GAGs present. This is in agreement with the literature (Dietrich et al. 1976; Dietrich et al. 1977). GBM is well reported to undergo marked thickening during diabetes, making it more porous to the macromolecules (proteinuria). This suggests that diabetes leads to alterations in its structure and function. Major alterations known are, increased collagen content, decreased heparan sulfate and laminin contents. Previous workers have reported a reduction in heparan sulfate in both GBM and renal cortex (Kanwar et al. 1983; Rohrbach et al. 1982; Rohrbach et al. 1983; Parthasarathy and Spiro, 1982, Kanwarra and Farquhar et al. 1979; Tamsma et al. 1994) This loss is thought to result in a loss of negative electrostatic charge and thus permit the passage of positively charged proteins such as albumin (Scandling and Myers, 1992). Total sulfated GAGs decreased by about 41% during diabetes (SFD) compared to control rats. The content of uronic acid and amino sugar, which are building block of GAG are also decreased during diabetes resulting in decreased content of GAG. Since this estimation was basically based on the sulfate content, the decrease in GAGs could also be due to under sulfation, which is known to occur during diabetes. The uronic acid content was also decreased. The decrease in sulfated GAGs was most probably due to decrease in GAGs as well as under sulphation. The decrease in

total GAG was due to decrease in both heparan sulfate and chondroitin sulfate. Reduction in heparan sulfate during diabetes has been observed by previous workers in both glomerular basement membrane and renal cortex. The decrease in GAG during diabetes was prevented by the presence of drumstick (%) and jambolana (%) in the diet as seen in DFD and JFD. The total GAGs in control animals were nearly the same. Heparan sulfate and chondroitin sulfates are known to be located on two different core proteins (Kanwar et al. 1981). Modulation of GAGs during diabetes in kidney tissue has been reported by earlier workers (Hatch and Cao, 1995). Lee Stanlee (1982) observed that addition of non-absorbable fiber to the diet of genetically diabetic mice improved glycemic control and retards the development of diabetic nephropathy. Barley has been found to have modulating influence in the altered metabolism of basement membranes in the diabetic rats. Earlier work in our lab provided experimental evidence to show that decreased synthesis of heparan sulfate in diabetes was ameliorated by dietary fibres such as wheat bran and guar gum (Mahdi et al. 1994; Nandini et al. 2003).

The beneficial effect of fibers in ameliorating the symptoms of diabetic nephropathy may be either indirectly by the production short chain fatty acids like acetate, propionate and butyrate or by direct consequences of controlling the blood glucose level by acting as insoluble matrix which is a primary step in the control of diabetes. It has been suggested that some of the effects of high carbohydrate and high fiber diets are due to altered carbohydrate and lipid metabolism by SCFA in the liver (Smith et al. 1994). Many authors have put forward the hypothesis that SCFA are involved in the fiber-induced metabolic changes (Smith et al. 1998; Cummings, 1983; Wolever et al. 1989). Butyrate is shown to modulate activities of many cellular enzymes including the enzymes involved in glycoconjugate metabolism like sialyl transferases (Shah et al. 1992) and sulphotransferases, (Jocobsson et al. 1985) etc. Such alteration in enzyme activities would lead to

alteration in the synthesis/degradation of many of the cellular components, including the glycoconjugates such as heparan sulfate. Our results demonstrated that abnormalities in kidney tissue as a result of diabetic condition are prevented to certain extent by the presence of drumstick and jambolana in diet. Both are ameliorating kidney nephropathy to a considerable extent and improved the metabolites of glycosaminoglycans or heparan sulfate in particular.

It is also known that during diabetes oxidative damage is increased in diabetic kidney and has been shown to be a state of increased free radical production (Bavnes, 1991). Mechanisms that contribute to the formation of free radicals in diabetes may include increased nonenzymatic and autoxidative glycosylation. It is known that free radicals have been suggested to be contributory factor in complications of diabetes. Recently, Cocharan and Robinson (2002) have demonstrated that in vitro glycation of glomerular basement membrane alters its permeability. There is also evidence that diabetes induces changes in the activity of antioxidant enzymes of various tissues. Jambolana seed powder showed a potent antioxidant activity with respect to DPPH and reducing power ability. Presence of gallic acid as major phenolic acid identified by HPLC and higher amount of phenolic acids content were noticed in chemical study in the next section. Any of these molecules may be involved in bringing down the severity of diabetes through antioxidant defence mechanism.

3.3.4. Summary and conclusion:

Studies were carried out on the effect of drumstick pulp and jambolana seeds on diabetic and diabetic nephropathy status with particular emphasis on kidney heparan sulfate in STZ induced diabetic rats. Results obtained can be summarized as follows.

1. Diabetic symptoms were assessed by general parameters such as diet intake, water consumption, and body weight. Drumstick fed diabetic rats

consumed more diet because of caloric dilution, whereas jambolana consumed less and may be because of its non-palatability nature. Loss in body weight during diabetes was better controlled by drumstick compared to jambolana.

2. Diabetic status was measured in terms of urine volume, urine sugar and fasting blood sugar, which were increased significantly. Supplementation of both drumstick pulp and jambolana in diet improved the diabetic status to various extents. However, jambolana was better in ameliorating diabetic status with respect to urine volume, urine sugar and fasting blood glucose when compared to drumstick.
3. Increased activities of intestinal disaccharidases and decreased activities of renal disaccharidases during diabetes were ameliorated by both the drumstick and jambolana to various extents.
4. Kidney weight and glomerular filtration rate (GFR) were significantly increased in diabetic group. Both drumstick and jambolana were effective in preventing the increase in GFR to various extents. However, jambolana was found to be better effective in preventing relative kidney weight gain and increased GFR.
5. Drumstick and jambolana in diet ameliorated the increased total sugar and uronic content. Jambolana in the diet prevented the decrease in protein content during diabetes.
6. Activities of some of the renal enzymes like L-glutamine fructose-6-phosphate aminotransferase (GFAT), N-acetyl-β glucosaminidase (NAG) and β-glucuronidase involved in glycoconjugate metabolism were elevated during diabetes. Increase in all the three enzyme activities were prevented by the presence of jambolana and drumstick in the diet. However jambolana was comparatively better than drumstick.

7. Glycosaminoglycans isolated from kidney were analyzed for their carbohydrate profile such as uronic acid, total sugar, amino sugar and sulfate content. The content of sulfate decreased during diabetes and was ameliorated by drumstick and jambolana feeding. This is of particular interest in improving the state of diabetic nephropathy.
8. Heparan sulfate and chondroitin sulfate were fractionated from normal and diabetic kidneys. The content of heparan sulfate decreased in diabetic kidneys as reported earlier and interestingly drumstick and jambolana feeding improved the content of heparan sulfate in diabetic rat kidney. Electrophoretic profile showed that there is an increase in heparan sulfate content in JFD and DFD group.

Conclusion

To conclude, dietary fiber rich source like drumstick and jambolana ameliorate the diabetic conditions in terms of FBS, urine sugar but also in terms of glycosaminoglycan metabolism and heparan sulfate in particular. This may occur either directly as a result of controlling blood glucose or by it metabolites like short chain fatty acids, which arise as a result of fermentation of fiber in colon or other bioactive component present in both the food materials.

3.4. Carbohydrate composition of polysaccharides from *Syzygium jambolana* and their antioxidant properties

3.4.1. Introduction

Syzygium jambolana fruit commonly known as jambolana has characteristic taste and flavor. The tropical tree is grown mainly in India and is evergreen and bears fruits during summer. Fruits when ripe are purple colored and the fruit is a delicacy. The ripe fruit is relished for its characteristic taste and flavour. It is generally sub-acidic and astringent to taste. Wine is prepared from the fruits in some parts of India. Juice made from the fruit is known for its characteristic color and flavour and its delicacy. Fruits are also used to make preserver, squashes and jellies. Good jelly can be made from purple fleshed fruits (Chada, 1976; Coombe et al. 1976). It has been attributed to posses several medicinal properties in the folklore system of medicine (Warrier, 1996). The plant has been extensively used to treat diabetes, constipation, leucorrhoea, stomachalgia, fever, gastropathy, strangury, dermopathy (Bhandary, 1995; Warrier, 1996) and to inhibit blood discharges in the faeces (Rastogi and Mehrotra, 1990.). Recently, the plant has been reported to poses acetyl oleanolic acid, triterpenoids, ellagic acid, isoquercitin, quercetin, kaempferol and myricetin (Martinez and Del Valle, 1981).

Considerable amount of work has been done on hypoglycemic property of various parts of plant materials (Achrekar, 1991; Teixeira, 1992; Daisy, 2004). In this study antidiabetic property of seed and its effect on kidney functions are investigated. It is known that hyperglycemia leeds to production of free radicals which are causative factors for diabetic complications (Boguslaw Lipinski, 2001). Correlations are made on the relationship between production of activated oxygen apecies and diabetes (Kakkar, 1997). Health beneficial effects of fruits and vegetables are particularly attributed to the phenolic compounds having antioxidant properties (Croft, 1998; Borchers, 1997; Masumorthy, 1995) This

property is due to their ability to donate hydrogen or electron but also because of their stable radical intermediate, which can effectively prevent the oxidation at cellular and physiological level. Since phenolic acids are distributed in free and bound form, it was our interest to see their profile and their contribution to antioxidant activity.

Pharmacological properties of polysaccharides are gaining lot of interest in recent years. Polysaccharides from various sources have been shown to have biological properties such as anti-tumor, anti-ulcer, immunological, anti-inflammatory, anti-coagulant, hypoglycemic and anti-viral activities (Shin, 1997; Borchers, 1997; Masumorthy, 1995). Sulphated polysaccharides in particular have been known to have antioxidant activity (Pilar upérez, 2002; Quanbin Zhang, 2003). Hence present works aims at,

A. Isolation and characterization of polysaccharides from *S. jambolona* fruit, seed and seed coat.
B. Antioxidant property of various polysaccharides and identification of phenolic acids.

3.4.2. Results

3.4.2.1. Carbohydrates of *S. jambolana*

In the present investigation *S. jambolana* fruit were separated in to pulp, seed, and seed coat.

Carbohydrate composition of *S. jambolana* fruit pulp and its isolated fractions

The yield and chemical composition of *S. jambolana* pulp and the various polysaccharide fractions is given in Table 38. The pulp did not contain starch. The fruit pulp was rich in carbohydrates and the content of protein was around 4%. The content of uronic acid was 8.8% and the uronic acid was identified as

galacturonic acid. Carbohydrate composition of the fruit pulp indicated it to be rich in pectic fraction as evidenced by high amount of arabinose (32%), galactose (9%) and uronic acid (8%). Glucose was the major sugar (47.6%) and xylose (7%) was the minor sugar identified. In water soluble polysaccharide fraction (WSP), the amount of carbohydrates was 84% and uronic acid content was 12%. Sugar analysis by GLC indicated it to be rich in arabinose (44.9%.), galactose (14.3%) and glucose (30.8%), which could indicate the presence of arabinogalactans and β-glucan type of polysaccharides. Xylose and mannose were present in small amounts. The pectic fractions (hot ammonium oxalate extracted) contained considerable amount of carbohydrates of which 36% uronic acid. Among the neutral sugars, arabinose (70%) was the major sugar identified. Presence of galactose, rhamnose/fucose along with high amounts of arabinose and galactouronic acid indicated the fraction to be rich in complex arabinogalactan type of polysaccharides with high amount of galactouronic acid content (Salimath and Tharanathan, 1982; Tharanathan, 1994).

Table 38. Carbohydrate composition (%) of *Syzygium jambolana* fruit pulp and its isolated fractions

Fractions	Yield	TS	UA	Fuc/ Rha	Ara	Xyl	Man	Gal	Glc
Flour	(100.0)	81.0	8.8	3.8	32.0	7.0	9.3	9.0	47.6
WSP	20.0	84.0	12.0	3.1	44.9	4.2	2.4	14.3	30.8
PPS	13.0	79.2	36.0	4.3	70.8	3.8	1.4	7.2	11.9
Hem A	14.5	82.0	8.0	2.2	22.9	16.6	3.0	11.7	43.4
Hem B	8.8	80.0	4.5	2.4	19.7	19.7	3.4	20.6	35.8
AIR	11.0	75.8	3.6	1.4	5.7	19.8	5.8	4.4	62.9

Note: WSP-Water soluble polysaccharide; PPS-Pectic polysaccharide; Hemi A- Hemi cellulose A; Hemi B-Hemi cellulose B; AIR-Alkali insoluble residue,
TS: Total Sugar, Rha: Rhamnose, Fuc: Fucose, Xyl: Xylose,
UA: Uronic acid, Ara: Arabinose, Man: Mannose, Gal: Galactose, Glc: Glucose.

The fruit pulp contained around 23% alkali-soluble polysaccharides of which precipitable hemicellulose A (Hem A) were present in higher amount. The Hem A was rich in carbohydrates and contained around 8% uronic acid. Glucose was the major sugar. High amounts of arabinose and galactose indicate the presence of arabinogalactan type alkali-soluble polysaccharides in fruit pulp. The alkali soluble hemicellulose B (Hem B) contained 80% carbohydrates and contain small amount of uronic acid. Glucose was the major sugar and may be coming from alkali soluble β-glucan type of polysaccharides along with arabinoxylans (Ramesh and Tharanathan, 1998). Presence of nearly equal amounts of arabinose and galactose indicate presence of arabinogalactan type of alkali soluble

polysaccharides being present in the fruit pulp. The content of alkali insoluble residue (10% NaOH) was 11%. The fraction contained 75.8% carbohydrates and had small amount of uronic acid. Glucose was the major sugar, indicating it to be cellulosic in nature. A large amount (36%) of non-cellulosic polysaccharide had still remained unextracted with 10% NaOH. This indicates strong association of cellulosic polysaccharides with non-cellulosic polysaccharides.

Table 39. Carbohydrate composition (%) of *Syzygium jambolana* seed and its isolated fractions

Fractions	Yield	TS	UA	Rha/ Fuc	Ara	Xyl	Man	Gal	Glc
Flour	(100)	83.3	5.0	0.9	6.8	18.8	1.7	2.3	70.4
WSP	9.7	88.0	19.9	8.2	52.8	3.2	1.0	10.7	23.3
PPS	1.4	84.4	28.0	2.5	9.4	2.2	3.2	56.7	26.0
Hemi A	3.6	80.0	9.4	0.5	2.4	5.3	1.7	1.7	88.4
Hemi B	3.7	89.2	5.6	1.6	3.0	7.4	-	-	88.0
AIR	6.3	83.0	3.1	1.5	6.8	19.5	1.9	4.8	65.5

Abbreviation as in Table 38

Carbohydrate composition (%) of *Syzygium jambolana* seed and its isolated fractions

The carbohydrate profile of the seeds and its isolated polysaccharide fractions is given in Table 39. The seeds contained 29% starch. Carbohydrate content of the seeds was 83% and small amount of uronic acid was present. Glucose was the major sugar (70%) identified and small amounts of arabinose and xylose were also present along with other sugars. Water-soluble polysaccharides were isolated in about 10% yield from the seeds. The water soluble polysaccharide fraction was rich in carbohydrates (88%), and contained about 20% uronic acid. The fraction contained high amount of arabinose (52%) and considerable amount of glucose and galactose. Xylose and mannose were identified in negligible amounts. Pectic polysaccharides were in small amounts (1.4%). Total sugar content was 84.4% but uronic acid content was high (28%). Galactose (56%) was the major neutral sugar along with small amounts of arabinose and rhamnose/fucose. The alkali-soluble (10% NaOH) hemicelluloses were isolated in about 8% yield. Both acetic acid prcepitable (Hem A) and non-precepitable (Hem B) fractions were present in nearly equal amounts. Both Hem A and Hem B were rich in glucose (80-90%) and may be β-glucan type (Ramesh and Tharanathan, 1998). The alkali insoluble residue was recovered in 6.3% yield. Carbohydrate content was 83%. Uronic acid was present in small amounts. Glucose was the major sugar (65%) identified, whereas xylose, arabinose and galactose were present in small amounts.

Table 40. Carbohydrate composition (%) of *Syzygium jambolna* seed coat and its isolated fractions

Fractions	Yield	TS	UA	Rha/ Fuc	Ara	Xyl	Man	Gal	Glc
Flour	(100)	86.0	12.0	1.5	5.4	16.6	1.4	3.4	69.3
WSP	9.8	87.0	26.0	1.6	10.9	0.6	0.5	7.1	79.3
PPS	2.2	79.0	36.5	3.0	15.6	2.2	-	20.4	58.8
Hem A	1.9	76.0	2.7	2.0	2.3	61.9	1.8	2.7	29.3
Hem B	3.2	82.5	23.1	3.5	5.6	22.5	9.0	15.0	44.4
AIR	35.5	75.0	4.8	1.4	6.9	31.4	1.5	3.0	55.8

Abbreviation as in Table 38

Carbohydrate composition (%) of *Syzygium jambolana* seed coat and its isolated fractions

The carbohydrate composition of jambolana seed coat and its isolated fractions is presented in Table 40. The seed coat contained 86 % carbohydrates. Uronic acid content was 12%. Starch was not detectable. Among sugars identified, glucose (69%) was in higher amount followed by xylose (16%) was next to glucose. All other sugars-arabinose, mannose and galactose were identified in small amounts. Water-soluble polysaccharides were isolated in 9.8% yield. The

fraction was rich in carbohydrates and also contained significant amounts of uronic acid (26%). Glucose was the major sugar (79%) identified. Arabinose (11%) and galactose (7%) were present in significant amounts, whereas mannose and xylose were in negligible amounts. The ammonium oxalate soluble pectic polysaccharides were isolated is 2.2% yield. The fraction was rich in uronic acid (36.5%) content and contained mainly galacturonic acid. Among neutral sugars, glucose was identified in higher amounts, whereas both galactose and arabinose were in considerable amounts. Small amount of rhamnose/fucose and xylose were also identified in this fraction. The yield of alkali soluble polysaccharide was 5.1%. Hemicellulose A was rich in carbohydrates and content of uronic acid was less. However it contained high amount of xylose (62%). Glucose present was next to xylose, which was around 29.3%. Hem B was rich in carbohydrates and showed good amount of uronic acid content (23%). Among neutral sugars, glucose, xylose and galactose were identified in higher amounts. The alkali-insoluble residue (AIR) was obtained in 35.5% yield. This fraction was rich in carbohydrates (75%) and contained small amounts of uronic acid. Glucose was the major sugar (56%) and contained xylose (31.4%) in significant amounts. Arabinose was present in small amounts. Presence of non-cellulosic polysaccharides indicated strong association of cellulose with noncellulosic polysaccharide in seed coat and this was more in seed coat than seeds or fruit pulp (Ramachandra Swamy and Salimath, 1991).

Table 41. Carbohydrate composition of dietary fiber of *Syzygium jambolana* fruit pulp, seed and seed coat.

Sample	TS	UA	Rha	Ara	Xyl	Man	Gal	Glc
PP - SDF	75.7	26.5	8.3	47.5	4.1	-	26.9	13.2
PP - IDF	70.5	9.3	-	3.7	41.7	4.8	11.8	37.5
SP - SDF	75.0	13.3	0.8	3.9	7.0	1.9	13.7	72.5
SP – IDF	77.5	4.5	0.4	4.3	80.8	5.6	0.9	7.94
SC - SDF	80.2	13.5	9.5	23.4	9.03	-	33.4	24.4
SC - IDF	71.8	12.4	8.5	21.4	5.2	-	9.2	55.6

SDF: Soluble, dietary fiber, IDF: Insoluble dietary fiber.

PP: Pulp powder, SP: Seed powder, SC: Seed coat.

Dietary fibers from *S. jambolana* fruit, seed and seed coat and their carbohydrate composition

Both soluble and insoluble dietary fibers of pulp, seed and seed coat were rich in carbohydrate content (70 - 80%) Table 41. However uronic acid contents were found to be in higher amounts in soluble dietary fibers (26.5, 13.3 and 13.5%) compared to insoluble dietary fiber, which was 9.3, 4.5 and 12.4% respectively.

Soluble dietary fiber (SDF) of pulp showed arabinose (47.5%) and galactose (27%) as major sugars, where as insoluble dietary fiber (IDF) showed xylose (42%) and glucose (37%) as major sugars. Glucose (72%) was the major

sugar identified in SDF of seed indicating it to be a β-glucan type of polysaccharide (Ramesh, 1998). Other major sugar found was galactose which was around 14%. IDF of seed powder seems to be xylan type of polysaccharide because of the presence of xylose in higher amounts (80%). Major sugars found in SDF of seed coat were arabinose (23.4%), galactose (33.4%) and glucose (24.4%) and may be coming from arabiogalactan type of polysaccharides. IDF of seed coat showed higher amount of glucose (55.6%) followed by arabinose (21.4%).

3.4.2.2. Discussion

Plant polysaccharides are gaining lot of interest now a days due to their various biological functions. Considerable amount of work has been done on polysaccharides from various sources and their hypoglycemic, anticancer, antiulcer, immunomodulatory property etc (Coombe, 1976; Warrier, 1996). *Syzygium jambolana* fruit has been studied extensively for their biological activities (Warrier, 1996; Martinez, 1991; Achrekar, 1991) and few bioactive compounds have been identified (Teixeira, 1992). Not much work has been done on non starchy polysaccharides and their beneficial role in health. Hence, attempts are made to isolate the various non-starchy polysaccharides and analyze their composition.

Various polysaccharides such as water soluble polysaccharides, pectic polysaccharides and hemicellulose A and Hemicellulose B were isolated sequentially from pulp, seed and seed coat and taken up for further compositional studies. Seed and seed coat showed higher amount of glucose and xylose indicating them to be rich in xyloglucan or xylan type of polysaccharides (Ramachandra Swamy and Salimath, 1991). Presence of glucose as major sugar in all the three flours was observed and they may be coming from β-glucan and cellulosic polysaccharides (Ramesh and Tharanathan, 1998). Presence of mannose

indicates mannan (Prasanna, 2003) or glucomannan (Salimath, 1982) type of polysaccharides to be present in the pulp.

Water soluble polysaccharides of pulp, seed and seed coat showed higher amount of uronic acid along with arabinose and galactose as major sugars indicating them to be arabinogalactan type of polysaccharides. Arabinane was characterized from red gram (Tharanathan, 1994). Water soluble glucans were identified in sorghum (Salimath, 1982). The fraction contained high amount of arabinose and galactose indicating it to be a mixture of water soluble acidic arabinogalactans and neutral arabinogalactans (Wankhede, 1979; Salimath, 1982). Pectic arabinogalactans have been isolated and characterized from roots of *Angeliva acutiloba* (Zhang, 1996). Small amount of glucose may be coming from unhydrolysed starch and water soluble β-glucan type of polysaccharides (Ramesh, 1998). Highly branched glucans having capillary permeability and phagocyte stimulating activity from the stems of *Cactus spicatus* are reported. Arabinose and galactose were also present in significant amounts and could be coming from water soluble acidic arabinogalactans.

Pectic polysaccharide is an important structural components of cell walls of plants and used widely in food industry as gelling, thickening and stabilizing agents. The composition, structure and physiological properties of pectic polysaccharides depend on extraction method, source and other environmental factors. In this investigation we have extracted the pectic polysaccharides with ammonium oxalate. Pectic polysaccharides have been isolated and characterized from other sources such as mango (Prasanna, 2003). Present data indicated that pulp has higher yield of pectic polysaccharides than seed and seed coat. Higher amount of uronic acid content was observed in these fractions. Pectic polysaccharides were characterized from field bean (Salimath, 1982) and black gram (Tharanathan, 1994). Seed pectic polysaccharides contain galactose as the

major neutral sugar along with small amounts of arabinose and rhamnose/fucose and indicate presence of complex acidic arabinogalactan type of polysaccharide (Bhagya Lakhsmi, 2002; Prasanna, 2003; Zhang, 1996). Anti-ulcer pectic polysaccharides are reported from *Bupleatum faclcatum* (Sakarai, 1996). Fine structure-function relationship of galactosyl chains attached to rhamnogalacturonans is reported from Chinese herbs (Yamada, 1994). High amount of glucose present in seed coat pectic polysaccharide fraction indicate the presence of associated β-glucan type polysaccharide (Ramesh, 1998).

Hemicellulose of pulp contains arabinose and galactose in equal amounts indicates the presence of arabinogalactan type of alkali-soluble polysaccharides being present in the fruit pulp (Bhagya Lakhsmi, 2002; Prasanna, 2003). Both the hemicelluloses A and B of seeds are rich in glucose (90%), indicating them to be principally glucan type of polysaccharides (25%, 33%). Xylose was rich in seed coat along with glucose indicating the presence of either xylan or xyloglucan type of polysaccharides. Glucose may be coming from alkali soluble glucan and xyloglucan type of polysaccharides (Ramesh, 1998; Salimath, 1982). Xyloglucans have been isolated and characterized from field bean (Salimath, 1982). Difference in their acetic acid precipitable character could be due to their association with other polysaccharides and may be acidic in nature along with its associated fractions (Tharanathan, 2002). Acidic xylans were isolated and characterized from red gram husk (Salimath, 1982).

Alkali-insoluble residue contained glucose as major sugar and indicated them to be cellulosic in nature. High amount of association of cellulose with non-cellulosic polysaccharides from field bean are reported (Salimath, 1982). Presence of high amount of xylose (32%) in seed coat indicates it to be xyloglucan type of polysaccharide and is present associated with cellulose (Salimath, 1982). This suggests strong association of xyloglucan type of polysaccharide with cellulose.

Arabinose is also observed in small amounts in alkali insoluble residue of pulp, seed and seed coat.

Dietary fibers were isolated from pulp, seed and seed coat and were fractionated in to soluble and insoluble dietary fibers based on their solubility and studied for their composition. Both soluble and insoluble dietary fibers of pulp, seed and seed coat are rich in carbohydrate content. Uronic acid content was higher in insoluble dietary fiber compared to soluble dietary fiber of all the samples. However the amount of insoluble dietary fiber of pulp was higher than seed and seed coat. Soluble dietary fiber of pulp contained mainly arabinose and galactose where as IDF contained mainly xylose and glucose as major sugars indicating it to be xyloglucan type of polysaccharide. SDF of seed contained glucose as a major sugar along with xylose and galactose. Presence of xylose (80%) as major sugar in IDF of seed indicates it to be the xylan type of polysaccharide. Arabinose and galactose were the major sugars in seed coat of SDF, while IDF contained glucose in higher amounts along with arabinose and galactose.

3.4.2.3 Summary and conclusion.

S. jambolana has been attributed to possess several medicinal properties in the folklore system of medicine. In recent years biological functions of polysaccharides is receiving much interest. Hence, the present study was undertaken to study carbohydrate property of jambolana pulp, seed and seed coat. All the parts of plant are useful. We have selected pulp, seed and seed coat separately to study their carbohydrate profile, the results obtained can be summarized as follows;

1. Water soluble polysaccharide of pulp and seed showed arabinose, glucose and galactose indicating it to be arabinogalactan or arabinan type of

polysaccharides whereas seed coat showed higher amount of xylose and glucose indicating it to be xylan or xyloglucan type of polysaccharides

2. Pectic polysaccharides contained higher amount of uronic acid along with arabinose and galactose as major sugars indicating it to be arabinogalactan type of polysaccharide.
3. Both Hem A and Hem B of seed are found to have higher amounts of glucose indicating them to be β-glucan type of polysaccharide. Seed coat hemicelluloses contained higher amount of xylose and glucose indicating them to be xylan or xyloglucan type of polysaccharide.
4. Presence of glucose as a major sugar in alkali-insoluble residue of pulp, seed and seed coat indicated them to be cellulosic in nature.
5. Among soluble and insoluble dietary fibers of pulp, seed and seed coat, soluble polysaccharides were coming in nature.

It can be concluded that the difference in carbohydrate profile of various polysaccharide fractions could be due to the presence of different types of polysaccharide. Further investigations are needed to study their biological activity and characterize the nature of polysaccharides.

3.4.3. Phenolic acid content and their antioxidant activity from polysaccharide fractions

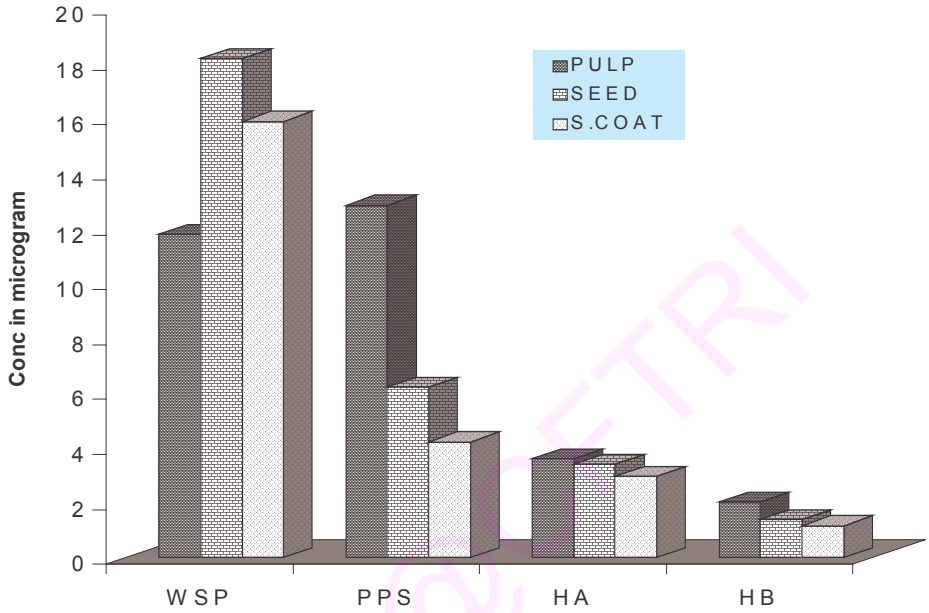


Fig. 30: Total phenolic acid content in various polysaccharide fractions.

Abbreviations as in footnote to Table 38

Total phenolic content in water solublepolysaccharides (WSP), pecticpolysaccharides (PPS) hemicellulose A (Hem A) and hemicellulose B (Hem B) polysaccharide fractions.

The total phenolic acid content of all the polysaccharide fractions is shown in Fig. 30. Among WSP fractions, seeds contained highest amount of phenolic acids (18 µg/ mg). WSP of seed coat had around 16 µg phenolic acids. WSP of pulp showed comparatively less amount of phenolic acid content. Among pectic fractions, pulp showed higher amount of phenolic acid content (12.8 µg) compared to seed coat and seed. Hem A and Hem B fractions of pulp, seed, and seed coat

showed marginal difference in their total phenolic content which was around 3-3.5 μg and 1.1-1.9 μg, respectively.

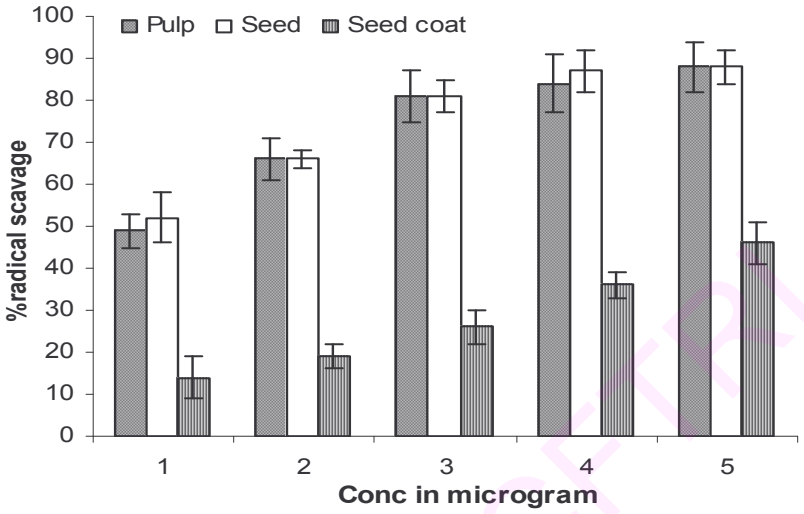


Fig. 31: DPPH scavenging ability of WSP of pulp, seed and seed coat.

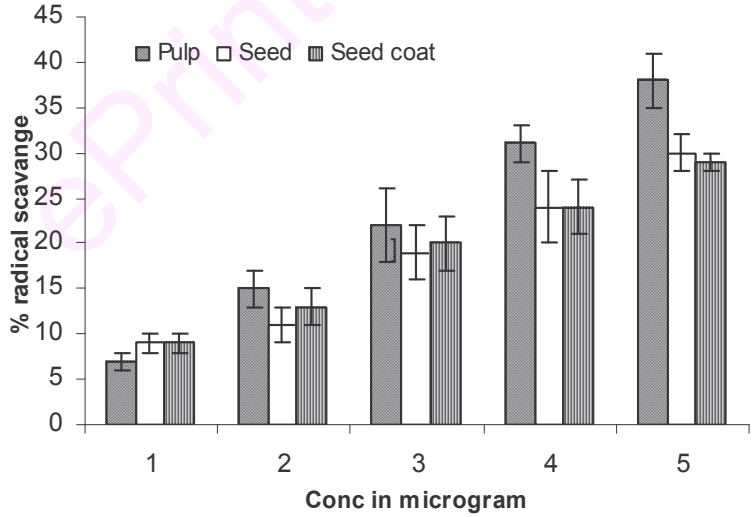


Fig. 32: DPPH scavenging ability of PPS of pulp, seed and seed coat.

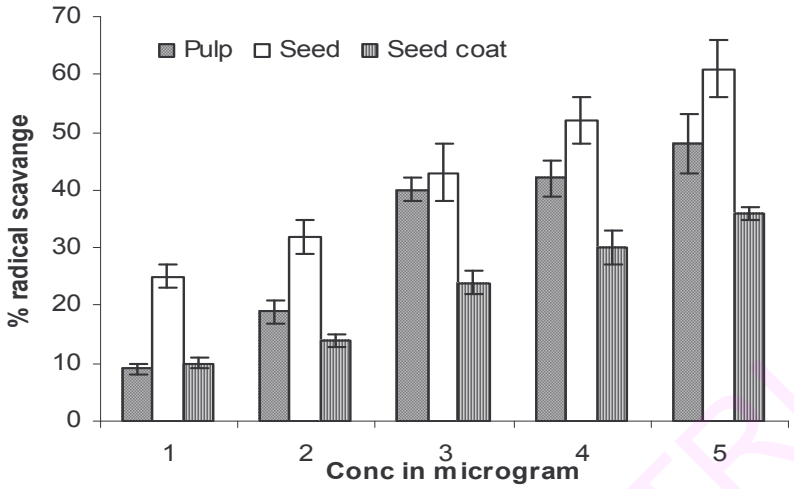


Fig. 33: DPPH scavenging ability of Hem A of pulp, seed and seed coat.

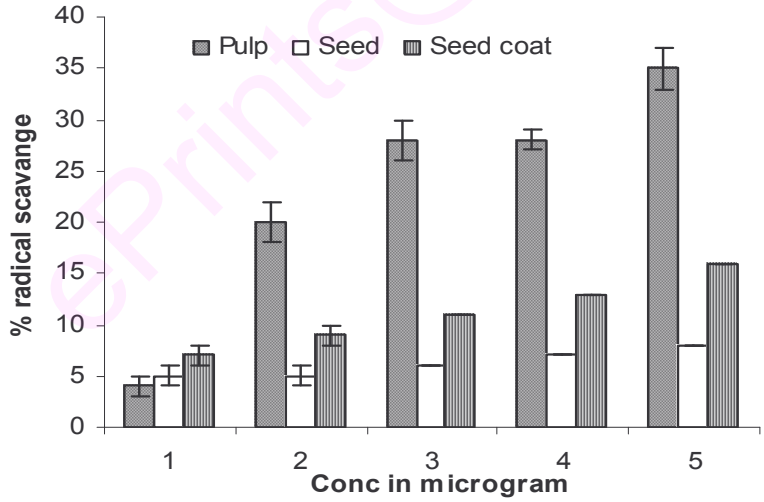


Fig. 34: DPPH scavenging ability of Hem B.

DPPH scavenging ability of WSP, Pectic, Hem A and Hem B

DPPH radical scavenging ability of water soluble, pectic, Hem A and Hem B of pulp, seed and seed coat of jambolana are shown in Figs. 30-34. Free radical scavenging potential of all the fractions was concentration dependent. Among the polysaccharide fractions, Hem B of pulp, seed and seed coat showed lesser activity. Higher activity was observed with WSP of both pulp and seed. However, WSP of seed was slightly more active than pulp. Among pectic fractions, free radical scavenging ability of them in order was pulp> seed > seed coat. Among Hem A fractions, seeds showed higher activity, whereas in Hem B, pulp showed higher activity.

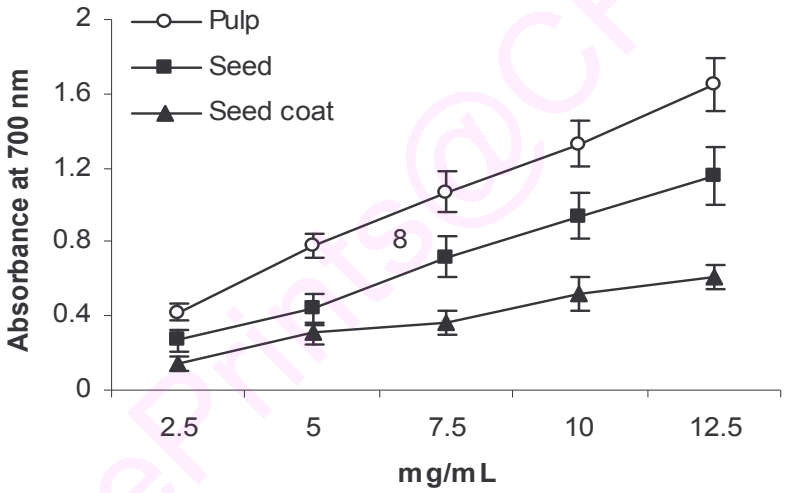


Fig. 35: Reducing power ability of WSP of pulp, seed and seed coat.

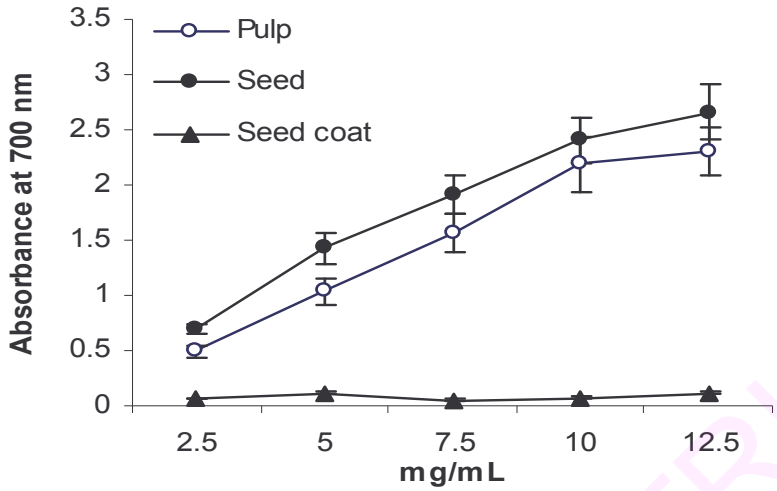


Fig. 36: Reducing power ability of PPS of pulp, seed and seed coat.

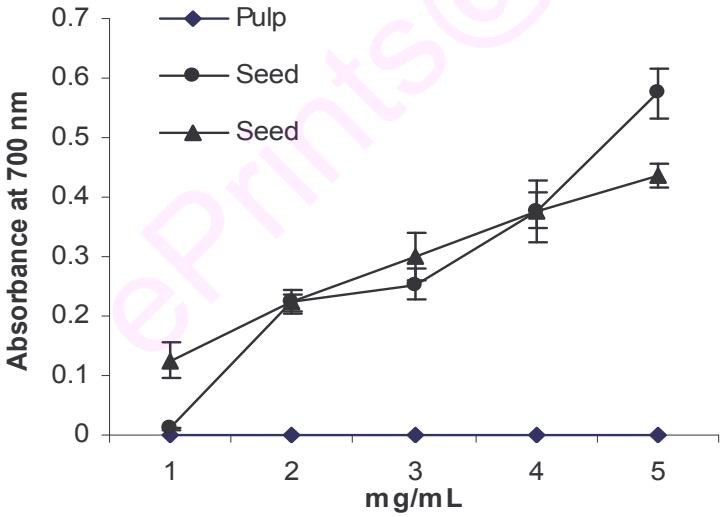


Fig. 37: Reducing power ability of Hem A of pulp, seed and seed coat.

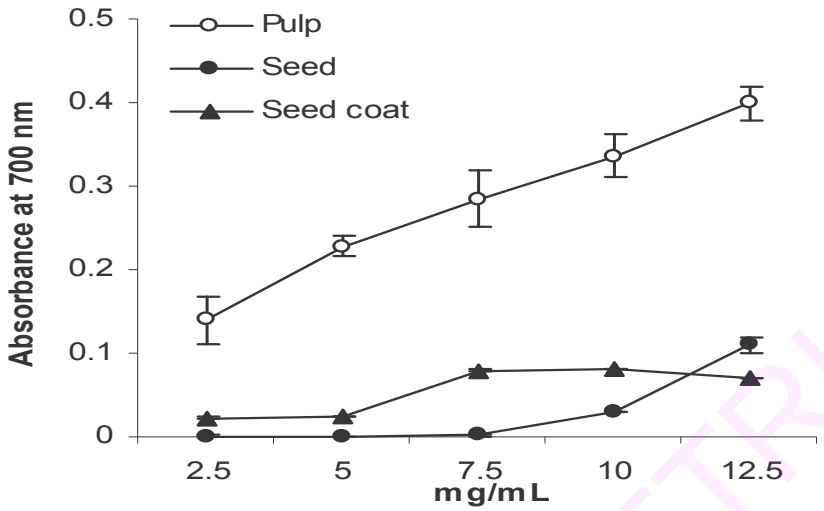


Fig. 38: Reducing power ability of Hem B of pulp, seed and seed coat.

Reducing power ability of WSP, Pectic, Hem A and Hem B

The reducing power ability of various polysaccharide fractions was in a dose dependent manner and correlated well with increasing concentration (Figs. 35-38). Between WSP of pulp (Fig. 35), PPS of seed (Fig. 36), Hem A of seed (Fig. 37) and Hem B of pulp (Fig. 38) showed relatively higher activities. However, WSP of seed was more active than pulp. Among pectic fractions, reducing power ability of pulp was found to be more active than seed and seed coat. Hemi A of pulp showed less activity and hemi B of pulp was more active.

HPLC analysis of free and bound phenolic acids

Free and bound phenolic acids were isolated from crude samples of pulp seed and seed coat. Their HPLC profiles are presented in Figs. 39-44. The elution profile of both bound and free phenolic acids shows that major peak was gallic acid in both

bound and free phenolics. Free phenolic acids fraction of pulp showed only gallic acid as the major phenolic acid which was around 96% and small amount of syringic acid was identified (Fig. 39). In seed, major free phenolic acid identified was syringic (33%) and gallic acid (21%). Other phenolic acids identified were caffeic acid, vannilic acid and protocetechuic acid (Fig. 40). Free phenolic acid fractions of seed coat showed almost same amount of gallic acid and syringic acid which was around 32%. Protocetechuic acid and vanillic acid were identified in small amounts (Fig. 41).

Bound phenolic acid fraction of pulp showed gallic acid (42%) as the major phenolic acid. Caffieic acid, vannilic acid, protocetechuic acids were other phenolic acids identified in this fraction (Fig. 42). In seed powder, gallic acid (78%) was identified as major bound phenolic acid. Ferulic acid found to be next to gallic acid amount of protocetechuic acid was in small amount (Fig. 43).

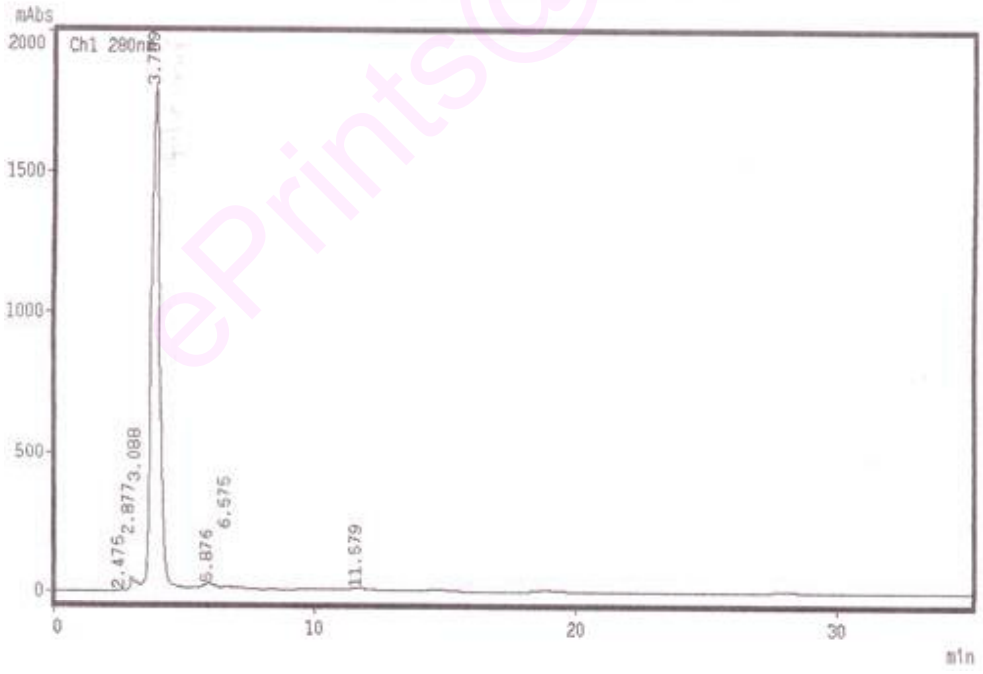


Fig. 39: HPLC profile of free phenolic acids from pulp seed and seed coat.

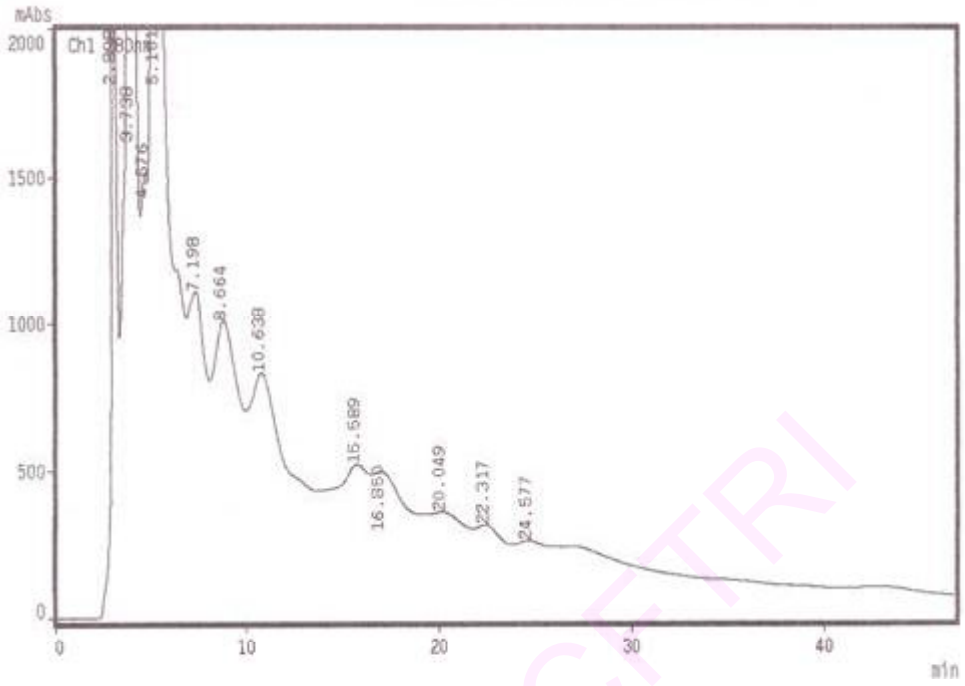


Fig. 40. HPLC profile of free phenolic acids from seed.

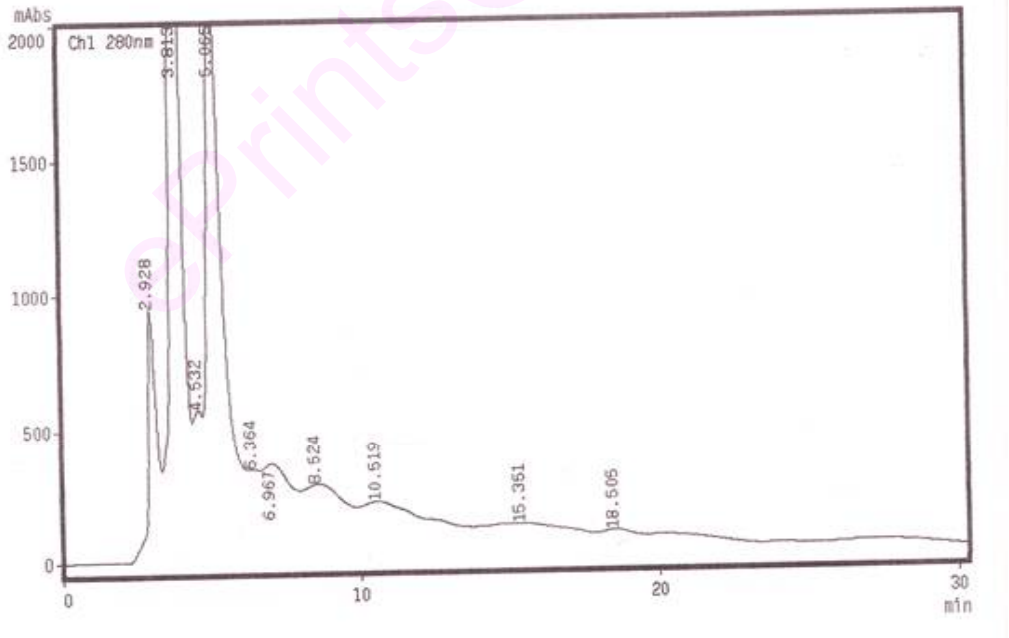


Fig. 41: HPLC profile of free phenolic acids from seed coat.

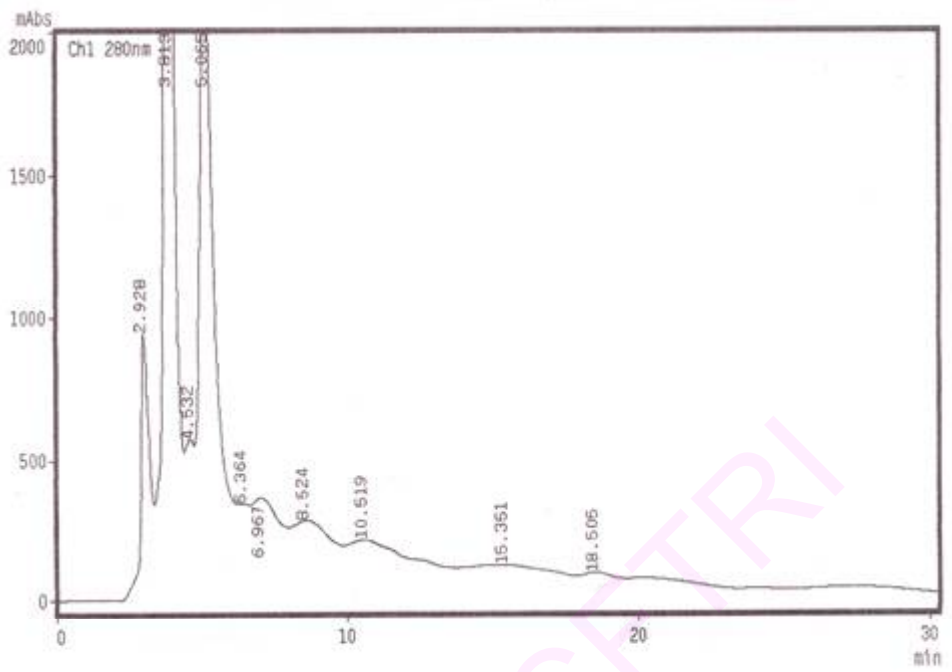


Fig. 42: Bound Phenolic acids from pulp.

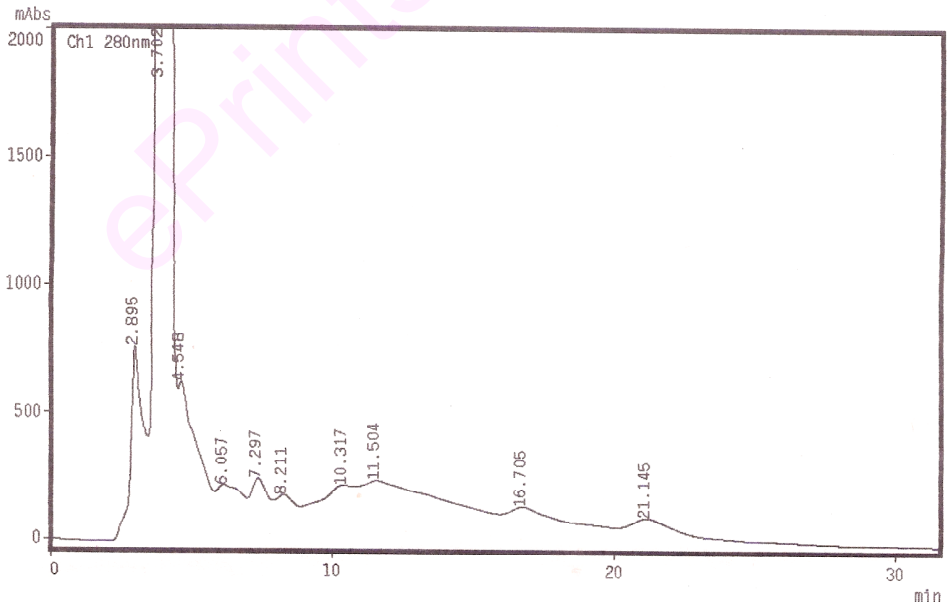


Fig. 43: Bound Phenolic acids from seed.

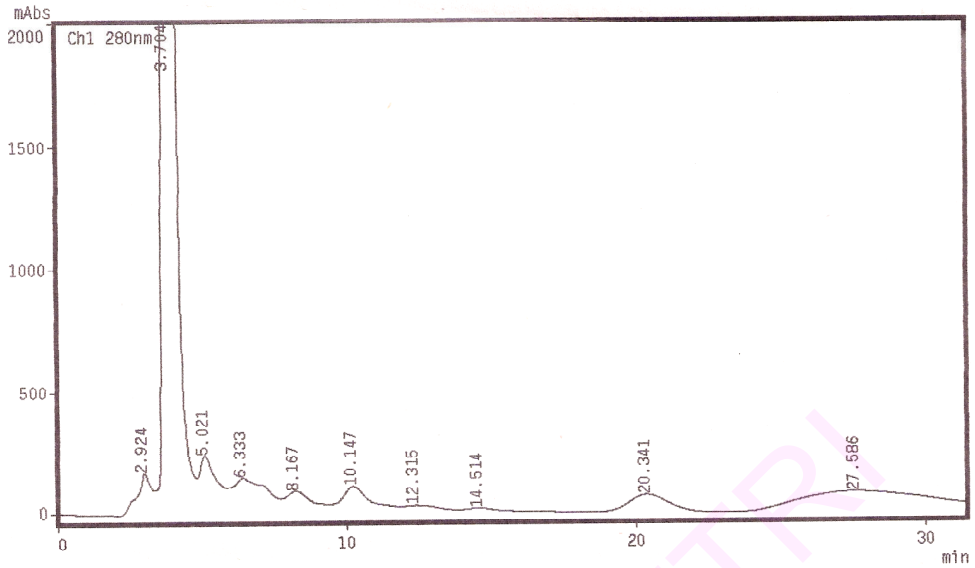


Fig. 44: Bound Phenolic acids from seed.

3.4.3.3. Discussion

Phenolic compounds are commonly found in the plant kingdom and they have been reported to have multiple biological effects, including antioxidant activity (Deshpande, 1984; Kahkonen, 1999). Antioxidant activity of plant extract is often associated with the phenolic content present in them. Hydrogen donating properties of the polyphenolic compounds are responsible for the inhibition of free radical induced lipid peroxidation. In this present investigation, we measured the total phenolic content by FC method (Singleton, 1965) in all the polysaccharide fractions. Among four different polysaccharides, WSP of pulp, seed and seed coat were showed higher phenolic acid contents and was 11, 18 and 16 µg/mg, respectively. Data clearly indicates that total content of phenolic acids goes on decreasing with sequential extraction of polysaccharides such as WSP, PPS, Hem A and Hem B. Interestingly, total phenolic acid content of WSP, PPS of pulp and seed are directly correlated with DPPH scavenging and reducing power ability. These results suggested that the effectiveness of the antioxidant activity of water

soluble polysaccharides in pulp and seed may be correlatable to their phenolic acid constituents. Similar observations also made (Nandini, 2001; Nordkvist, 1984; Onyeneho, 1992). In Hem A and Hem B (total phenolic acid) of pulp and seed coat power and DPPH scavenging ability could not be correlated. However, it is known that non-phenolic antioxidants could also contribute to the antioxidant activity of the extract.

DPPH is a stable radical that accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The reduction in DPPH was determined by decrease in absorbance at 517 nm induced by antioxidants. Hence, DPPH is generally used as a substrate to evaluate the antioxidant activity of antioxidants or plant extracts containing antioxidants The DPPH assay provides information about the intrinsic free radical scavenging power in solution irrespective of the physicochemical environments encountered in biological systems (Brand-Williams et al. 1995). The model system of scavenging DPPH free radical is a simple method to evaluate the antioxidant activity of antioxidants. DPPH was used to determine the proton-radical scavenging activity of polysaccharide fractions as it possesses a proton free radical and shows a characteristic absorption at 517 nm. The purple color of DPPH solution would fade rapidly when it encounters proton-radical scavengers. Figs. 31-34 illustrate the scavenging effect of various polysaccharide fractions. Among four polysaccharides, water soluble polysaccharides of pulp and seed exhibited higher free radical scavenging activity and IC_{50} of them were 2.1 and 2.4 µg respectively. Further it is well accepted that the DPPH free radical scavenging by antioxidants due to their hydrogen donating ability (Chen, 1995). DPPH scavenging activity of pectic polysaccharides of pulp, seed and seed coat can be directly correlated with total phenolic content. In the present study, most of the sample showed a concentration dependent activity. This may be attributable to their hydrogen-donating ability.

Antioxidant activity and reducing power are related (Duh, 1998; Duh, 1999; Tanaka, 1988). Reducing properties are generally associated with the presence of reductants and have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom (Gordon, 1990). In our study reducing power of water soluble and pectic polysaccharide of pulp and seed showed significant effect towards antioxidant activity. However, water soluble polysaccharide of seed and pulp were found more active than other polysaccharides. Therefore, the significant antioxidant activity of WSP of both pulp and seed may be related to their reducing power ability.

Phenolic acids are known to have anticarcinogenic activity (Aggarwal, 2003) and are believed to be an important part of the general defense mechanism of many plants to infections. Hence, both bound and free phenolic acids were isolated and characterized by HPLC.

Various plants have been analyzed with respect to phenolic acids by HPLC (Liu and Yao, 2007; Maillard, 1995; Mathew, 2004; McCallum, 1990). HPLC profile of both bound and free phenolic acids of pulp showed gallic acid 95% and 86%, respectively and is the major phenolic acid present. Syringic (33%) and gallic acid (21%) were identified as free phenolic acids and gallic acid is the main bound phenolic acid in seed. Free and bound phenolic acids were extracted with petroleum ether in earlier study (Suresh Kumar, 2006). Free phenolic acid fractions of seed coat showed almost same amount of gallic acid and syringic acid, which was around 32%. Protocetechuic acid and vanillic acid were identified in small amount.

Summary and conclusion

S. jambolana pulp, seed, seed coat and their isolated fractions were found to differ in their carbohydrate profile as described in earlier section. Phenolic acid and biological activity of these polysaccharides with respect to antioxidant activity was studied and results obtained are summarized as follows.

1. Total phenolic acid content was found to be higher in water soluble polysaccharides and goes on decreasing with sequential extracted fractions such as pectic, hemi A and hemi B.
2. Among all the polysaccharide fractions water soluble fraction of pulp and seed exhibited higher free radical scavenging activity. Both water soluble polysaccharides and pectic polysaccharide fractions of pulp and seeds showed better reducing power ability compared with Hem A and Hem B.
3. Gallic acid was found to be major phenolic acid present in bound form in seed and pulp.

Conclusion

It can be concluded that antioxidant property of polysaccharides could be due to Phenolic acids and or associated polysaccharides.

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