INVESTIGATIONS ON THE CARBOHYDRATE DIGESTIBILITY OF FINGER MILLET (*Eleusine coracana*) WITH SPECIAL REFERENCE TO THE INFLUENCE OF ITS SEED COAT CONSTITUENTS

A Thesis submitted to the University of Mysore, Mysore

For the award of the degree of

DOCTOR OF PHILOSOPHY

IN

BIOCHEMISTRY

By

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DECLARATION

I here by declare that the Ph. D. thesis entitled **"Investigations on the carbohydrate digestibility of finger millet (*Eleusine coracana*) with special reference to the influence of its seed coat constituents"** submitted to the University of Mysore, Mysore, for the award of Degree of "**Doctor of Philosophy**" is the result of the work carried out by me under the guidance of Dr. N. G. Malleshi, former Head, Department of Grain Science & Technology, Central Food Technological Research Institute, Mysore, during the period 2005-2009.

I further declare that the results of the work presented in the thesis have not been previously submitted for any other Degree, Diploma or any other similar titles.

Place: Mysore (S. Shobana)
Date:

Dr. N. G. Malleshi
Former Head
Department of Grain Science & Technology

CERTIFICATE

This is to certify that the Ph. D. thesis entitled **"Investigations on the carbohydrate digestibility of finger millet (*Eleusine coracana*) with special reference to the influence of its seed coat constituents"** submitted to the University of Mysore, Mysore for the award of Degree of "**Doctor of Philosophy"** is the result of work carried out by Mrs. S. Shobana, CSIR-SRF in the Department of Grain Science & Technology, Central Food Technological Research Institute, Mysore - 570 020 under my guidance during the period, April 2005 to October 2009.

Place: Mysore
Date:

(**N. G. MALLESHI)**
GUIDE

Dedicated to.....

***My Parents** who encouraged me....*

*My guide **Dr. N. G. Malleshi** who inspired and supported me....*

and

*The **Almighty** who enabled me.....*

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Above all, I believe that nothing is possible without ***God's*** *grace.*

SHOBANA

CONTENTS

ABBREVIATIONS

α	Alpha
ADP	adenosine diphosphate
AG	Aminoguanidine
AGE	advanced glycation endproducts
$AlCl_3$	aluminium chloride
APS	ammonium per sulphate
AR	aldose reductase
ATP	adenosine triphosphate
AU	arbitary units
β	Beta
BSA	bovine serum albumin
BSS	British standard sieves
BU	Brabender units
Ca_2^+	calcium ion
$CaCl_2$	calcium chloride
CHD	coronary heart disease
cm	centimeter
CO_2	carbon dioxide
$CuSO_4$	copper sulphate
^0C	degree centigrade
dl	decilitre
DNA	deoxyribonucleic acid
DMSO	dimethyl sulphoxide
DM	decorticated millet
DNS	2, 4 dinitro salicylic acid
DPPH	2,2' diphenyl-1-picrylhydrazyl
DPPH·	2,2' diphenyl-1- picrylhydrazyl radical
EDTA	ethylene diamine tetra acetic acid
EC	enzyme commission
Em	emission
ERF	endosperm rich fraction
ESI-MS	electrospray ionization mass spectrometry
Ex	excitation
$FeCl_3$	ferric chloride
FFA	free fatty acid
$FeSO_4$	ferrous sulphate
g	gram
GI	glycemic index
GL	glycemic load
GPC	gel permeation chromatography
GR	glycemic response
h	hour
HbA_1c	glycosylated hemoglobin
HCl	hydrochloric acid
HDL	high density lipoprotein
H_2O	water
H_2O_2	hydrogen peroxide

HPLC	high performance liquid chromatography
H_2SO_4	sulphuric acid
HTM	hydrothermally processed millet
[I]	inhibitor concentration
ICA	iron chelating ability
i.p.	intraperitoneal
Kg	kilogram
K_m	Michaelis constant
KOH	potassium hydroxide
L	litre
LB plot	Lineweaver-Burk plot
LDL	low density lipoprotein
mg	milligram
µ	micron
µg	microgram
µm	micrometer
µmol	micromole
µl	microlitre
ml	millilitre
M	molar
min	minute
MSCP	millet seed coat polyphenols
N	normality
Na^+	sodium ion
NAD	nicotinamide adenine dinucleotide
$NaHCO_3$	sodium bicarbonate
$NaNO_2$	sodium nitrite
NaOH	sodium hydroxide
nm	nanometer
NM	native millet
NSP	non-starchy polysaccharides
$O_2^{\cdot-}$	superoxide radical
$OH^{\cdot}$	hydroxyl radical
%	percent
PMSF	phenyl methane sulpfonyl fluoride
PNP	*p*-nitrophenol
PNP- glycoside	*p*-nitrophenyl-alpha-D-glucopyranoside
RF	refined flour
ROS	reactive oxygen species
rpm	revolutions per minute
RSA	radical scavenging ability
RVU	rapid visco-analyzer units
SCM	seed coat matter
SCM-HTM	seed coat matter from hydrothermally processed finger millet
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	standard error of mean
STZ	streptozotocin

[S]/V	substrate concentration Vs velocity
TAA	total antioxidant activity
TCA	trichloroacetic acid
TEMED	N,N,N',N'- tetramethylethylenediamine
TRIS	tris (hydroxymethyl) aminomethane
UV	ultraviolet
Vis	visible
V/V	volume/volume
VLDL	very low density lipoprotein
V_{max}	velocity maximum
WB	white bread
weight/volume	W/V
WM	whole meal
wt	weight

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Synopsis

of a Ph.D. thesis

on

INVESTIGATIONS ON THE CARBOHYDRATE DIGESTIBILITY OF FINGER MILLET (*Eleusine coracana*) WITH SPECIAL REFERENCE TO THE INFLUENCE OF ITS SEED COAT CONSTITUENTS

For

Ph.D. Degree in Biochemistry

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Finger millet (*Eleusine coracana* L.) or ragi is one of the important minor cereals in the Indian subcontinent and also in many of the African countries. The millet foods are known for their higher sustaining power, lower glycemic response and higher satiety scores compared to other cereal foods. The incidence of diabetes and duodenal ulcer are known to be lower among the population using the millet as staple. These health beneficial properties of the millet may be attributed to the special features of its carbohydrates and also to some of its phytochemical contents.

The millet contains 6 - 9% protein, 65 - 70% starch, 12 - 18% non-starchy polysaccharides, 1.2 - 1.5% lipids, 2.5 - 3% minerals, 340 - 360 mg% calcium and 270 - 290 mg% phosphorus. It is also a rich source of dietary fibre (15 - 20%), polyphenols (0.3 - 2%) and phytate (0.2 - 0.3%). The seed coat which forms about 15% of the seed matter contains about 70% of the kernel polyphenols and the constituents of the polyphenols are mainly phenolic acids, flavonoids, anthocyanins and tannins. Usually, the millet is pulverized and the whole meal including the seed coat is utilized for the preparation of traditional foods. Since, there are no reports of any adverse effect even on prolonged consumption of the millet foods, it is strongly believed that, the millet seed coat is safe for human consumption, and its constituents contribute for the nutritional benefits of the millet.

Diabetes mellitus is a common endocrine disorder characterized by hyperglycemia and it predisposes to some of the chronic complications such as retinopathy, cataract, neuropathy, nephropathy and atherosclerosis. One of the therapeutic approaches to decrease the postprandial hyperglycemia is to slow down the digestion of carbohydrates through inhibition of the carbohydrate digesting enzymes, namely the α-amylase and α-glucosidase and thereby delaying the absorption of glucose. Hyperglycemia plays an important role in the pathogenesis of diabetic complications and one of the most important complication is the protein glycation and advanced glycation end-product (AGE) formation. In the case of uncontrolled hyperglycemia, formation of the AGEs augments the free radical generation leading to the biomolecular damage and subsequently increases the severity of diabetes. Hence, it is highly desirable to

provide foods containing antioxidants, modulators of postprandial blood glucose and also inhibitors of protein glycation.

The polyphenols, phytate and the carbohydrates of the millet may probably, contribute for the slow digestion and other health beneficial qualities of the millet foods. The scientific information about the role of the millet polyphenols on slowing down its carbohydrate digestibility and also their ability to ameliorate the complications of diabetes are scanty. Hence, generation of scientific information on these aspects would be helpful in identifying the pharmaco-nutritional uses for the millet and also for the development of a special dietary regimen for the diabetic subjects. This could also augment the consumption of the millet thereby increasing its agro-economics.

Accordingly, investigations on the nature as well as the nutraceutical potentials of the millet in general and its seed coat in particular with special reference to their influence on the carbohydrate digestibility of the millet were undertaken with the following objectives;

1. Localization of the polyphenols in the millet kernel and preparation of the polyphenols rich fraction of the millet (seed coat matter abbreviated as SCM), by developing a milling protocol and also isolation and characterization of polyphenols,
2. Assessment of the influence of the millet SCM as well as the hydrothermal processing on the *in vivo* carbohydrate digestibility of millet foods
3. Investigations on the inhibitory properties of the millet polyphenols against α-amylase, α-glucosidase, protein glycation and hydroxyl radical ($OH^{\cdot}$) mediated protein fragmentation in *in vitro* model systems,
4. Evaluation of the effect of the millet SCM on the hyperglycemia and its associated complications in diabetic animal models.

The experiments conducted and the results obtained with these objectives are presented in the form of the thesis. The subject matter on the special features of the millet carbohydrates, phenolics and the nutritional aspects of the millet forms the **Introduction** to the thesis. Localization of polyphenols in the millet kernel by histochemical techniques and preparation of the polyphenols rich fraction of the millet following a special milling protocol, and also the extraction as well as characterization of polyphenols in the SCM are

dealt in **Chapter I**. The influence of the SCM from the native millet and also from the hydrothermally processed millet on the carbohydrate digestibility of millet foods by *in vitro* and *in vivo* (in normal and type II diabetic subjects) methods are described in **Chapter II.** The mode of inhibition of the millet polyphenols against α-amylase and α-glucosidase (the key enzymes involved in blood glucose homeostasis) and besides, the inhibitory properties of the millet polyphenols against fructose induced protein (albumin) glycation as well as AGE formation are elaborated in **Chapter III**. The salient features of the ameliorative properties of the millet SCM on the complications associated with diabetes such as hyperglycemia induced protein glycation and the AGE formation, dyslipidemia, kidney dysfunction and also cataractogenesis are reported in **Chapter IV**. Finally, the details of the reference cited in all the chapters are compiled under the reference section.

A brief account of the above are as follows;

INTRODUCTION

The literature on the scientific and technological aspects, including the information on the health benefits of finger millet published in the peer reviewed scientific journals, proceedings of the scientific conferences, book chapters and the popular articles, are reviewed in this section. The information on the millet kernel morphology, its processing and utilization and the nutraceutical properties as well as its carbohydrate profile and also the factors influencing the carbohydrate digestibility of foods including the possible role of cereal polyphenols in amelioration of the complications of diabetes are briefly covered under the introduction section.

CHAPTER I

LOCALISATION OF POLYPHENOLS IN FINGER MILLET KERNEL AND CHARACTERIZATION OF THE POLYPHENOLS ISOLATED FROM THE MILLET SEED COAT MATTER

One of the main objectives of the research program was to prepare the fraction rich in phytochemicals with the nutraceuticals qualities and in view of that, the localization of the polyphenols and phytate in the millet kernels was carried

out following the histochemical techniques. The millet sections were stained with ferric chloride, acriflavine-HCl and alizarin red S for the localization of the polyphenols, phytate and calcium associated phytate reserves respectively and it was observed that, the major portion of the polyphenols are concentrated in the seed coat including aleurone layer and germ, whereas, the presence of phytates including the calcium associated phytate reserves were noticed mainly in the germ and the aleurone layer. Based on these observations, fractionation of the millet kernels into seed coat rich and endosperm rich fractions were carried out following a sequential milling and sieving method. The SCM thus prepared formed about 22% of the seed mass and contained 11.2 ± 1% total polyphenols, 2.3 ± 0.3 % tannins, 0.3 ± 0.05 % flavonoids and also 0.7 ± 0.1 % phytate. Subsequently the polyphenols of the SCM were extracted with HCl-methanol solvent system and the isolated polyphenols were fractionated into the constituent phenolics by HPLC. The HPLC elution profile indicated that, the millet polyphenols are a complex mixture of several phenolics and the phenolics were also characterized by the direct infusion electrospray insertion mass spectrometry (ESI-MS) and the results revealed that, the phenolics belong to the category of simple phenols, phenolic acids, flavonoids, tannins and anthocyanins. Although 19 phenolic compounds were identified, gallic acid, ferulic acid, apigenin, trimers as well as tetramers of catechin were prominent and yet a good number of the phenolics still remained unidentified.

CHAPTER II

CARBOHYDRATE DIGESTIBILITY OF FINGER MILLET AS INFLUENCED BY ITS SEED COAT MATTER AND HYDROTHERMAL PROCESSING

The glycemic index (GI) of the foods is normally influenced by the nature of food as well as by the processing it has undergone. The hydrothermal processing to the millet has been recently applied and hence, it was felt desirable to evaluate the influence of hydrothermal processing on the millet carbohydrate digestibility. Since, the seed coat of the millet is a rich source of polyphenols and dietary fibre, it could also influence the carbohydrate digestibility of the millet foods. Hence, the influence of the millet seed coat matter on the carbohydrate digestibility of the millet foods were studied. Generally, the *in vivo* carbohydrate digestibility of foods

are assessed by their GI, as they indicate the rate of carbohydrate digestibility. The influence of the millet seed coat polyphenols (in isolation) on the *in vitro* starch digestibility was studied using the starch isolated from the millet. In the presence of the millet seed coat polyphenols, the maltose equivalents released by the pancreatic amylase digestion of the millet starch decreased. The decrease was proportionate to the concentration of the polyphenols present in the reaction mixture. Hence, to understand the effect of the millet SCM on its carbohydrate digestibility, the millet diets prepared from the whole meal and its counter part free from the seed coat matter were tested for their glycemic response (GR). For the purpose, dumpling or stiff porridge (*mudde*) prepared from the whole meals of the native (NM) as well as the hydrothermally processed millet (HTM) and their counterparts devoid of the seed coat matter namely, the refined flour (RF) and the decorticated millet (DM) were tested for their GR on normal and type II diabetic subjects (n=6 in each group). The GI values of NM and RF were 69 ± 3 and 100 ± 3 in the normal subjects, and 73 ± 4 and 104 ± 4 in the type II diabetic subjects respectively. Similarly, the GI values of HTM and DM were 101 ± 3 and 81 ± 5 in the normal and 107 ± 2 and 78 ± 6 in the type II diabetic subjects respectively. The significantly lower GI of the whole meal compared to the refined flour clearly brought out the beneficial effect of seed coat matter with respect to slowing down the carbohydrate digestibility. However, among the HTM and DM, the lower GI for the DM could be due to its intake in the form of grains even though it was free from the seed coat matter. These observations also indicated that, the nature of processing as well as the form in which the food material is consumed influence the GI values. The study clearly brought out the beneficial effect of the millet polyphenols with respect to slowing down the carbohydrate digestibility of the millet foods.

CHAPTER III
INVESTIGATIONS ON THE NUTRACEUTICAL ABILITY OF FINGER MILLET SEED COAT POLYPHENOLS

Polyphenols are well known modulators of postprandial hyperglycemia and inhibitors of protein glycation. The hyperglycemia associated complications of diabetes are a consequence of accelerated non-enzymatic modification of

proteins as well as other biomolecules by glucose or its metabolic intermediates. Hence, the modulatory characteristics of the millet polyphenols on the glucose homeostasis and also on the inhibition of the protein glycation were studied in the *in vitro* model systems. The results indicated that, the polyphenols exhibit very strong inhibition against porcine pancreatic amylase (IC_{50} 23.5 µg) and rat intestinal α-glucosidase (IC_{50} 16.9 µg). The studies on the mode and the kinetics of enzyme inhibition using Michaelis-Menton and Line-weaver Burk plots clearly revealed that, the K_m remain constant and the V_{max} decrease in the presence of the polyphenol extract indicating that, the inhibition of pancreatic amylase and α-glucosidase by the millet polyphenols were of non-competitive type. The protein glycation inhibitory properties of the millet polyphenols was studied with fructose-BSA model system and it was observed that, the polyphenols are potent inhibitiors of protein glycation. This was observed by the restoration of tryptophan fluorescence (nearly 24%) and also by the reduction of AGE formation (by about 40%) in the presence of 45 µg of the millet polyphenols. The anti-protein glycation property of the millet polyphenols may be due to its interference in the first phase of glycation cascade and hence subsequent inhibition of down stream reactions involved in the AGE formation. The polyphenols were also found to be effective free ($DPPH^{\cdot}$) radical scavengers (IC_{50} 2 µg). In addition, investigations on the inhibitory properties of the millet polyphenols against the hydroxyl radical ($OH^{\cdot}$) mediated BSA fragmentation was carried out in BSA - $H_2O_2/CuSO_4$ catalyzed oxidation model system, which clearly brought out that, the millet polyphenols are potential inhibitors of $OH^{\cdot}$ mediated BSA fragmentation.

CHAPTER IV
AMELIORATION OF HYPERGLYCEMIA AND ITS ASSOCIATED COMPLICATIONS BY FINGER MILLET SEED COAT MATTER IN STREPTOZOTOCIN INDUCED DIABETIC RATS

In continuation of the studies on the nutraceutical ability of the millet seed coat polyphenols, investigations were undertaken towards the ameliorative properties of the millet SCM on the hyperglycemia as well as its associated complications such as dyslipidemia, AGE formation, kidney dysfunction and cataractogenesis by animal feeding trials. The albino rats (*Wistar*) were rendered

diabetic by i.p. injection of streptozotocin (35 mg/kg b.wt.). The experimental diet contained 20% of the millet SCM whereas, the control diet was corn starch based semi-synthetic diet. The diabetic animals were divided into two groups, for providing the control diet and the experimental diet. Parallely, two groups of healthy animals receiving the control as well as the experimental diets were also included in the experiment. The duration of the experiment was of six weeks. During the course of the experiment, the body weight and the excretion of urinary metabolites (glucose, urea, creatinine and protein) were monitored at weekly intervals whereas, the fasting blood glucose levels were determined at fortnightly intervals. At the end of the experimental period, slit lamp examination of eyes, the blood biochemical investigations, and the histological examination of the kidneys were carried out.

It was observed that, the urinary excretion of glucose (4.5 g/24 h), protein (15 g/24 h), urea (132 mg/24 h) and creatinine (18 mg/24 h) in the diabetic animals receiving the experimental diet (diabetic experimental, DE) were significantly lower than the corresponding group receiving the control diet (diabetic controls, DC). The glomerular filtration rates (GFR) in the former group of animals were lower than the latter group. The fasting hyperglycemia and other abnormalities in the serum protein, albumin, urea as well as creatinine and the hypercholesterolemia and hypertriglyceridemia observed in the DC group of animals were significantly lower in the DE group. Also a significantly lower LDL+VLDL cholesterol fraction and the atherogenic index (AI) but slightly higher HDL cholesterol levels were observed in the DE group compared to DC group. Mature cataract with significant lenticular opacity and corneal vascularization found in the DC group was almost absent as only immature subcapsular cataract with mild lenticular opacity was observed in the DE group. This was also substantiated by the lower levels of the aldose reductase (AR) activity in the eye lens of the DE group compared to that of DC group of animals. In addition, the AGE and HbA1c levels in the DE group were considerably lower compared to the DC group. The histological examination of the kidney sections of the DE group of animals showed normal glomerulus and tubules compared to shrunken glomerulus, tubular vacuolations, clarifications and significant mucopolysaccharide depositions observed in the DC group.

The animal feeding trials clearly brought out the ameliorative qualities of the millet SCM on the hyperglycemia and its associated complications in the streptozotocin (STZ) induced diabetic rats. These observations may be helpful in drawing a scientific reasoning for the common belief of better glycemic control with millet consuming population.

The relevant references cited in the thesis are compiled as BIBLIOGRAPHY in the alphabetical order.

SUMMARY AND CONCLUSIONS

Finger millet foods are known for their slow carbohydrate digestibility and high sustaining ability but, the scientific reasoning for these characteristic features of the millet are scanty. The millet contains complex carbohydrates and higher proportion of dietary fiber and several other phytochemicals with nutraceutical characteristics. The polyphenols are prominent among the millet phytochemicals. The polyphenols as well as the dietary fiber are concentrated in the millet seed coat matter (SCM) and because of this, it is hypothesized that, the SCM may exert hypoglycemic, hypocholesterolemic and antioxidant activities and contribute towards slowing down the carbohydrate digestibility of finger millet foods. Hence, to understand the role of the millet SCM on the carbohydrate digestibility of the millet, R&D work were undertaken and the salient features of the outcome of the studies are;

- The polyphenols of the millet kernels were localized by histochemical techniques and it was observed that, they are concentrated in the seed coat, aleurone layer and germ but are sparingly present in the endosperm cell walls. Accordingly the seed coat matter (SCM) rich in polyphenols (11.2%) and dietary fiber (48%) was prepared following a special milling protocol.
- The polyphenols of the SCM were isolated using acidic methanol solvent and characterized by HPLC, ESI-MS and 19 phenolic compounds belonging to simple phenols, phenolic acids, tannins, flavonoids and anthocyanins were identified.
- The carbohydrate digestibility of the whole meals from the native millet (NM), hydrothermally processed millet (HTM) and also the refined flour

(RF) and the decorticated millet (DM) determined in terms of glycemic index (GI) in normal and type II diabetic subjects indicated the beneficial role of the SCM on lowering the carbohydrate digestibility of the millet foods. The GI values of the NM, RF, HTM and DM were 69 ± 3, 100 ± 3, 101 ± 3 and 81 ± 5 in the normal and 73 ± 4, 104 ± 4, 107 ± 2 and 78 ± 6 in the type II diabetic subjects respectively. From these observations, it could be inferred that, nature of processing and also the form in which the food is consumed play a vital role in determining the GI of the foods.

- The mode of inhibition of pancreatic amylase and intestinal α-glucosidase by the millet seed coat polyphenols investigated by the enzyme kinetic studies revealed that, the K_m remains constant and V_{max} decreases in the presence of the polyphenols, indicating that the polyphenols are non-competitive type of inhibitors. The millet seed coat polyphenols exhibited higher inhibitory potency for both intestinal α-glucosidase (IC_{50} 16.9 µg) and pancreatic amylase (IC_{50} 23.5µg).
- The bioefficacy of the polyphenols as inhibitors of fructose induced protein (albumin) glycation (45 µg produced 24% restoration in tryptophan fluorescence and 40% inhibition in AGE formation) and also the hydroxyl radical ($OH^{\cdot}$) mediated protein fragmentation were ascertained in *in vitro* model systems. The higher DPPH· scavenging property observed for the polyphenols extract may also account for these nutraceutical properties of the millet polyphenols.
- The ameliorative qualities of the millet SCM on the hyperglycemia and its associated complications were examined in streptozotocin induced diabetic rat models. The diabetic animals maintained on the millet SCM (experimental) diet (DE group) was found to exhibit lower blood glucose, lipids, urea and creatinine levels compared to the diabetic animals maintained on the control/corn starch based semi-synthetic diet (DC group). The atherogenic index (AI), excretion of the urinary metabolites, glomerular filteration rate (GFR), AGE (advanced glycation end products), HbA1c levels and also the occurrence of mature cataract were relatively lower in the DE group compared to the DC group. The histological examination of the kidneys indicated normal glomerular and tubular

structures in the DE group compared to the shrunken glomerulus, tubular vacuolations and mucopolysaccharide depositions in the DC group of animals.

From the studies, it may be concluded that, the seed coat of the millet which is a rich source of polyphenols and dietary fibre imparts a considerable health beneficial effect with respect to slowing down the millet carbohydrate digestibility. This characteristic feature of the SCM may synergistically contribute with the complex nature of the millet carbohydrates to the ameliorating properties of the millet on hyperglycemia and its associated complications.

Since, the millet SCM is an edible matter, it has very high potential for its utilization in the development of antioxidant rich functional foods.

Synopsis

Introduction

Bibliography

Publications

CHAPTER I

Localization of polyphenols in finger millet kernel and characterization of the polyphenols isolated from the millet seed coat matter

CHAPTER II

Carbohydrate digestibility of finger millet as influenced by its seed coat matter and hydrothermal processing

CHAPTER III

Investigations on the nutraceutical ability of finger millet seed coat polyphenols

CHAPTER IV

Amelioration of hyperglycemia and its associated complications by finger millet seed coat matter in streptozotocin induced diabetic rats

Millets are minor cereals of the grass family, *Poaceae*. They are small seeded, annual cereal grasses, many of which are adapted to tropical and arid climates and are characterized by their ability to survive in less fertile soil. The most important cultivated species of millets in India are finger millet (*Eleusine coracana*), foxtail millet (*Setaria italica*), pearl or cat tail millet (*Pennisetum glaucum*), proso millet (*Panicum miliaceum*), Japanese/barnyard millet (*Echinochola crusgalli*), brown top millet (*Panicum ramosum*) and kodo or ditch millet (*Paspalum scrobiculatum*).

The annual global production of all the millets stands at 28-31 million metric tones from about 45 million hectare land. It is reported that, more than 400 million people in the world depend on millets for subsistence. They are important food crops of India, China, Japan, Ethiopia, Kenya, Tanzania, Zambia and few other African countries (Figure 1). The annual production of millets in India amounts to about 12 million metric tones and out of this pearl millet accounts to 60%, finger millet 25% and other small millets form 15%. Table 1 details the production of millets in different parts of the world.

Finger millet (*Eleusine coracana* L.) or Ragi (Figure 2), one of the important minor cereals, forms the staple food for a large segment of the population in the Indian subcontinent namely India, Nepal, Srilanka, and also in many of the African countries. About the origin of the millet, there are conflicting reports while, Werth (1937) was of opinion that, the millet originated in India and spread to Africa. Whereas Chandola (1959) opined that, it developed simultaneously in India and Africa. Since the mention of ragi has been made in Sanskrit literature, it is generally believed that, it is of Indian origin, however, the studies made by taxonomists and genetists suggest that, the millet is of African origin and latter migrated to India in pre-aryan times (Kennedy O'Byrne 1957). The millet is one of the oldest crops and referred as "Artta-kandaka" in the ancient Sanskrit literature, which means "Dancing grain".

Finger millet is cultivated in Karnataka, Tamil Nadu, Andhra Pradesh, Maharastra, Orissa, Uttar Pradesh and some parts of Uttarkhand, out of which Karnataka state is the largest producer of the millet followed by Tamil Nadu.

Figure 1. Millet producing countries in the world

Table 1. Top twenty countries producing millets (pearl, finger and other millets)

Country	Area (HA)	Yeild (Hg/Ha)	Production (MT)
India	11,000,000	8,182	9,000,000
Nigeria	NA	NA	7,694,000
Niger	NA	NA	2,500,000
China	1,070,420	18,225	1,950,800
Burkina Faso	1,500,000	8,096	1,214,419
Russian Federation	500,000	19,500	975,000
Mali	1,245,480	6,544	815,000
Sudan	2,440,000	2,541	620,000
Uganda	390,000	14,974	584,000
Senegal	820,000	5,488	450,000
Chad	706,935	6,083	430,000
Ethiopia	300,000	11,667	350,000
Nepal	NA	NA	288,000
Tanzania	250,000	10,800	270,000
United states of America	250,910	10,350	259,680
Pakistan	NA	NA	180,000
Myanmar	NA	NA	162,000
Ghana	198,000	7,575	150,000
Ukraine	200,000	6,200	124,000
Angola	200,000	5,000	100,000

Source: Food and Agriculture Organization of United Nations, http://faostat.fao.org

A

B

Figure 2. Finger millet: (A) ear head and (B) grains

It is known by different names in different regions of India and also in other countries (Table 2). The botanical classification of the millet is given in Table 3.

The millet kernel is an utricle and not a true caryopsis of globose shape, with a granulated surface texture and of 1.2 - 1.8 mm in diameter. Utricles are of yellow, white, tan, red, brown or violet, but red colored is commonly cultivated worldwide. It is characterized by a shallow depression at the germ portion and a characteristic protruding ridge around the depression. The pericarp is membranous and surrounds the entire seed, but it is not fused to the testa or seed coat. The major portion of the pericarp comes off during harvesting and can be easily removed by slight rubbing or soaking in water.

Morphology

The testa is slightly undulated on its surface, is multilayered (5 layers) and tightly bound to unilayered aleurone. The first layer of the testa is 1.5μm thick, is composed of sections of tissue that “interlocked’ like pieces of jigsaw puzzle. Each section is composed of 2 - 4 dimpled mounds. Beneath the first layer, lies the second thickest layer (5.5 - 17.5 μm thick) with striated look. The third and fourth layers are approximately 1.4 - 2.1 μm thick, with distinct wave like structures, predominantly straight wave patterns. The fifth layer being about 1 μm thick, differs distinctly in color from the previous layers. The aleurone layer is one cell layer thick tissue, and surrounds the entire endosperm, and is similar to the aleurone tissues in corn, sorghum and pearl millet. The cells being small (18×7.6 μm) are packed with aleurone bodies ranging from 0.9 - 2.2 μm in diameter (McDonough et al 1986).

Table 2. Common names of finger millet

Country/Language	Common name
Arabic	Tailabon
Chinese (Pinyin)	Cănzi
Denmark	Fingerhirse
English	Finger millet, African millet, Ragi, Koracan
Ethiopia	
Amharic/Sodo	Dagussa
Amharic	Tokuso
Oromo	Barankiya
French	Eleusine cultivee, Coracan, Koracan
German	Fingerhirse
India	
Kannada, Telugu	Ragi
Tamil	Kezhvaragu, Aariyam
Malayalam	Panjapule
North India	Maduva
Marathi	Nachani
Kenya	
Swahili	Wimbi
Dholuo	Kal
Kikuyu	Ugimbi
Nepal	Koddo
Srilanka	Kurakkan
Sudan	
Bari	Ceyut
Tanzania	Mbege (Swahili), Mwimbi, Wimbi, Ulezi
Uganda	Bulo
Vietnam	Hong mi, Chi ke
Zambia	Kambale, Lupoko, Mawele, Majolothi, Amale, Bule
Zimbabwe	Rapoko, Zviyo, Njera, Rukweza, Mazhovole, Uphoko, Poho

Source: http://www.answers.com/topic/finger-millet

Table 3. Botanical classification of finger millet

Kingdom	Plantae – Plants
Sub Kingdom	Tracheobionta – Vascular plants
Super division	Spermatophyta – Seed plants
Division	Mangnoliophyta – Flowering plants
Class	Liliopsida - Monocotyledons
Subclass	Commelinidae
Order	Cyperales
Family	Pocaceae – Grass family
Genus	*Eleusine* Gaertn, - Goose grass
Species	*Eleusine coracana* (L.) Gaertn. – Finger millet

Source: United States Department of Agriculture (USDA), National resources conservation service, Plants data base, http://plants.usda.gov

The endosperm cells are usually long and narrow in some areas, and short and thick in the other area. Simple granules are mostly packed in between compound granules. Thin patches of protein matrix is also present in the corneous endosperm cells. The floury endosperm contains the starch granules with little semblance of organization. The diameter of the compound starch granules ranged from 11 - 21 µm. Both compound and simple starch granules exhibit strongly birefringence (McDonough et al 1986).

Germ or embryo is located in a depression surrounded by a characteristic ridge, whereas, the hilum is situated just adjacent to the germ in a separate but somewhat shallower depression. The scutellum cells were of smooth round appearance with 25-35 µm diameter and the scutellum was separated from the floury endosperm by the scutellar epithelium. The protein bodies in the scutellum and the scutellar epithelium are visible as small spheres beneath the cell walls. The size of the protein bodies range from 1.5-6.0 µm in diameter (McDonough et al 1986). The schematic diagram of the millet kernel is presented in Figure 3.

Processing and utilization

In India, usually the millet is pulverized and the whole meal is utilized for the preparation of traditional foods, such as *roti* (unleavened breads or pancake), *mudde* (dumpling or stiff porridge) and *ambali* (thin porridge) (Figure 4). In addition to these traditional foods, the millet is also processed to prepare popped, malted, fermented products. The non-conventional products from the millet are papads, noodles, soup etc.,. A brief account of the nature of processing and the quality characteristics of the products are given below;

Milling: The millet grain invariably needs processing for food preparation. The most commonly practiced primary processing is pulverization or milling for preparation of flour. The millet is pulverized in stone, iron or emery coated disc mills and generally the whole meal is used for food preparation. Generally, the whole meal millet based foods are darker and unattractive.

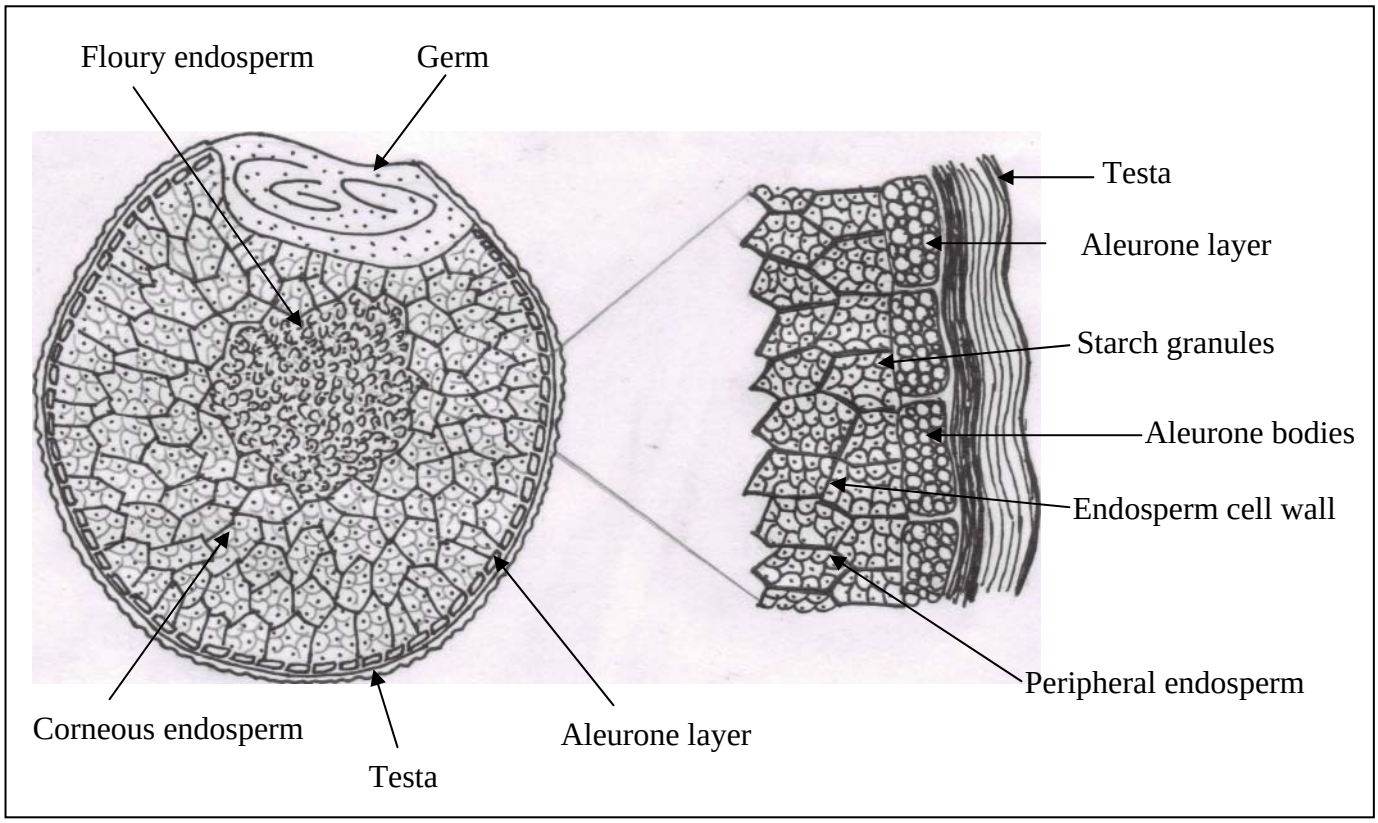


Figure 3. Schematic diagram of finger millet section

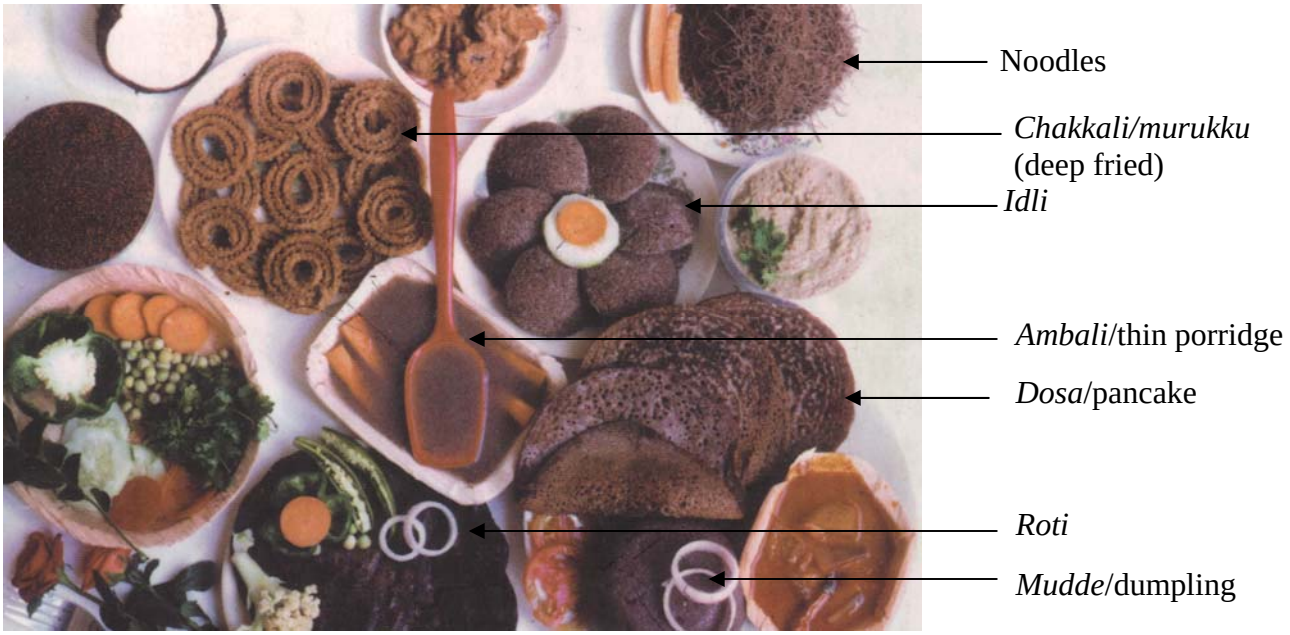


Figure 4. Prominent traditional foods from finger millet

Hence, to prepare refined flour that is largely free from seed coat matter (SCM), the grains are sprayed with 5 - 7% additional water, tempered for about 10 min, pulverized in any of the cereal pulverizers and sifted through about 85 mesh (B.S.S) sieve to separate the coarse seed coat as over tails (Malleshi and Desikachar 1981). The flour (refined flour) thus obtained is comparatively whiter and fairly free from the SCM. This fraction being a rich source of millet starch finds usage in bakery products, noodles and composite flour mixes. Whereas, the SCM being a rich source of edible phytochemicals, dietary fiber and minerals may form an adjunct in functional food formulations.

Decortication: This is a very recent process developed for the millet. The debranning or decortication methods followed for most of the cereals are not effective in the case of finger millet owing to the intactness of bran or the seed coat with highly soft and friable endosperm. Hence to decorticate, the millet is hydrothermally processed (hydration, steaming and drying) to harden the millet endosperm. The decorticated millet is cream colored and devoid of seed coat and could be cooked as discrete grains similar to rice (Malleshi 2006). The SCM forms the major by-product of the decortication process, is a rich source of health beneficial phenolic compounds, minerals and dietary fibre.

Malting: Among the various tropical cereals, finger millet has good malting characteristics. For malting, the millet is soaked in water for 8 - 12 h, germinated for 36 - 48 hrs and dried and kilned. The malt flour is a good source of amylases and hence termed as “Amylase rich food”. When the malt flour is heated in water to boiling, the amylases partially hydrolyze the starch to lower molecular weight carbohydrates such as oligo- and disaccharides and there by reducing the water holding capacity and thus the nutrient density of the food is increased. Due to its easily digestible nature of the carbohydrates, higher nutrient density, the refined millet malt flour has scope for its utilization in health foods for target population requiring easily digestible foods such as infants foods, weaning foods, enteral foods and geriatric foods (Malleshi 2002a), besides its utilization in the milk based beverages and confectionary.

The refined malt flour has also been utilized in composite flour mixes to improve the aroma and texture of the products. Thus the millet malt is likely to find usage as a new ingredient in the food industry.

Popping: It is one of the important processing techniques widely used to prepare ready-to-eat products, which involves high temperature short time (HTST) treatment for the millet using sand or salt as heat transfer media, where the millet endosperm bursts open. The popped millet possesses a desirable flavor and aroma. It is used as such or as a snack after seasoning with spice or after pulverizing (*hurihittu*). It is rich in major nutrients and dietary fiber and is mainly utilized in the preparation of nutritious food supplements for growing children. In recent days, the millet is popped using air popper also. Recently, expanded millet similar to that of rice *puri* has also been developed from the millet (Ushakumari et al 2007).

Now-a-days, the **noodles** made out of finger millet flour are gaining popularity. The process involves pretreatments (pre-gelatinization) to the millet flour to facilitate extrusion and retain the texture of noodles (to prevent fissuring and subsequent solid loss when cooked in water). **Papads** are flattened and dried dough products which are toasted and used as adjuncts with meal. Even though, the millet papads appear dark and less appealing, their expansion characteristics are very good and deep frying, toasting or microwaving turns the product to light colored product with good consumer acceptability. Roller drying of refined millet flour has been attempted successfully and the product may finds its application as base for baby foods and soup mixes (Guha and Malleshi 2006). Millet based bakery products such as bread, cookies and biscuits are also being prepared and marketed locally. The biscuits prepared by replacing 20% of the biscuit flour with millet seed coat matter have also been successfully attempted.

The millet flour has versatility and could be used for the preparation of traditional and contemporary food products that are prepared from rice and wheat. Now-a-days due to increased awareness on the health benefits of the millet, its nutritional advantages are overtaking the drawbacks associated with the dark color of its products.

Nutrient composition of finger millet

The millet contains about 5 - 8 % protein, 1 – 2 % ether extractives, 65 – 75 % carbohydrates, 15 – 20 % dietary fibre and 2.5 – 3.5 % minerals. Its is a very good source of dietary fibre, micronutrients and phytochemicals such as, polyphenols, pigments and phytates (Hulse et al 1980). The sulfur based amino acids (0.35mg/g protein) content of the millet protein is much higher, compared to other cereal proteins and happens to be a good source of tryptophan also. Prolamins are the major fractions of the millet protein and similar to other cereal proteins, lysine, one of the essential amino acids, forms the limiting amino acids of its protein. The leucine/isoleucine quotient of the millet proteins being about 2 is almost equivalent to rice and wheat. Albumins and globulins constitute 8 - 15% whereas, prolamins and glutelin like proteins form 12 - 18% of the proteins in the millet (Hulse et al 1980).

The millet is a poor source of lipids (1-2%). Oleic (49%), linoleic (25%) and palmitic (25%) acids are the predominant fatty acids. Major component of the free lipids are triglycerides (Mahadevappa and Raina 1978). The lower lipid contents could be one of the contributing factors for the extremely good storage properties of the millet. The detailed nutrient, vitamin, mineral and essential amino acid composition of the millet is given in Table 4.

The millet carbohydrates comprise of free sugars (1 - 2%), starch (75 - 80%) and non-starchy polysaccharides (15 - 20%). Glucose, fructose, maltose and sucrose are the main constituents of free sugars. The non-starchy polysaccharides (NSP) largely consists of cellulose and hemicellulose. The NSP forms the major component of its dietary fiber (Table 5). The cellulose contributes towards the major part of insoluble fiber whereas, the hemicellulose forms soluble fiber. The ratio of pentoses to hexoses in the millet NSP is 1.5 to 1, whereas, the ratio of arabinose to xylose is 1:1 (Malleshi et al 1986). Since, the dietary fiber exerts several physiological benefits such as binding of toxic components, ease of bowel movement and removal of low density lipoproteins, consumption of the millet helps in lowering the cholesterol formation and also contributes towards slow digestibility of millet carbohydrates or in other words, imparts the hypoglycemic and hypocholesterolemic qualities to the millet.

Table 4. Nutrients, vitamins, essential amino acid and minerals composition of finger millet

Nutrients (g/100 g)	Content	Minerals (mg/100 g)	Content
Protein	7.3	Calcium	344
Carbohydrates	72	Phosphorus	283
Fat	1.3	Copper	1.0
Diet ary fiber	18.8	Magnesium	173
Ash	2.7	Manganese	1.7
Vitamins (mg/100 g)		Molybdenum	0.01
Carotene	0.04	Aluminum	0.4
Riboflavin	0.19	Barium	2.2
Niacin	1.1	Beryllium	<0.05
Choline	16.9	Bismuth	<0.05
Folic acid	18.3	Boron	0.05
Essential amino acids (g/100 g protein)		Cobalt	0.01
Isoleusine	4.4	Chromium	0.02
Leusine	9.5	Gallium	<0.01
Lysine	2.9	Potassium	300
Methionine	3.1	Nickel	0.02
Cystine	2.2	Lead	0.6
Phenyl alanine	5.2	Rubidium	0.2
Threonine	3.8	Sulfur	122
Tryptophan	1.6	Tin	0.006
Valine	6.6	Strontium	3.3
		Titanium	0.03
		Vanadium	0.04
		Lithium	0.2
		Zinc	1.5

Table 5. Free sugars, starch and non-starchy polysaccharide (NSP) content of finger millet

Carbohydrate fraction	Content (g%)	Carbohydrate constituents (%)
Free sugars	1.04	Glucose (18.3), fructose (13.5), maltose (3.9), sucrose (63.5), raffinose (0.96)
Starch	75	Amylopectin (82), amylose (18)
NSP		
WSNSP*	1.05	Pentoses (63.5), hexoses (27.1), uronic acid (9.4)
Hemicellulose- A	2.3	Pentoses (60.2), hexoses (36.8), uronic acid (3.0)
Hemicellulose- B	2.7	Pentoses (64), hexoses (33.1), uronic acid (2.3)
Cellulose Type	6.1	Pentoses (26.1), hexoses (72.1), uronic acid (1.8)

* Water soluble non-starchy polysaccharide

Adapted from: Malleshi et al (1986)

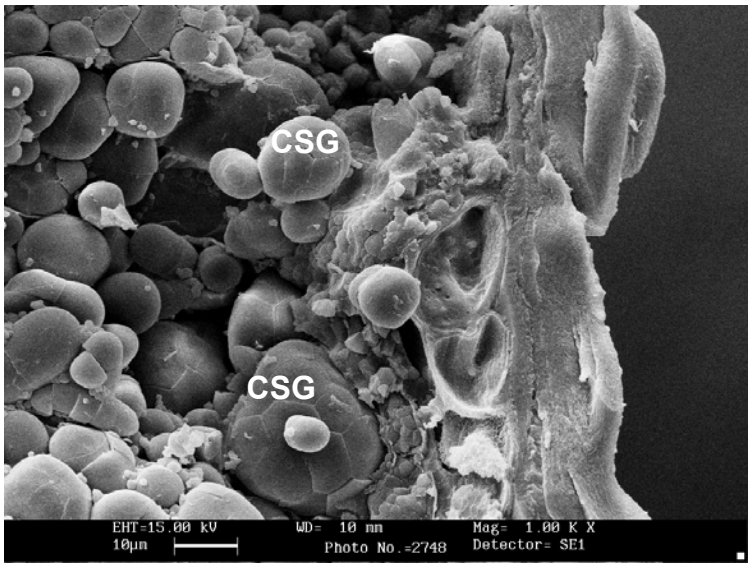
The millet starch comprises of amylose and amylopectin, normally present in the ratio of 25:75, which is comparable to the Indian rice varieties and other cereals. There are no reports of very low or very high amylose containing millet cultivars. The millet endosperm contains both compound (Figure 5a) and simple starch granules and the compound granules were 8-16.9 µm in diameter (McDonough et al 1986). The starch granules in the millet are generally hexagonal, diamond shaped (Figure 5b) and typically different from wheat and rice starch granules. Most of the granules are compacted in the cells in the corneous endosperm whereas, the granules present in the floury endosperm are loosely packed. Mohan et al (2005) reported a higher degree of crystallinity for the millet starch (30.09%) compared to that of rice starch (21.69%). The authors also indicated that, the quantum of heat flow recorded by DSC (differential scanning calorimetry) in terms of energy to gelatinize the millet starch was nearly 30% higher for the millet compared to that of rice. Moreover, the molecular weight of the human salivary amylase digests of the millet starch were >500 KDa compared to 415 KDa of the rice starch digest which clearly indicate the higher degree of crystallinity and lesser susceptibility characteristics of the millet starch to the digestive enzymes. Similar observations were also reported by Singh and Ali, (2006) who indicated that, among the cereal starches such as rice, wheat, maize, sorghum and finger millet, the millet starch was the most resistant to the enzymatic hydrolysis by fungal α-amylase and suggested that, the granular form of starch in addition to the molecular architecture of the millet starch may contribute to their lesser enzymatic susceptibility characteristics.

The nature of carbohydrates in processed foods and the influence of processing on the *in vitro* and *in vivo* carbohydrate digestibility are dealt subsequently.

Nature of finger millet phenolics

Phenolic compounds or polyphenols constitute one of the most numerous and widely distributed groups of substances in the plant kingdom. They are products of secondary metabolism in plants. They arise biogenetically from two main synthetic pathways: the shikimate pathway and the acetate pathway. Plants may contain simple phenols, phenolic acids, coumarins, flavonoids, stilbenes, hydrolysable and

A



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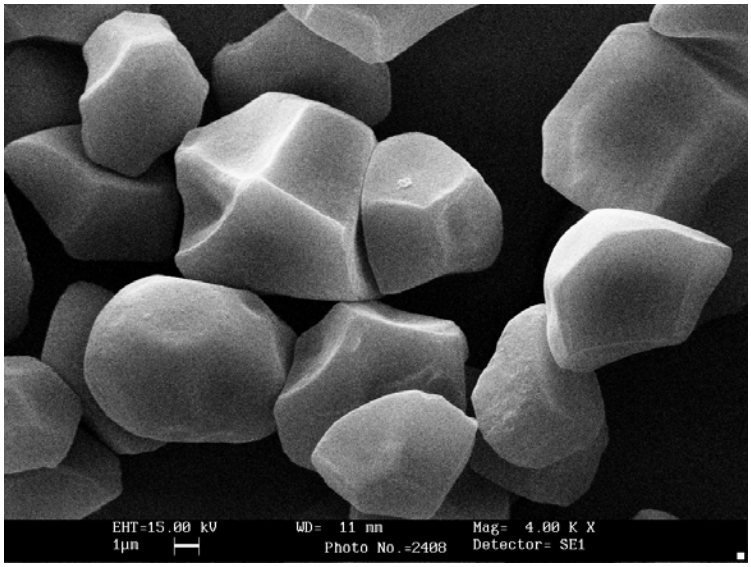


Figure 5. Scanning electron photomicrographs of finger millet starch

A. Compound starch granules (CSG) in the peripheral endosperm (1.00 KX)
B. Starch granules in the starch isolate (4.00 KX)

condensed tannins, lignans and lignins. Phenolic acids and flavonoids are present in cereals in free and conjugated forms. The highest concentration of the phenolic acid and flavonoids are present in the bran, aleurone and germ of cereal grains. The millet is a very good source of polyphenols but very little information on the subject is available. Similar to many other cereals and legumes, varietal variations with respect to the polyphenol content of finger millet has been reported. Ramachandra et al (1977) analyzed 32 varieties of the millet comprising of both brown and white seed coat material of Indian and African source and reported that, the white varieties contained lower levels of polyphenols (0.04 – 0.09 %) than the brown varieties (0.08 – 3.47 %). The total polyphenol (as chlorogenic acid equivalents) and tannins (as catechin equivalents) contents of the Indian varieties ranged from 0.08 – 0.96% and 0.04 – 1.05% respectively whereas, in the African varieties the corresponding values were 0.54 – 2.44 and 0.5 – 3.47% respectively.

Rao and Prabhavathi (1982) in an unspecified variety of finger millet reported 0.36% tannin (catechin equivalents) whereas, McDonough et al (1986) in 3 millet varieties recorded 0.55 - 0.59 % total polyphenols and 0.17 - 0.32 % tannins (catechin equivalent) and Shankara (1991) analyzed a large number of finger millet varieties (n = 85) from the Indian state of Karnataka, and reported a wide variability in the total polyphenol contents assayed as chlorogenic acid (0.06 – 0.67 %), tannic acid (0.03 – 0.57 %) and catechin (0.03 – 2.37 %) equivalents. According to Sripriya et al (1996), the total polyphenol contents of a brown variety of the millet (0.1 %) was higher than the white variety (0.003 %). Very recently Chethan and Malleshi (2007a) analyzed five brown and two white varieties and reported 1.3 – 2.3 % and 0.3 – 0.5 % polyphenols as gallic acid equivalents respectively in brown and white varieties.

This information on the polyphenol contents of the millet gives an indication that, considerable variations exists among different genotypes of the millet. However, the values have to be taken on their face value, because the method of extraction, as well as the method of assay and also the standards used vary considerably among different reports.

Hilu et al (1978) characterized the millet flavonoids by HPLC and identified orientin, isoorientin, isovitexin, saponarin, violanthin, leucenin-1 and tricin.

McDonough et al (1986) identified ferulic (405 µg/g), coumaric (67 µg/g), gentisic (53 µg/g), cinnamic (35 µg/g), caffeic (15 µg/g), vanillic (15 µg/g), protocatechuic (14 µg/g), *p*-hydroxy benzoic (9 µg/g), syringic (7 µg/g) and sinapic (4 µg/g) as component phenolics in the millet. Subba Rao and Muralikrishna (2002) reported that gallic, protocatechuic and vanillic acids are present in millet only in the free form whereas, caffeic, coumaric and ferulic acids exists both in the free as well as bound forms. Very recently, Chethan and Malleshi (2007b), screened a few polar and non-polar solvents for extraction of the millet polyphenols and found that 1% HCl methanol was very effective extractant. Accordingly, they extracted the polyphenols of the millet seed coat in HCl methanol and characterized the phenolics by HPLC. They identified nine phenolic compounds namely, the benzoic acid derivatives (gallic acid, protocatechuic acid, p-hydroxybenzoic acid, vanillic acid, syringic acid), and cinnamic acid derivatives (ferulic acid, trans-cinnamic acid, *p*-coumaric acid) including a flavonoid compound namely, quercitin. They also indicated that, benzoic acid derivatives accounted for about 85% of the total phenolic compounds. They also observed that, the millet polyphenols irrespective of the solvent used for extraction, appear to be colored and the color varies from pink to dark red. The polyphenols contents of the millet in the extract (6.4±1.0%) were pH sensitive, and remained constant from highly acidic to near neutral pH (6.5) but highly unstable in the alkaline pH range (the polyphenol content dropped to 2.5±0.3% at pH 10). The constituent phenolics in the acidic pH were gallic acid, proto-catechuic acid, *p*-hydroxy benzoic acid, *p*-coumaric acid, syringic acid, ferulic acid, trans-cinnamic acid and quercitin. Whereas, only gallic acid and proto-catechuic acids were detected in a highly alkaline condition (pH 10). The stability of the phenolics in acidic and alkaline conditions is reversible to a large extent. However, very little information is available on the mechanism of the stability and also the structural changes of the polyphenols in different acidic and alkaline pH. The detailed study on this aspect would be very useful towards understanding the chemistry and bioavailability of the millet polyphenols.

Health benefits of finger millet

The millet is known for several health benefits, and the information reported on the subject is listed in Table 6. Some of the known health benefits of the millet, could be attributed to its polyphenol contents because of their hypoglycemic or the anti-diabetic properties. The information on the health benefits of the millet and the possible role of the polyphenols towards imparting the health benefits are dealt here in detail.

Diabetes mellitus

Diabetes mellitus is characterized by chronic hyperglycemia and disturbances in carbohydrate, fat and protein metabolism resulting from the defects in insulin secretion as well as defective insulin action or both. When fully expressed, diabetes is characterized by fasting hyperglycemia, but the disorder can also be recognized during less overt stages, usually by the presence of glucose intolerance. The adversities of diabetes include long term damage, dysfunction and failure of various vital organs such as eyes, kidneys, heart and blood vessels. The characteristic symptoms of diabetes are thirst, polyuria, blurring of vision, weight loss and polyphagia. In the case of severe diabetes, ketoacidosis or non-ketotic hyperosmolarity occur, which in the absence of effective treatment lead to stupor, coma and mortality (Bennett and Knowler 2005). The etiological classification of diabetes is given in Table 7.

Prevalence of diabetes worldwide and in India: Worldwide, 2.8% of the total population are diabetic (Wild et al 2004). The diabetic population in India in 2004 was around 66.58 million out of which, 37.73 million were in the urban areas and 28.85 million in the rural areas. It has been well documented that, diabetes is directly responsible for 9% of AMI (acute myocardial infarction) cases, 4% of stroke cases, 2% of neuropathy, and 32% of cataract cases (www.whoindia.org/SCN/AssBOD/06-diabetic.pdf). The global prevalence of diabetes is given in Figure 6.

Table 6. Some of the known health beneficial properties of finger millet

Property	References
• Cholesterol lowering (*in vivo*)	Pore and Magar (1976), Hegde et al (2005a)
• Anti-ulcerative (*in vivo*)	Tovey (1994)
• Free radical scavenging (*in vitro*)	Sripriya et al (1996), Subba Rao and Muralikrishna (2002), Hegde and Chandra (2005)
• Anti-microbial (*in vitro*)	Antony et al (1998), Chethan and Malleshi (2007a), Varsha et al (2009)
• Anti-cataractogenesis (*in vitro*, eye lens aldose reductase inhibition)	Chethan et al (2008)
• Inhibition of phospholipases A_2 from snake venom (*in vitro*)	Chethan (2008)
• Inhibition of intestinal α-glucosidase and pancreatic amylase (*in vitro*)	Shobana et al (2009)
• Anti-collagen glycation (*in vitro* and *in vivo*)	Hegde et al (2002), Hegde et al (2005a)
• Blood glucose lowering property (*in vivo*)	Hegde et al (2005a)
• Wound healing property (*in vivo*)	Rajasekaran et al (2004), Hegde et al (2005b)
• Lower glycemic index values for millet diets (*in vivo*)	Ramanathan and Gopalan (1957), Geetha et al (1990), Lakshmi kumari and Sumathi (2002)

Table 7. Etiological classification of diabetes

S. No	Etiological classification of diabetes
1.	Type I or IDDM (β – cell destruction, usually leading to absolute insulin deficiency) A. Autoimmune B. Idiopathic
2.	Type II or NIDDM (may range from predominantly insulin resistance with relative insulin deficiency to a predominantly secretory defect with or without insulin resistance)
3.	Other specific types • Genetic defects of β-cell function • Genetic defects of insulin action • Diseases of the exocrine pancreas • Endocrinopathies • Drug or chemical induced • Infections • Uncommon forms of immune mediated diabetes • Other genetic syndromes sometimes associated with diabetes
4.	Gestational diabetes

Source: Bennett and Knowler (2005)

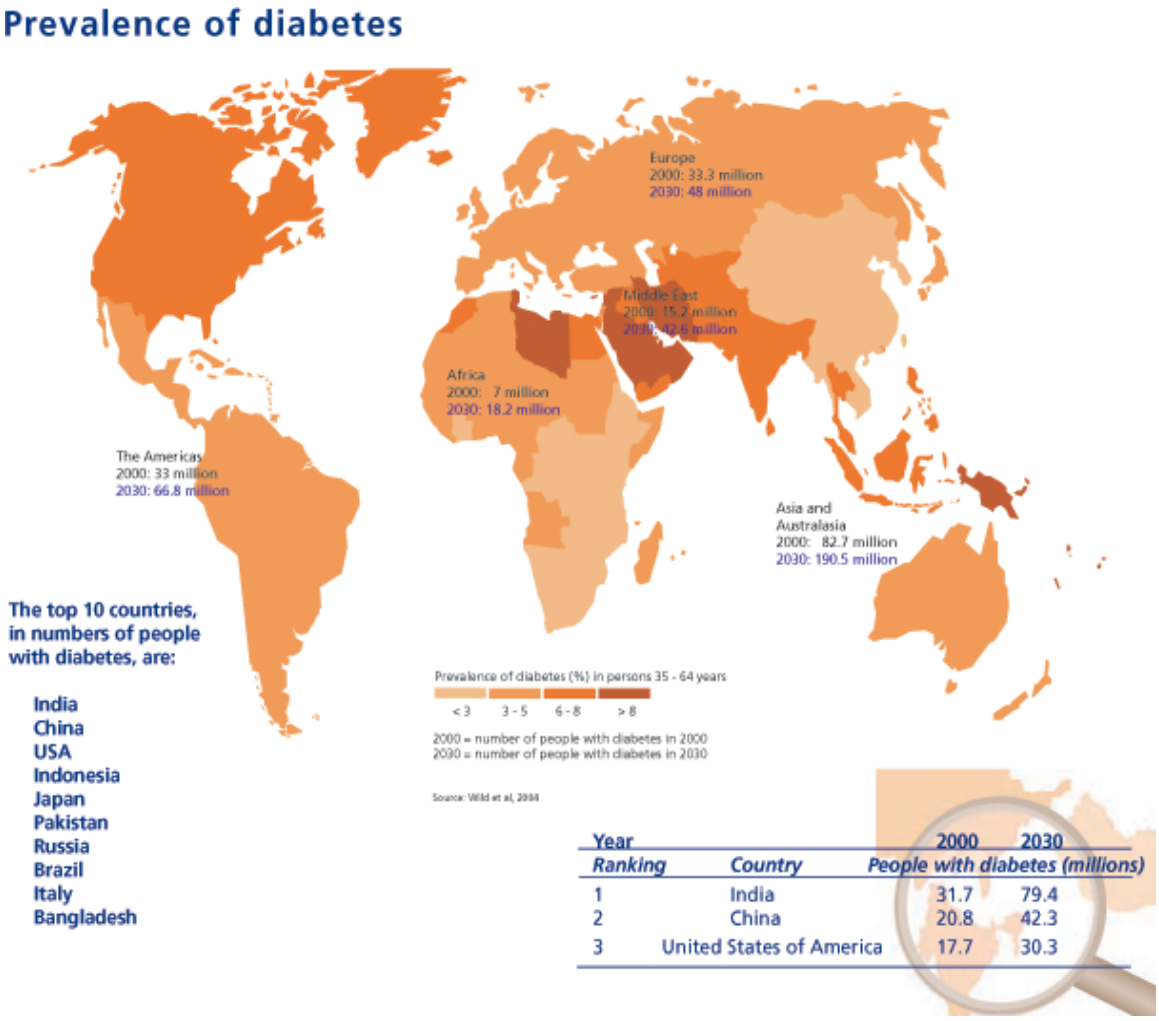


Figure 6. Global prevalence of diabetes

Source: www.who.int/diabetes/actionnow/en/mapdiabprev.pdf

Insulin treatment is very much essential for the clinical management of IDDM subjects, however, foods containing phenolics and other nutraceuticals with insulin-like properties and insulin secretogogue properties such as found in bitter gourd and cinnamon (Yeh et al 2003) may be recommended for IDDM subjects. But however, reports of cereal polyphenols and their role to this effect are scanty.

The deficiency in insulin action results in hyperglycemia and other metabolic disturbances in NIDDM. It is frequently asymptomatic, but may present with classical symptoms such as polyuria, polyphagia, pruritus and weight loss but these usually appear only after a long asymptomatic period as a result, other complications of diabetes such as retinopathy, nephropathy, artherosclerotic heart disease or neuropathy may be the first clinical indications of the disease.The subject of dietary intervention in minimizing the progression of the complications of NIDDM, and also its clinical management are well studied. Among the various dietary constituents, the importance of complex carbohydrates, dietary fiber and the nutraceuticals such as polyphenols, which are known to inhibit digestive enzymes and reduce the absorption of glucose in the small intestine, or help in insulin signaling, or act as antioxidants are well documented (Scalbert et al 2005).

Since finger millet is a good source of complex carbohydrates, dietary fiber, polyphenols and micronutrients, the role of the millet diet in the prevention as well as management of diabetes deserves intense investigations. However, the millet being a minor cereal and localized in certain parts of the globe, the scientific information on the finger millet, with respect to their anti-diabetic properties have been studied to a limited extent.

In the normal process of metabolism, the digestible carbohydrates are converted into simpler form viz glucose which is absorbed by the intestine and pass on to liver through blood circulation where the glucose is converted to glycogen. Thus, in well controlled metabolism, this glycogenic function of the liver keeps the blood sugar (glucose) at a nearly constant level 80-100 mg/dl. The chief and most obvious defect in diabetes is that, the liver would have lost the power of oxidation of glucose there by increasing the blood glucose levels (hyperglycemia). When sugar levels raise above the renal threshold levels i.e., 180 mg/dl, it appears in the urine,

the first and most obvious sign pointing to diabetes. Increased blood glucose levels induce transition metal catalyzed oxidation. This also results in the generation of H_2O_2, reactive oxidants equivalent to reactivity of the hydroxyl radical and protein reactive dicarbonyl compounds. The hyperglycemia also leads to the formation of sorbitol from glucose catalyzed by aldose reductase in tissues of diabetic subjects and results in the development of diabetic complications (Ahmed 2005). The antioxidant property of the millet polyphenols are well established and hence, the millet polyphenols may prevent hyperglycemia induced oxidation reactions.

In diabetic condition, due to the non-availability of glucose for energy formation, the proteins are broken down in excess, causing decrease in serum protein levels. Moreover in diabetic nephropathy, large amounts of protein (albumin) are excreted (albuminuria). Besides this, hyperglycemia causes structural modifications in proteins to form AGEs (advanced glycation end products). The anti-protein (collagen) glycation property of the millet is reported earlier and this property may be attributed to the polyphenol contents of the millet as polyphenols posses protein glycation inhibitory property, hence the millet polyphenols may prevent hyperglycemia triggered protein glycation reactions.

Fats are broken down into fatty acids and glycerol during digestion but reformed for storage after absorption. Their metabolism is more like that of carbohydrate than of protein and they are eventually used up by complete oxidation to CO_2 and H_2O. Due to the impairment of carbohydrate metabolism in diabetes, the dependence is more on fat for the energy requirements. Because of this increase in fat combustion, excessive ketone bodies are produced that ultimately leads to ketoacidosis. One of the most prominent features of insulin deficiency is rapid mobilization of fatty acids from adipose tissue. The major metabolic disturbance in diabetes relates to the handling of free fatty acids (FFA). The effect of this increased production of FFA increases the production of VLDL which accounts for hypertriglyceridemia, the major disturbance of lipids found in poorly controlled diabetes irrespective of type I or type II. Triglycerides, LDL, VLDL are elevated whereas HDL decrease in diabetic condition. LDL is the most important lipoprotein related to the development of atherosclerosis. Hence diabetic patients with

lipoprotein abnormalities have more tendency to develop atherosclerosis when compared to diabetics with normal lipid profiles. The millet is a rich source of dietary fiber and polyphenols. The hypocholesterolemic property of dietary fiber is well established, moreover polyphenols are known for their HMG-CoA reductase (key enzyme involved in cholesterol synthesis) inhibitory properties and hence may lower cholesterol formation. Hence intake of the millet may help in the management of dyslipidemia found in diabetic condition.

Several secondary complications such as neuropathy, retinopathy, nephropathy, gangrene and heart diseases occur as a consequence of persistent hyperglycemia in long term diabetes mellitus. These complications develop as a consequence of non-enzymatic glycosylation and hardening of vital tissue proteins, thickening of blood vessels and accumulation of sorbitol, the reduced product of glucose in nerves and cells causing osmotic imbalance. Under normal physiological conditions, bulk of glucose is metabolized through glycolytic pathway and the pentose shunt. When hyperglycemia occurs, an increased amount of glucose is converted to sorbitol by aldose reductase (AR) enzyme via polyol pathway. Sorbitol formed in this pathway accumulate in cells and disturbs osmotic homeostasis. Intra lenticular accumulation of sorbitol has been suggested as the main causative factor for formation of sugar cataract. There exists evidence with respect to the anti-protein glycation and anti-cataract (millet polyphenols are potential inhibitors of aldose reductase) properties of the millet polyphenols (Table 6). Hence the millet based diets may benefit diabetics in minimizing the hyperglycemia induced protein glycation reactions, AGE formation and cataractogenesis that arise in the diabetic condition.

Carbohydrate digestibility

Renewed interest in recent years towards carbohydrates in the dietary management of diabetes mellitus has spurned a new area of research mainly directed towards measuring differences in plasma glucose and insulin response to various carbohydrate containing foods. There is a good deal of current interest in the complex carbohydrates which are remerging as an important dietary component for the general population. It was assumed that, simple sugars are being rapidly absorbed inducing a higher glycemic response than complex carbohydrates like

starch. The carbohydrate digestibility of the foods is normally assessed in terms of glycemic response (GR) or glycemic index (GI). The Glycemic Index (GI) is a scale that ranks carbohydrate-rich foods by their ability to raise blood glucose levels when ingested compared to a standard food (glucose or white bread). Based on GI, foods are classified as low GI ($\leq$ 55), intermediate GI (56 - 69) and high GI ($\geq$ 70). The possible role of high GL foods in the development of diabetes are given in Figure 7.

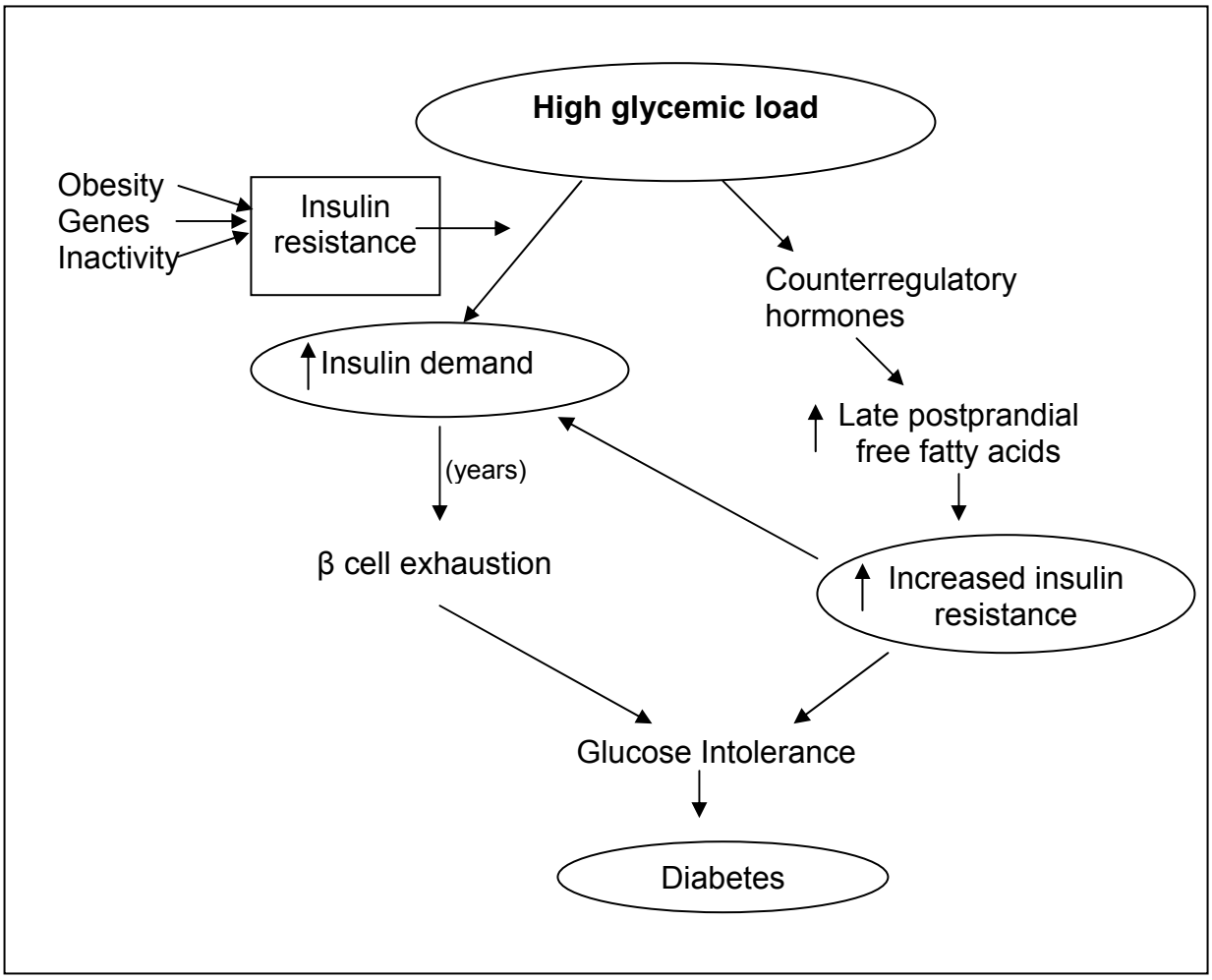


Figure 7. Possible role of high glycemic load diets in the development of type II diabetes

Source: Willett et al (2002)

Recently, it has been realized that starches derived from various foods are not handled identically in the gastro-intestinal tract. Large differences have been found to exist in the degree to which different starch containing foods affect the blood glucose levels of both normal subjects and diabetics. These differences appear to relate to the digestibility of starch. Infact many factors have been identified to affect starch digestibility and ultimately the GR. Research studies have revealed that, chemical form of starch and its food source can influence its rate of digestion. In addition, structural changes due to storage, processing (including cooking), chemical modifications and the gastro-intestinal effects like gastric emptying time are known to affect the digestibility and thus the postprandial glucose levels. Some of the important cereal processing methodologies and other factors that influence the carbohydrate digestibility is discussed in length in the subsequent pages.

Factors affecting carbohydrate digestibility

The carbohydrate digestibility of a food is reported to be affected by a number of factors namely, nature and source of the carbohydrates, processing, dietary fiber content, presence of amylase inhibitors such as polyphenols and phytate etc., These factors are discussed in detail in the subsequent lines.

Normally high amylose starch has been shown to be digested more slowly than high amylopectin starch (Goddard et al 1984). Among the cereals, rice carbohydrates are known to be easily digested compared to others, besides the varietal variations, the agro-climatic conditions of harvest as well as the type of rice namely, normal white, red, black, aromatic etc., also influence the carbohydrate digestibility. A marginal decrease of glycemic response in high amylose barley was reported by Granfeldt et al (1994). The apparent difference in the glycemic response of amylose and amylopectin starches has been attributed to the larger surface area per molecule of amylopectin. In addition, it is reported that glucose chains of amylose starch are more strongly bound to each other by hydrogen bonds. Thus making them less available for amylolytic attack than amylopectin, which has many branched chains of glucose.

The starch in general, both amylose and amylopectin in starch granules undergo transition due to food processing.

Processing as well as the physical form of the food influences the carbohydrate digestibility. The form in which the starch exists in the food and also the particle size and surface area to starch ratio are also important factors in determining the availability of starch to the hydrolytic enzymes. This has been demonstrated clearly for both raw and cooked cereals (Snow and O'Dea 1981). The method of cooking of cereal flours namely gelatinizing, freezing, thawing and repetition of this cycle enhanced the resistant starch (RS) content (Mangala et al 1999). Cooking–cooling cycles are known to reduce the GI of rice (Guha and Malleshi 2007). The digestibility of starch is largely influenced by the disruption of starch granular structure which may lead to the release of the starchy matter from the protein and cellular matrix thereby increasing its availability to the digestive enzymes of the GI tract. Apart from these, environment *in situ* or by incorporation of external constituents such as monosaccharides, lipids, gelling fibers such as guar gum, organic acids such as acetic acids, polyphenols, phytates and other amylase inhibitors largely influence the carbohydrate digestibility which may be favorable or otherwise (Brouns et al 2005).

All the food processes which introduce modifications in the starch such as those which produce obvious hydration of the granules, changes in chemical nature, disruption of the organized granule structure have been found to alter digestibility. By grinding, rolling, milling, pressing or even thoroughly chewing a kernel or other starchy foods can disrupt the starch granules (Granfeldt and Bjorck 1991). Chemically modifying a food also affects its GI. For instance, 1-2% acetylated potato starch decreases the GI as does the addition of β cyclodextrins to stabilize the carbohydrate. Gelatinization (irreversible loss of the crystalline regions in starch that occur upon heating in the presence of water) dramatically increases the availability of the starch for digestion by amylolytic enzymes thus increasing the GI. Retrogradation of starch increases the resistant starch content and decreases the carbohydrate digestibility. Parboiling of rice facilitates amylose to retrograde and or to form inclusion complexes with polar lipids there by lowers its carbohydrate digestibility or GI and in addition, alters the cooking properties of rice (Unnikrishnan and Bhattacharya 1986, Wolever et al 1986). Similarly lower GI was reported for

parboiled and parboiled-quick cooking rice compared to polished rice (Casiraghi et al 1993).

Extrusion cooking in general is known to decrease the carbohydrate digestibility. Lower GI has been reported for high amylose rice noodles and the lower GI was attributed to the retrogradation of starch (Panlasigui et al 1992). However severe extrusion conditions produced a higher blood glucose and insulin response compared to the samples prepared by boiling and mild extrusion cooking conditions (Bjorck et al 1984).

The lower peak blood glucose levels and GI of starch in pasta products such as macaroni, sphagetti and sphagetti porridge (cooked spaghetti mixed in a food processor) was reported by (Granfeldt and Bjorck 1991). They attributed the 'lente' properties of the pasta and compact food structure to restricted enzyme availability. Thus processing conditions and the particle size of foods play a critical role in the carbohydrate digestibility.

Higher rate of starch digestion and higher plasma glucose levels for gelatinized foods have been indicated in the literature (Holm et al 1988). A higher glycemic response for drum dried flour compared to incompletely gelatinized steam flaked and dry autoclaved whole grain wheat products (Holm et al 1989) are indicative of the role of gelatinization in modulating the carbohydrate digestibility rate. Whereas, flaking and expansion is known to increase the GI. Formation of resistant starch during processing has also been reported by several workers and the subject has been reviewed extensively (Sajilata et al 2006).

While several processing methods enhance the resistant starch there by lowering the *in vivo* carbohydrate digestibility, some of the processes such as malting, fermentation, micronizing increase the carbohydrate digestibility as during these processes the enzymes developed partially hydrolyze the starch or cause extensive mechanical damage to the starch during the various unit operations followed to prepare these products (Lakshmi kumari and Sumathi 2002).

Whole grains are rich sources of fermentable carbohydrates such as **dietary fibre**. The dietary fiber in the foods exists in soluble and insoluble forms. Soluble fibers are reported to coat the starch molecules and are known to form a barrier and

reduce its availability for the amylolytic enzymes. Current evidence suggests that a generous intake of fibre lowers fasting, postprandial plasma glucose, insulin levels and reduces hyperlipidemia among patients with type II diabetes (McIntosh and Miller 2000). The low glycemic response of high fibre meals was thought to be due to the reduced rate of gastric emptying and small intestine absorption. This effect was more marked with isolated viscous fibers like guar or pectin or both in healthy volunteers and diabetics. It is emphasized that timing of ingestion of the fibre is important as fibre in one meal appears to improve glucose tolerance to the next meal (second meal effect).

Amylase inhibitors are present in many raw foods including legumes, wheat and finger millet. In the case of some of the cereals such as finger millet, presence of heat stable amylase inhibitors have been reported (Chandrasekher et al 1981). Large amounts of purified amylase inhibitors have dramatic effects in reducing postprandial glycemic and insulinemic responses and may induce carbohydrate malabsorption. This beneficial effect of amylase inhibitors are utilized in the development of slow digesting carbohydrate foods by incorporating purified amylase inhibitors.

Phytate, myo-inositol hexakis phosphate is found in mature legumes and cereal grains. The term phytin implies a calcium magnesium salt of the phytic acid, whereas, phytate would mean the mono-dodeca anion of phytic acid. In legumes, phyate is uniformly distributed and associated with protein but, in cereal grains it is present in both the bran and the germ (Maga 1982). The phytate is absorbed and metabolized in the GI tract (Sandberg et al 1987; Sakamoto et al 1993). Phytate is a powerful chelator of metal ions. Three mechanisms have been proposed recently by which phytate could inhibit amylases and modulate carbohydrate digestibility. Amylases are calcium dependent enzymes whose activity could be reduced by limiting the availability of calcium due to binding of calcium to phytate, or by binding of phytate to the enzyme bound calcium. Alternatively, phytate could bind to starch by hydrogen bonding, or phytate could bind to the protein portion of a starch-protein complex, may reduce the ability of starch to reach the active site of amylase (Thompson 1986). Reports indicate that, inositol hexa phosphate inhibits serine/threonine protein phosphatases type I (PP1), type 2A (PP2A) and type-3

(PP3) in a concentration dependent manner and controls the activity of the voltage gated L-type Ca^{2+} channels in the insulin secreting cells and regulates the Ca^{2+} influx and there by primes the Ca^{2+} induced insulin exocytosis (Efanov et al 1997).

Polyphenols are powerful inhibitors of amylases and α-glucosidases (key carbohydrate digesting enzymes) and hence reduces carbohydrate digestibility. Several phenolic compounds have been studied for their amylase and alpha glucosidase inhibitory properties (Tadera et al 2006). An inverse relationship has been reported with polyphenol ingestion and glycemic response (Thompson et al 1983).

Cereal polyphenols

Polyphenols are essential compounds with benzene ring with one or more hydroxyl groups. Phenolic acids, flavonoids, condensed tannins, coumarins and alkyl resorcinols are the examples. The phenolic compounds belonging to different classes have their own characteristic health benefits such as antioxidant, anti-carcinogenic, hypocholestrolemic, anti-diabetic and several other health benefits and each one of the constituent group of the phenolics present in the cereals and the health benefits associated with them are discussed in length in the subsequent pages.

Several phenolic compounds reported in fruits and vegetables have also been identified in cereals. However, some of the phenolic compounds are unique to cereals such as avenanthramides in oats.

There are two classes of cereal **phenolic acids** namely, hydroxybenzoic acids and hydroxycinnamic acids (Table 8, Figure 8). Hydroxybenzoic acids include gallic, *p*-hydroxy benzoic, vanillic, syringic, and protocatechuic acids. The hydroxycinnamic acids have a C_6-C_3 structure and include coumaric, caffeic, ferulic and sinapic acids. The phenolic acids reported in cereals occur in both free and bound forms. Sorghum and millet have the widest variety of phenolic acids. Free phenolic acids are located in the outer layer of the pericarp and are extracted using aqueous medium or organic solvents. Bound phenolic acids are esterified to cell walls, acid or base hydrolysis is required to release these bound compounds from cell matrix (Dykes and Rooney 2007). The major phenolic acids present in cereals are ferulic and *p*-coumaric acids and cereal bran are rich source of phenolic acids (Table 9).

Table 8. Phenolic acids reported in different cereals

Phenolic acid	Cereals
Hydroxy benzoic acids	
Gallic	Finger millet, rice, sorghum
Protocatechuic	Barley, maize, finger and pearl millets, teff, oat, rice, rye, sorghum, wheat
p-hydroxy benzoic	Barley, maize, finger, pearl and foxtail millets, oat, rice, rye, sorghum, wheat
Gentisic	Finger, pearl and foxtail millets, teff, sorghum
Salicylic	Barley, sorghum, wheat
Vanillic	Barley, maize, finger, pearl and foxtail millets, teff, oat, rice, rye, sorghum, wheat
Syringic	Barley, maize, finger, pearl and foxtail millets, teff, oat, rice, rye, sorghum, wheat
Hydroxy cinnamic acids	
Ferulic	Barley, maize, finger, pearl and foxtail millets, teff, oat, rice, rye, sorghum, wheat
Caffeic	Maize, finger, pearl and foxtail millets, teff, oat, rice, rye, sorghum, wheat
o-Coumaric	Barley
m-Coumaric	Barley
p-Coumaric	Barley, maize, finger, pearl, and foxtail millets, teff, oat, rice, rye, sorghum
Cinnamic	finger, pearl, and foxtail millets, teff, sorghum, wheat
Sinapic	Barley, finger and pearl millets, oat, rice, rye, sorghum

Source: Dykes and Rooney (2007)

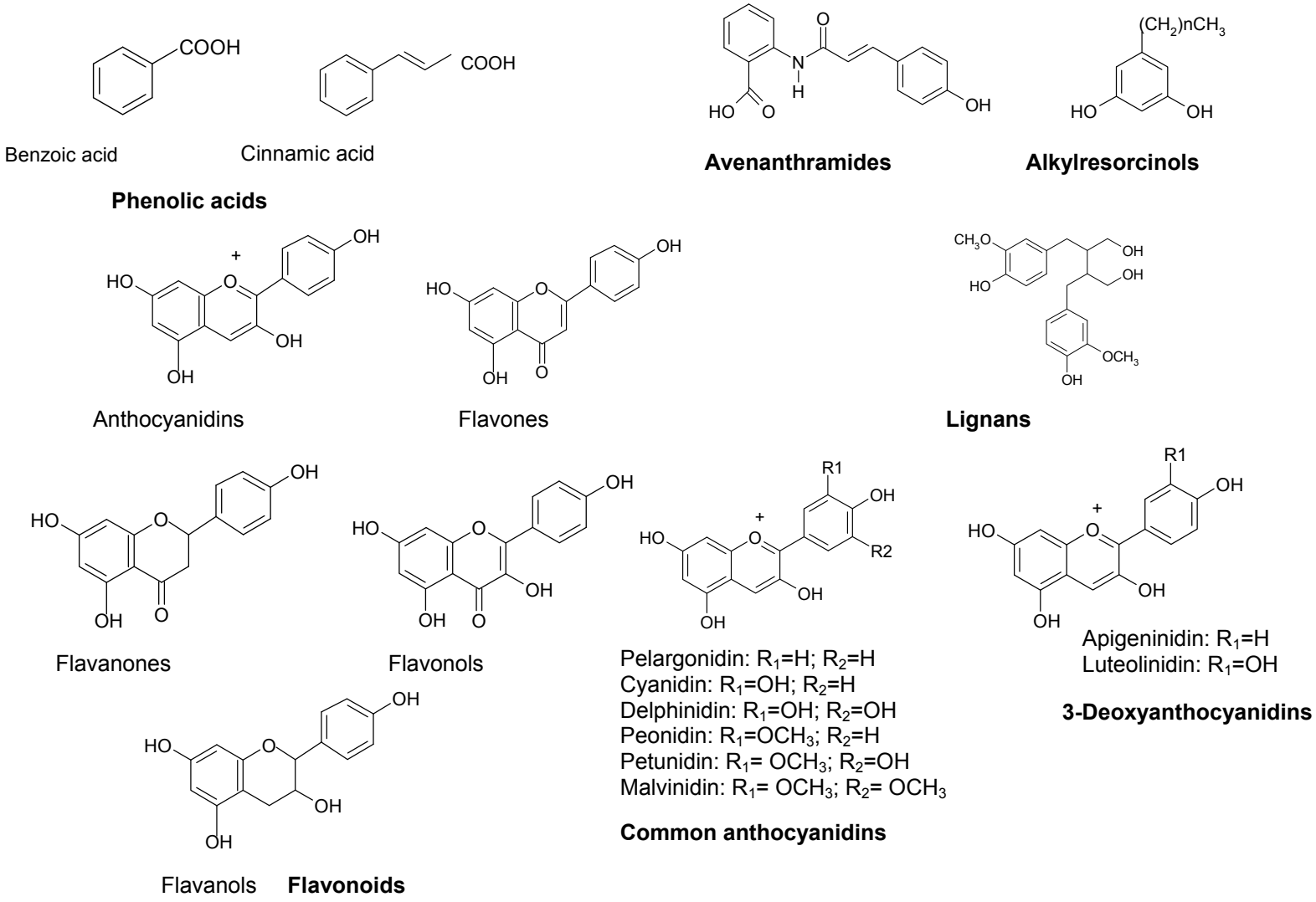


Figure 8. Structures of some common phenolic compounds present in cereals

Source: Dykes and Rooney (2007)

Table 9. Phenolic acids content in different cereals

Cereals	Phenolic acids content (µg/g)
Whole grains	
Barley	450-1346
Finger millet	612
Foxtail millet	3907
Maize	601
Oat	472
Pearl millet	1478
Rice	197-376
Rye	1362-1366
Sorghum	385-746
Wheat	1342
Bran	
Oat	651
Rye	4190
Wheat	4527

Source: Dykes and Rooney (2007)

Flavonoids are compounds with a C_6-C_3-C_6 skeleton that consists of two aromatic rings joined by a three carbon link; they include anthocyanins, flavanols, flavones, flavanones and flavonols (Figure 8). More than 5000 flavonoids have been identified in nature. Flavonoids are located in the pericarp of all cereals. Among the cereals, sorghum has the widest variety of flavonoids (Table 10).

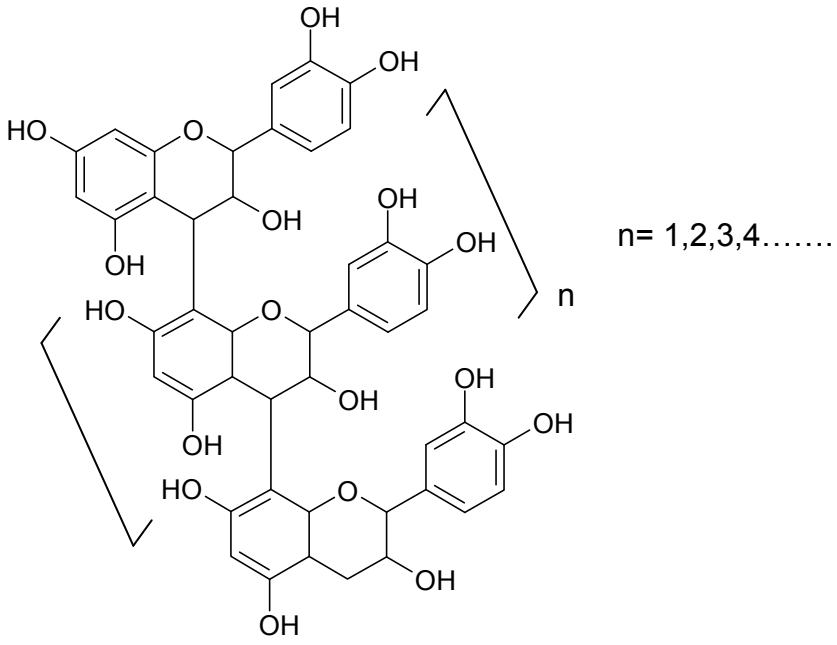
Anthocyanins are water soluble pigments that contribute to the blue, purple and red colour in plant foods (blue berries, black berries and straw berries) and are the major flavonoids studied in cereals. The six common anthocyanidins found in nature are cyanidin, delphinidin, malvinidin, pelargonidin, petunidin and peonidin, which are also normally present in the pericarp of pigmented varieties of barley, maize, rye, sorghum and wheat (Table 10). They are present in the coloured testa of cereals and their contents vary from 4 – 2,268 µg/g. Purple maize, black sorghum and black rice contain 965, 944 and 2,268 µg/g anthocyanins respectively (Dykes and Rooney 2007).

Condensed tannins, which are also called proanthocyandins or procyanidins, consist of polymerized flavanol units (Figure 9), and they contribute to the astringency in foods. These compounds are found in sorghum with pigmented testa layer, red finger millet, and barley (Dykes and Rooney 2007).

Table 10. Flavonoids reported in cereal grains

Grains	Compounds
Sorghum	Apigeninidin 5-glucoside, Luteolinidin, Luteolinidin 5-glucoside, 5-Methoxyapigeninidin, 7-Methoxyapigeninidin, 7-Methoxyapigeninidin 5-glucoside, 5-Methoxyluteolinidin, 5-Methoxyluteolinidin 7-glucoside, 7-Methoxyluteolinidin, Apigenin, Luteolin, Eriodictyol, Eriodictyol 5-glucoside, Naringenin, Kaempferol 3-rutinoside 7-glucuronide, Taxifolin, Taxifolin 7-glucoside, Apiforol, Luteoforol, Catechin, Procyanidin B-1
Barley	Cyanidin, Cyanidin 3-glucoside, Delphinidin, Pelargonidin, Petunidin 3-glucoside, Chysoeriol, Leucocyanidin, Leucodelphinidin, Procyanidin B-3, Prodelphinidin B-3, Pelargonidin glycosides
Maize	Cyanidin 3-glucoside, Cyanidin 3-galactoside, Cyanidin 3-rutinoside, Pelargonidin 3-glucoside, Pelargonidin glycosides, Peonidin 3-glucoside, Kaempferol, Quercitin, Leucocyanidin, Leucopelargonidin
Rice	Cyanidin 3-glucoside, Cyanidin 3-rutinoside, Peonidin 3-glucoside
Wheat	Cyanidin 3-glucoside, Cyanidin 3-galactoside, Cyanidin 3-rutinoside, Delphinidin 3-rutinoside, Peonidin 3-glucoside, Petunidin 3-glucoside, Petunidin 3-rutinoside, Apigenin glycosides, Tricin
Rye	Cyanidin 3-glucoside, Delphinidin 3-glucoside, Delphinidin 3-rutinoside, Peonidin 3-glucoside
Oat	Apigenin, Luteolin, Isovitexin, Tricin, Vitexin, Homoeriodictyol, Quercitin, Quercitin 3-rutinoside
Pearl millet	Glucosylorientin, Glucosylvitexin, Vitexin
Fonio millet	Luteolin, Apigenin
Japanese barnyard millets	Luteolin, Tricin
Finger millet	Tricin, Vitexin, Quercitin, Orientin, Isoorientin, Isovitexin, Saponarin, Violanthin, Leucenin-1, Quercitin

Adapted from: Hilu et al (1978), Dykes and Rooney (2007b), Chethan and Malleshi (2007b)



Condensed tannins

Figure 9. Structure of condensed tannins present in cereals

Source: Dykes and Rooney (2007)

Avenanthramides consists of an anthranilic acid derivative linked to a hydroxyl-cinnamic acid derivative (Figure 8). The three major avenanthramides reported in oat are avenanthramides 1, 3 and 4 which are also known as avenanthramides B, C, and A, respectively. Level of avenanthramides range from 40-132 µg/g in the grain, they are heat stable and are not denatured during common methods of cereal processing (Dykes and Rooney 2007).

Lignans are a class of phytoestrogens (Figure 8) that are predominant in flaxseed, but they are also found in cool season cereal grains (barley, oat, rye, triticale and wheat). The amount of lignans in these cereals ranges from 8-299 µg/ 100g (Dykes and Rooney 2007). The two plant lignans, secoisolariciresinol and matairesinol when ingested, are converted to mammalian lignans eneterodiol and enterolactone, respectively, by microbial enzymes in the colon (Thompson 1994). These compounds are bioavailable and are believed to reduce the risk of hormone-dependent cancers (breast and prostrate), colon cancer and heart disease.

Alkylresorcinols are 1,3-dihydroxybenzene derivatives with an odd-numbered *n*-alkyl side-chain at C-5 position on the benezene ring (Figure 8), are found in the bran of wheat, rye, triticale and barley (Dykes and Rooney 2007).

Nutraceutical ability of cereal polyphenols

Cereal polyphenols are known for their potent **antioxidant activity**. The antioxidant activity of different cereal grains in relation to their phenolic content have been reviewed extensively by Dykes and Rooney (2007). Aguilar-Garcia et al (2007) reported higher antioxidant capacities to rice bran compared to brown rice powder and attributed the antioxidant activity to total polyphenols, c-oryzanol, a- and c-tocopherols and a-, c- and d-tocotrienols contents. Whereas, the prevention of reactive oxygen species induced supercoiled DNA strand scission by cyanidin 3-glucoside from black rice was reported by Hu et al (2003). In the case of wheat, Pathirana et al (2006) reported the inhibition of oxidation of human LDL (low density lipoprotein) and hydroxyl radical mediated cleavage of DNA by its phenolics. The antioxidant activity of the whole grain cereals are attributed to their polyphenol contents. Even though, cereals exert considerable anti-oxidant activity, the seed coat matter or bran layer in their concentrated forms, form extremely good source of edible grade antioxidant components.

The **anti-carcinogenic properties** of sorghum and wheat have been reported by several workers. Van Rensburg (1981) and Chen et al (1993) reported that, populations consuming sorghum and millet had lower incidences of esophageal cancer compared to those consuming wheat or maize. In a recent study, black and tannin sorghum bran has been found to reduce colon carcinogenesis in rats due to its antioxidant effect (Turner et al 2006). In a different study, Gomez-Cordoves and co-workers (2001) demonstrated the therapeutic effect of sorghum proanthocyanidins against human melanoma. The anti-tumor activity of sorghum flavan-4-ols was due to the enhancement of immune response of the host animals through the actions on tumor cells and some immunocytes. In the case of anti-human colonic cancer property of wheat bran, Qu et al (2005) hypothesized that, phytochemical lignans (enterolactone and enterodiol) may account for the decreases in cell numbers by cytostatic and apoptotic mechanisms. Whereas, Drankhan et al (2003) indicated that, orthophenolic acids contents in the wheat predicted its anti-tumor activity *in vivo.*

Foods based on sorghum varieties rich in proanthocyanidins (PAs) are known for their **hypocholestrolemic property** and can be suggested to obese people and diabetic patients by analogy with the 50% weight loss observed with animals (rabbits, pigs, etc.) fed with sorghums containing high levels of PAs (Ambula et al 2001). The low digestibility of high PAs containing sorghums through the inhibition of hydrolytic enzymes, together with their high antioxidant activities may be interesting from a nutritional standpoint for obese persons. Rooney et al (1992) reported that, pearl millet brans were excellent bulking agents with better cholesterol-lowering properties compared to wheat, white sorghum or brown sorghum brans. Cereal brans are richer source of phenolics in addition to dietary fiber. Synergistic effect of phenolics with dietary fiber in cholesterol reduction can be expected as phenolics are proven HMG-CoA reductase inhibitors.

Sorghum tannins are known for their **anti-diabetic property,** as they are slowly digested and have potential applications in foods for diabetics (Awika and Rooney 2004). A negative correlation between the glycemic response and polyphenol intake has been reported by Thompson et al (1983). They also reported that large polymeric type or condensed tannins may be involved in

lowering of glycemic response of foods. Oryzanols and other polyphenols from the stabilized rice bran are very much effective in the management of type I & II diabetes as it was found to lower serum cholesterol, postprandial hyperglycemia, glucosuria by enhancing tissue sensitivity without insulin treatment (Qureshi et al 2002).

The **cardio-protective properties** of the cereal polyphenols are well studied and the intake of polyphenols along with diet rich in cereal grains lowers the risk of coronary diseases. Ling et al (2001) reported 50% reduction on the atherosclerotic plaque formation in red and black rice fed rabbits compared to the ones fed with white rice. They indicated that, the red or black rice consumption retarded the progression of atherosclerotic plaque formation induced by cholesterol, in addition, it increased serum HDL – cholesterol levels. The anti-atherogenic effect of the red and black rice may be attributed to their phenolic contents. In the case of oats, avenanthramide-c (Avn-c), inhibited the serum induced proliferation of vascular smooth muscle cells (SMC), which is an important process in the initiation and development of atherosclerosis (Nie et al 2006).

Alkylresorcinols are known for their **anti-mutagenic property** as they markedly decreased mutagenic activity of four standard mutagens examined in the Ames test (Gasiorowski et al 1996). In the Sister Chromatid Exchanges (SCEs) test with cultured *in vitro* human blood-derived lymphocytes, a significant decrease of SCEs frequency induced by benzo[a]pyrene was observed in the presence of alkylresorcinols. Moreover, polymeric tannins from sorghum had higher anti-mutagenic activity compared to lower molecular weight tannins (Grimmer 1992).

The **anti-aging properties** of the cereal polyphenols are reported by Álvarez et al (2006), who inidicated an improvement in the leucocyte functions in prematurely aging mice after five weeks of diet supplement with polyphenol rich cereals such as wheat germ, buckwheat flour, fine rice bran and wheat middlings (containing different amounts of gallic acid, *p*-hydroxybenzoic acid, vanillic acid, sinapic acid, *p*-coumaric acid, ferulic acid, quercetin, catechin, rutin and oryzanol as major polyphenols). The study emphasized that, regular intake of cereals rich in phenolic compounds could delay normal aging and improve quality of life.

The **immune enhancing property** of the cereal polyphenols are studied by several workers and Akbay et al (2003) in their study showed that, three flavonoid glycosides from *Urtica dioica* L., quercetin-3-*O*-rutinoside, kaempferol-3-*O*-rutinoside and isorhamnetin-3-*O*-glucoside increased, *in vitro*, the chemotaxis and the intracellular killing activity of neutrophils. Wheat middling was able to enhance macrophage chemotaxis, whereas, all the cereal fractions were able to show a strong stimulatory effect on this function in lymphocytes. These facts suggests that, the polyphenols present in cereal fractions could exert a cell-dependent effect on migration capacity and also could increase the microbicidal activity of peritoneal leukocytes.

There are few reports on the **antimicrobial property** of cereal polyphenols. Chethan and Malleshi (2007a) reported the antimicrobial property of finger millet seed coat phenolics against *Helicobacter pyroli, K. pneumoniae, Y. enterocolitica, S. pyrogenes, P. mirabilis* and *S. marcescens.* The antimicrobial effect of the acidic methanol extract may be due to the anthocyanins, tannins, flavonoids and phenolic acids present in the extract. Also, proanthocyanidin is an important phenolic compound present in the cereals. The binding of proanthocyanidins with proteins participates in their antibacterial activity. Proanthocyanidins have been shown to inhibit the growth of human immunodeficiency virus 1 (HIV-1), influenza virus, and herpes simplex virus by blocking their entry in the host cells (Lu et al 2004, Hamauzu et al 2005).The mechanism of proanthocyanidins toxicity against microbes is related to inhibition of hydrolytic enzymes, interactions to inactivate microbial adhesions and cell envelope transport proteins, and non-specific interaction with carbohydrates (Cowan 1999).

Flavonoids are known for their **anti-thyroid properties** as they are potent inhibitors of iodothyronine-deiodinase, a key enzyme of thyroid hormone metabolism, both in the liver and in the brain of rats. Flavonoids are capable of displacing bound thyroxine from transthyretin, its specific plasma carrier-protein, hence contributing to increase the free hormonal pool available to peripheral tissues. Sartelet et al (1996) found potent anti-thyroid flavonoid compounds namely apigenin and luteolin from fonio millet (*Digitaria exilis*) and these compounds exhibited strong anti-thyroid peroxidase (TPO) activities, resulting in a

significant reduction of the hormonogenic capacity of this enzyme. In addition, luteolin significantly depressed the cyclic AMP phosphodiesterase, implying a concomitant overproduction of the thyrotropin-dependent nucleotide. Hence fonio millet diets can be comfortably recommended for thyroid patients.

Morimitsu et al 2002 reported the **anti-cataract properties** of Japanese black and red rice wherein, the authors indicated that, the methanol extract of coloured rice was found to show potent inhibitory activity against rat lens opacity. They attributed the anti-cataract properties to cyanidin 3-glucoside, petunidin 3-glucoside, ferulic acid, caffeic acid and protocatechuic acid present in the rice samples. Chethan et al (2008) studied the inhibitory effect of finger millet polyphenols on aldose reductase (the key enzyme involved in cataract formation) extracted from cataracted human eye lenses. They reported that, finger millet polyphenols are non-competitive inhibitors of the enzyme. Hence the cereals rich in phenolic compounds can be recommended for diabetics as they help in delay of cataractogenesis.

SCOPE OF THE WORK

Finger millet foods are known for their lower glycemic index and higher satiety scores compared to other cereals and from good old days, the millet foods are usually recommended for diabetics. However, scientific reports on the health beneficial aspects of the millet are scanty. The millet is a rich source of dietary fiber and phytochemicals such as polyphenols and phyate, and these phytochemicals are believed to be concentrated in the seed coat matter of the millet. Generally, these phytochemicals are known to influence the carbohydrate digestibility of foods. Hence it was hypothesized that, the dietary fiber, polyphenols and phytate of the millet may help in slowing down the carbohydrate digestibility of the millet foods. Accordingly, the investigations on the influence of the millet seed coat phytochemicals as well as the seed coat matter as such on the carbohydrate digestibility of the millet foods and also their ameliorative potential towards hyperglycemia and associated complications in diabetic animal models were carried out with the following objectives;

1. Localization of polyphenols and phytate in the millet kernel and preparation of the millet fraction rich in these phytochemicals and also isolation as well as characterization of the constituent phenolics,
2. Evaluation of the influence of the millet seed coat matter and also the effect of hydrothermal processing of the millet on the carbohydrate digestibility of millet foods by *in vitro* and *in vivo* methods,
3. Investigations on the nutraceutical properties of the millet seed coat polyphenols with special reference to their inhibitory properties against carbohydrases, fructose induced protein glycation and $OH^{\cdot}$ mediated protein fragmentation in the *in vitro* model systems, and
4. Assessment of the effect of the millet seed coat matter on hyperglycemia and its associated complications in diabetic animal models.

The research work conducted with these objectives are presented in four chapters in the thesis.

INTRODUCTION

Now-a-days consumption of whole grain products is increasing advocated health benefits such as lower incidence of diabetes (Montonen et al 2003), coronary heart disease (CHD) and cancer (Jones et al 2002). The health benefits of the whole grain products may be attributable to the synergistic effects of dietary fibre, phytochemicals and micronutrients, which are generally concentrated in the peripheral part of the food grains (Jones et al 2002, Montonen et al 2003). The bran and germ components of the food grains are rich in fibre, vitamins, minerals, phytoestrogens, polyphenols (Naczk and Shahidi 2006) and phytate (Oberleas 1983) and an inverse relationship is observed with the whole grain consumption and incidence of diabetes and heart disease (Jensen et al 2006). Whole grain based foods are known to be associated with improved insulin sensitivity and lower concentrations of serum triacylglycerol, as well as total and LDL cholesterol. Hence, there is an increasing demand for whole grain products among the health conscious population and as a sequel to that, several whole grain and high fiber products rich in antioxidants, hypoglycemic and hypocholestrolemic constituents are marketed worldwide. Since bran is normally a good source of health beneficial components of grains, its utilization in functional food formulations is gaining importance, accordingly several methods have been developed for the efficient separation of nutraceutical rich components of cereal grains. In the case of some of the cereals and millets, to separate the seed coat matter, milling after incipient moist conditioning (Malleshi and Desikachar 1981) and decortication (Awika et al 2005) are the most frequently adopted methods.

Finger millet or *ragi* (*Eleusine coracana* L.), is known for its health benefits such as hypoglycemic (Ramanathan and Gopalan 1957, Hegde et al 2005a), hypocholesterolemic (Pore and Magar 1976) and antiulcerative (Tovey 1994) properties. Although, definite reasons attributing to the health benefits of millet are not reported, probably the complex nature of its carbohydrates, higher levels of dietary fibre contents besides the presence of phytochemicals with nutraceutical qualities could be the reasons.

Finger millet seed coat constituting 15-20% of the kernel matter contains considerably higher proportion of non-starchy polysaccharides, minerals, polyphenols, pigments and phytates (Hulse et al 1980) compared to many other cereals. Generally it has been reported that, the seed coat matter of the cereals contains considerable amount of polyphenols and phytate. The millet seed coat is a reserve of polyphenols and several researchers have worked on the extraction of polyphenols. Chethan and Malleshi (2007b) studied the extractability of the millet seed coat polyphenols with a good number of polar and non-polar solvent systems and reported 1% HCl-methanol solvent system as the best solvent system to extract higher proportion of polyphenols. They also studied the stability of the polyphenols in the acidic and alkaline pH and reported that, the polyphenols were stable at acidic to near neutral pH. However, their study identifies only 12 compounds in the polyphenol extract, but indicates that, the millet polyphenols contain a large number of constituent phenolics which remained unidentified. Besides, the reports on the distribution of polyphenols, phytate and other important phytochemicals between the different tissues of the millet are scanty. Hence, the investigations were undertaken to; (a) localize the polyphenols and phytate in the millet kernel, (b) to prepare the millet fraction rich in dietary fiber, polyphenols and phytate namely the seed coat matter and (c) to characterize the millet seed coat polyphenols. Details of the experimental part and the results are reported in this chapter.

MATERIALS AND METHODS

Finger millet: Finger millet (GPU 28, variety) procured from University of Agricultural Sciences, Bangalore, India, was deglummed in Engleburg rice huller and the deglummed material, was passed through destoner - aspirator to remove foreign matter, impurities, immature kernels and was used for the studies.

Chemicals and reagents: Gallic acid, protocatechuic acid, gentisic acid, vanillic acid, caffeic acid, syringic acid, *p*-coumaric acid, ferulic acid, trans-cinnamic acid, *p*-hydroxy benzoic acid, genistein, diadzein, kaempferol, catechin and phytic acid were purchased from Sigma-Aldrich chemical company (St. Louis, USA). All the reagents

used were of analytical grade, and glass (double) distilled water was used for the preparation of the reagents.

Microscopic examination

a. Light microscopy: The procedure followed for the fixation of kernels (Berlyn and Miksche 1976) are given in Figure 10, and the steps involved are given below.

Fixation: The kernels were soaked in 0.025 M phosphate buffer (pH 7.0) containing 0.5% glutaraldehyde for 48 h with two changes of 24 h intervals.

Dehydration: The glutaraldehyde treated kernels were dehydrated successively by passing through series of ethanol of different concentration followed by ethanol-xylene series as shown in the flow diagram (Figure 10).

Infiltration: The dehydrated grains were infiltrated with paraffin wax and then suspended in xylene, saturated with paraffin wax and the contents were left overnight at ambient temperature.

Embedding: The infiltrated gains were incubated at 58^0C. The melted wax was decanted and replenished with fresh wax and the process was repeated (four to five times), till the material was free from xylene. The material was then embedded in a mixture of paraffin wax and bee wax (95:5) to make the blocks.

Sectioning: The blocks were cut open to expose the kernels, and soaked in 20% aqueous glycerol for 24 h at 4^0C and about 5µ thick sections were taken in a rotary microtome using stainless steel knife.

Dewaxing: The sections were suspended in xylene to dewax and the dewaxed sections were passed through xylene : ethanol series followed by ethanol series in the ascending order as shown in Figure 10.

The section of the millet thus prepared were used for localization of polyphenols and phytate by staining with suitable stains and taken for light and fluorescence microscopy.

Staining

i. Polyphenols: The localization of the polyphenols was done by staining with $FeCl_3$ and also by autofluorescence. The sections were flooded with 2% $FeCl_3$ in 95% ethanol solution and were left in the staining solution for 5 min and immediately

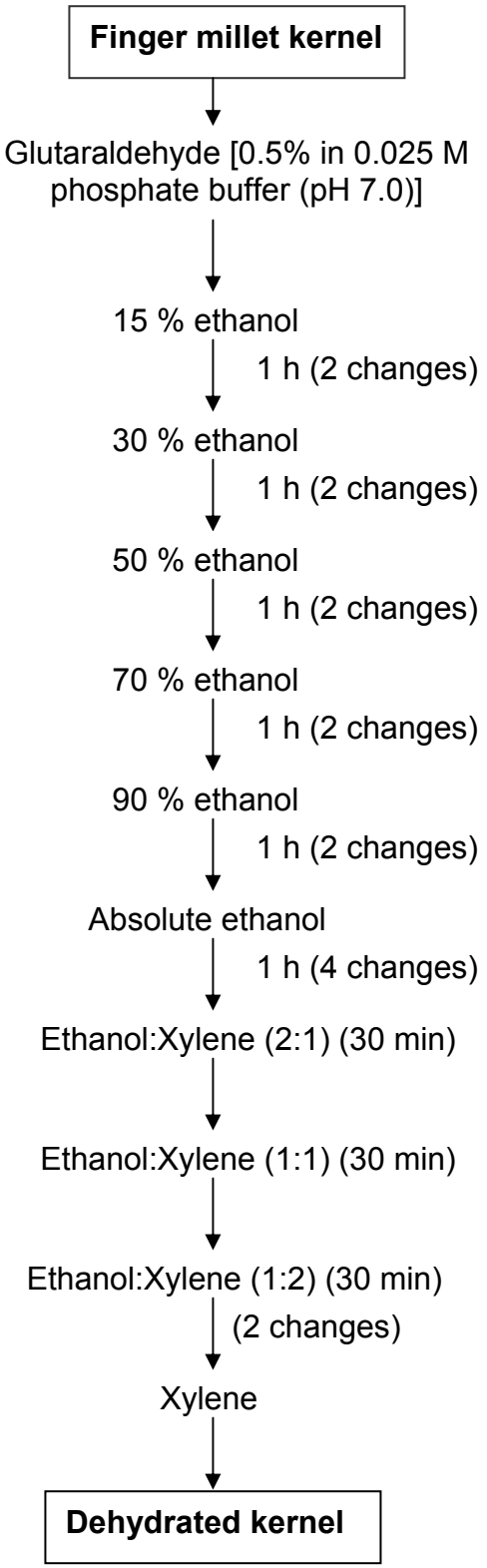


Figure 10. Schematic representation of the protocol followed for the fixation and dehydration of the millet kernels for microscopy

rinsed with 95% ethanol, hydrated and mounted in glycerol and examined for polyphenols according to the method of Gahan (1984).

The unstained sections were also exposed to ammonia vapors, mounted in glycerol and were examined for the autofluorescence emitted by the millet polyphenols in a fluorescence microscope under excitation 510 nm and emission 550 nm (Fulcher 1982).

ii. Phytate: To localize the phytate reserves, the sections were stained for 15 min in 0.01% (w/v) aqueous acriflavine-HCl at pH 3.1, rinsed with ethanol and mounted in glycerol and the fluorescence was observed at excitation 510 nm and emission 550 nm (Tanke and VanIngen 1980).

iii. Calcium associated phytate reserves: The millet sections were flooded with freshly prepared aqueous solution of Alizarin red S (1% w/v) containing 1% (w/v) ammonium hydroxide at pH ranging from 6.2 to 6.5 for 1 - 2 min, rinsed briefly, mounted in distilled water and examined for calcium associated phytate reserves using bright field optics (Pearse 1972).

b. Scanning electron microscopy: The native millet kernels were cut open to expose the endosperm and mounted on metallic stubs with the aid of double side scotch tape and were gold coated (about 100A^{o}) in a KSE 2 AM evaporation Seevac gold sputter. The samples were scanned in LEO 435 scanning electron microscope and the selective portions of the seed coat, aleurone layer, cell walls and the endosperm with the special reference to the granular organization of the starch, and also the topography of the millet kernels were photographed at different magnification.

The seed coat matter from the millet was prepared by following two different methods;

A. Sequential milling and sieving.
B. Decortication of hydrothermally processed millet.

Since each of these methods have unique features, the milling methods, the quality characteristics including fractionation of polyphenols and antioxidant activities for each of the fraction prepared by both the methods are described separately.

A. Processing of the millet for preparation of seed coat matter by sequential milling and sieving: The millet was sprayed with 7% additional water, tempered for about 10 min and pulverized in a carborundum disc mill (SABKO, Rajkot, India), wherein the gap between the plates were adjusted suitably to minimize the pulverization of seed coat. The meal was sifted through 85 mesh (BSS sieve, 180 μ openings) to separate the flour (endosperm) from the seed coat. Soon after sieving, the tailings (+ 85 fraction) were again pulverized and the process was repeated two more times in the same mill. The throughs from each stage and the tailings from the third stage grinding namely, throughs of the first (ERF1), second (ERF2) and third passes (ERF3) and also the tailings (seed coat matter, SCM) were collected separately, equilibrated, and weighed, to record their relative yields. The endosperm rich fractions, namely the ERF1, ERF2 and ERF3 were mixed together to prepare endosperm rich fraction or refined flour (RF). The milling flow for preparation of RF and SCM is given in Figure 11. The whole meal (WM), RF and SCM (Figure 12) were analyzed for nutrient, phytochemical contents and antioxidant properties. The milling fractions were also taken for isolation and characterization of polyphenols.

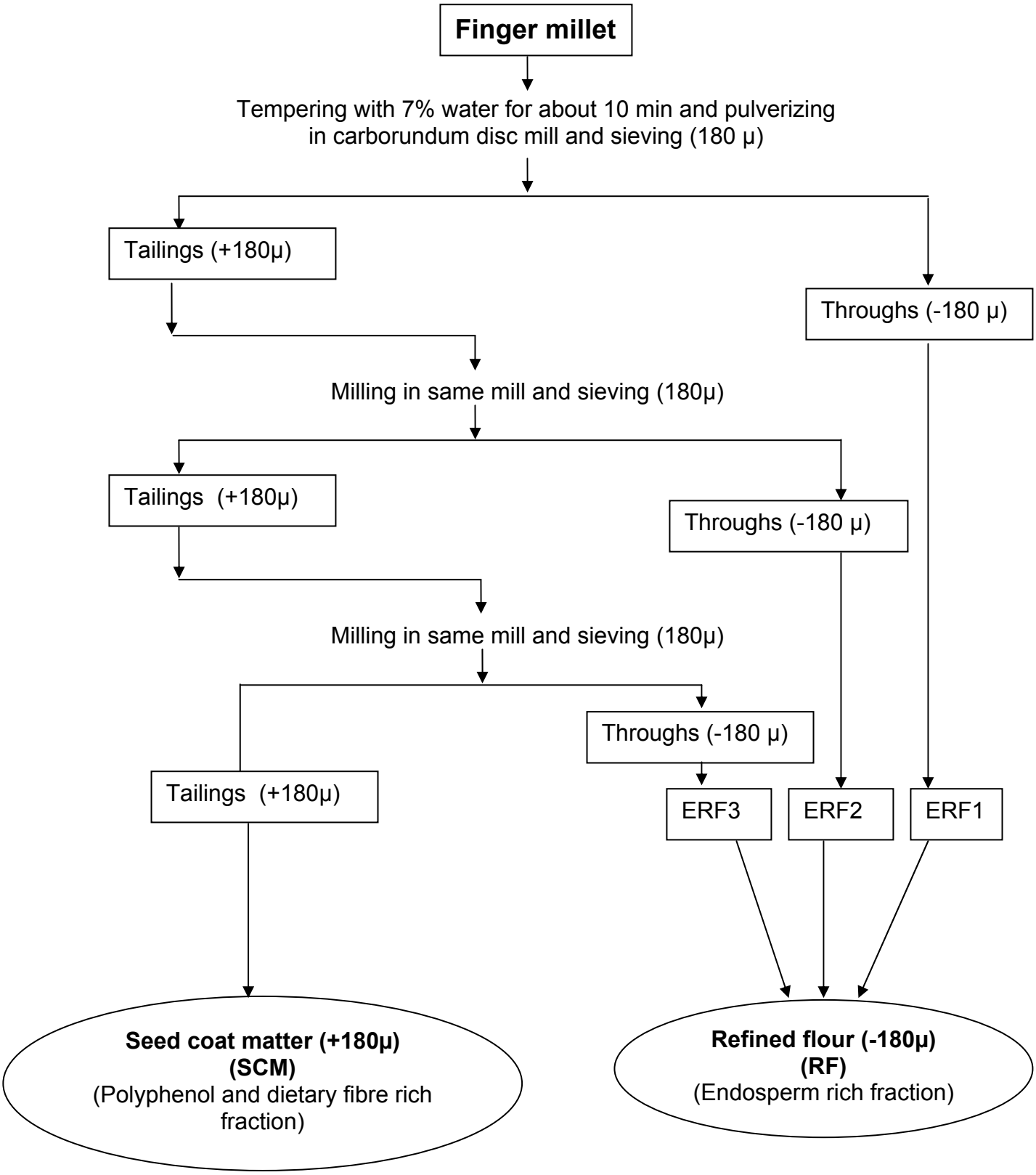


Figure 11. Flow chart for the preparation of finger millet seed coat matter (SCM)

Figure 12. a. Finger millet grains, b. refined flour and c. seed coat matter

Nutrient composition: The millet WM, RF and the SCM were analyzed for protein, fat and ash contents following AACC (2000) procedures, whereas, the starch content was estimated according to the enzymatic procedure of Holm et al (1986). The calcium and phosphorus contents were determined according to AOAC (2000), and Fiske and Subba Row (1983) respectively. Some of the minerals such as iron, zinc, copper and potassium levels were determined in atomic absorption spectrometer (Model AA-670F, Shimadzu, Singapore).

Color measurement: The apparent color was measured and recorded in terms of L* a* b* and ΔE (deviation from the standard) values were recorded as per CIE Lab scales in a Hunter Lab color measuring system (Labscan XE, Reston, Virginia)

Dietary fibre: The total (TDF), soluble (SDF) and insoluble (IDF) dietary fibre contents of the WM, RF and SCM were determined by the enzymatic method of Asp et al (1983). The details of the procedure followed for the determination of dietary fibre content is given in Figure 13.

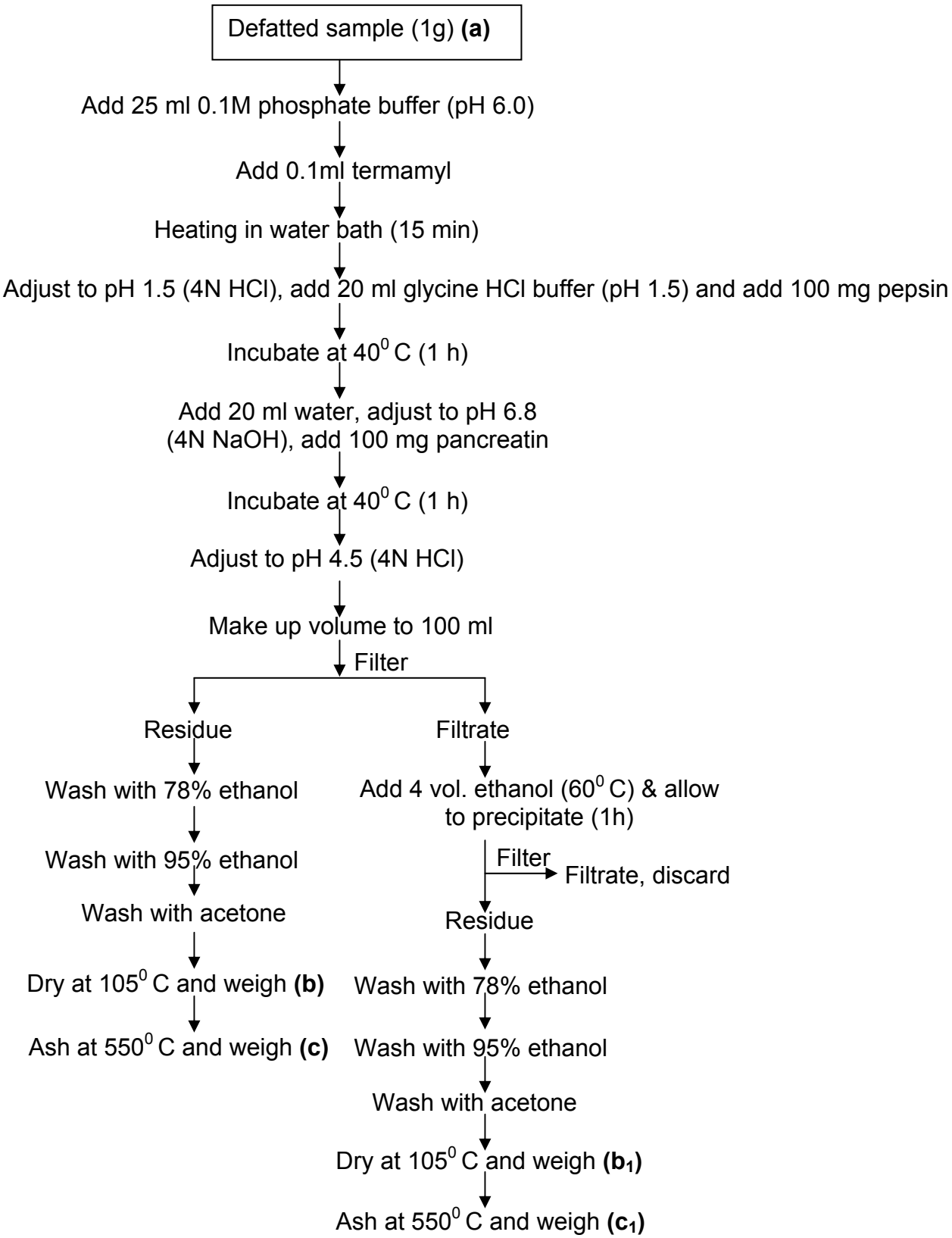


Figure 13. Procedure for estimation of soluble and insoluble dietary fibre

The dietary fibre content was calculated as follows;

$$\text{IDF (\%)} = \frac{b - c}{a}$$

$$\text{SDF (\%)} = \frac{b_1 - c_1}{a}$$

TDF = IDF + SDF

a- Wt. of sample

b- Wt. of the crucible with insoluble fibre before ashing

c- Wt. of the crucible after ashing

b_1- Wt. of the crucible with soluble fibre before ashing

c_1- Wt. of the crucible after ashing

The millet WM, RF and the SCM were defatted using petroleum ether (60 - 80^{0}C) and the defatted samples were used for the estimation of starch, polyphenols and also for the isolation and characterization of the polyphenols.

Estimation of starch: The samples (100 mg) were added to 15 ml of water containing 0.1 ml of thermostable α-amylase (termamyl) and the contents were heated in a water bath for 15-20 min to gelatinize the starch and cooled to room temperature. To the contents, 15 ml of acetate buffer (0.05M, pH 4.8) containing 10 mg amyloglucosidase were added and mixed well. The flasks were incubated at 55^{0}C for 16 h and at the end of incubation time, the contents were made up to 100 ml with water and filtered. The glucose content of the filtrates were estimated using glucose oxidase method. The glucose content of the sample was multiplied by 0.9 to get the starch content of the sample (Holm et al 1986).

Phytochemicals

a. Total polyphenols: The samples (1g) were refluxed with acidic methanol (99 ml of methanol mixed with 1 ml of concentrated HCl) solvent system (100ml×4) using a heating mantel maintained at 60^0 C for 10 min. The extracts were concentrated under vacuum in a rotary flash evaporator and taken for the assay. Briefly, 0.1 ml of the extract was mixed with a few ml of water in a 50 ml volumetric flask and to that 2.5 ml of Folin-Ciocalteau reagent (diluted 1: 2 with water) and 7.5 ml of 15% sodium carbonate solution were added, mixed thoroughly and made up to 50 ml. The blue color developed was read against the reagent blank at 760 nm after 30 min. Gallic acid served as the reference standard and the total polyphenols content was expressed as gallic acid equivalents (Singleton et al 1995).

b. Tannins: The tannin content was assayed following vanillin-HCl method of Price et al (1978). The samples (1g) were extracted in 10 ml of acidic methanol solvent system by agitation in a rotary shaker at ambient temperature for 20 min, were centrifuged at 5000 rpm and the clear supernatants were taken for the estimation of tannins. Briefly, to 1 ml of the supernatant, 5 ml of vanillin-HCl reagent [prepared freshly by mixing equal volumes of 4% vanillin solution (4g vanillin in 100 ml of methanol) and acidic methanol (8 ml concentrated HCl made up to 100 ml with methanol)] was added, followed by 5ml of 4% concentrated HCl in methanol and the

absorbance of the resultant solution was read at 500nm after 20 min of incubation at room temperature. Catechin served as the reference standard and the tannin contents of the samples were expressed as mg of catechin equiv/100g of sample.

c. Flavonoids: The samples were extracted with methanol for 6 h and the extracts were centrifuged at 5000 rpm and the clear supernatants were taken for the estimation. An aliquot (0.25 ml) of the supernatant was pre-diluted with 1.25 ml of distilled water, then 75µl of a 5% $NaNO_2$ solution was added to the mixture. After 6 min, 150µl of 10% $AlCl_3$ solution was added, and the mixture was allowed to stand for 5 min, after which 0.5 ml of 1M NaOH solution was added and the volume was made upto 2.5 ml with distilled water. The solution was mixed well and the absorbance was measured against the reagent blank at 510 nm. Catechin was used as the reference standard and the results were expressed as milligrams of catechin equivalents (Zhishen et al 1999).

d. Phytate: The samples (80 mg) were extracted in 10 ml of 0.2 M HCl with agitation in a rotary shaker for 1 h at room temperature, centrifuged at 5000 rpm and the clear supernatants were used for the assay of phytate content. The supernatant (0.5 ml) was pippetted into a test tube fitted with a ground-glass stopper after which 1 ml acidic ammonium Iron (III) sulphate dodeca hydrate (0.2g in 100 ml of 0.2 M HCl and made up to 1000 ml with distilled water) was added. The contents were boiled for 30 min and cooled rapidly to 25^0C in an ice-water bath, and to that 2 ml of 2'2' bipyridine solution (10 g 2'2' bipyridine and 10 ml thioglycollic acid in 1000 ml water) was added and mixed well. The absorbance was read after 1 min at 519 nm against distilled water. Sodium phytate (3-75 µg) was used as the reference standard and the phytate contents were expressed as mg phytate/100 g sample (Haug and Lantzsch 1983).

Isolation and characterization of polyphenols

a. Isolation: The defatted samples (1g) were refluxed with acidic methanol solvent system using a heating mantel maintained at 60^0 C for 10 min, the extract was cooled, filtered on Whatman No. 1 filter paper and the residue was re-extracted with acidic methanol and the process was repeated until the extract tested negative for polyphenols. The extracts were pooled and neutralized to 2.5 pH with 0.1 N NaOH,

concentrated under vacuum in a rotary flash evaporator, centrifuged and the supernatants were taken for characterization.

b. Characterization: The extracted polyphenols were fractionated and characterized by HPLC and direct infusion electrospray insertion mass spectrometry (ESI-MS).

Polyphenols from the extracts were fractionated through HPLC (model LC-8A, Shimadzu, reversed phase Shimpak C_{18} column fitted with diode array detector, operating at 280 & 320 nm) using isocratic solvent system consisting of water:acetic acid:methanol (80:5:15) as a mobile phase at a flow rate of 1 ml/min. Caffeic, coumaric, ferulic, gallic, gentisic, protocatechuic, syringic and vanillic acids were used as standards.

Parallely, the extracts (after centrifugation to separate out any suspended particles) were fractionated using Alliance, Waters 2695 mass spectrometer (Waters corporation, Micromass Ltd, UK) operating at ESI (-ve mode), maintained 3.0 kV capillary voltage, 120^0C and 300^0C source and desolvation temperatures respectively using cone gas (argon) and desolvation gas (nitrogen) at flow rates 50 litre h^{-1} and 500 litre h^{-1} respectively. The m/z values of the spectra obtained were matched with the m/z values obtained for different polyphenol standards and also from the m/z values available in the literature for the identification of polyphenols (Markowicz et al 2007). The polyphenol standards used were caffeic acid, coumaric acid, ferulic acid, gallic acid, gentisic acid, protocatechuic acid, syringic acid, vanillic acid, transcinnamic acid, p-hydroxy benzoic acid, genistein, diadzein, kaempferol and catechin.

Antioxidant properties: The antioxidant activity was determined in terms of (i) total antioxidant activity (TAA), (ii) DPPH radical scavenging activity (RSA) and (iii) Iron chelating activity (ICA). The flour samples were extracted with agitation in methanol (0.1g/ml methanol) for 6 h and the extracts were centrifuged at 5000 rpm and the clear supernatants were taken for the assay of TAA and RSA, whereas, the flour samples extracted with agitation in 1% HCl methanol (0.1g/ml) was taken for the assay of ICA.

The TAA of the extracts were measured in terms of their ability to reduce Mo (VI) to Mo (V) and subsequent formation of green phosphate/Mo(V) complex at acidic

pH (Prieto et al 1999). An aliquot (0.1 ml) of the extracts added to 1 ml of molybdate reagent (0.6M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate), in eppendorf tubes were capped and incubated in a water bath at 95^0 C for 90 min, cooled and the absorbance of the solution was read at 695 nm against a blank. Ascorbic acid served as the standard and the TAA was expressed as mM Ascorbic acid equiv TAA/g of samples.

For the assay of RSA, to 3.9 ml of 6×10^{-5} mol/L $DPPH^{\cdot}$ solution, 0.1 ml of the extracts were added and the decrease in the absorbance indicating the reaction kinetics of the extracts with DPPH radical was monitored at 515 nm at every 10 min for 90 min until the reaction reached a plateau. A control without any antioxidants was also included. The $DPPH^{\cdot}$. scavenging ability was estimated and expressed as % $DPPH^{\cdot}$ remaining using ascorbic acid as a reference standard (Brand Williams et al 1995).

The ICA was measured by 2,2' bipyridyl complexing assay (Yamaguchi et al 2000). Briefly, 0.25 ml of $FeSO_4$ solution (1 mM) and 0.25 ml of the extracts (both containing 1% SDS) were mixed and to that, 1 ml of Tris-HCl buffer (pH 7.4) and 2,2' bipyridyl solution (0.1% in 0.2 M HCl) were added along with 0.4 ml of 10% (w/v) hydroxylamine HCl and 2.5 ml of ethanol. The absorbance of the resulting reaction mixture was read at 522 nm. EDTA served as the reference standard. ICA values were expressed as mM EDTA equiv ICA/g of sample.

B. Preparation of the seed coat matter of the millet by decortication

Finger millet has peculiar endosperm texture, which is not conducive for decortication similar to other cereals because, its endosperm is highly fragile in nature to which seed coat is rigidly attached, hence the millet endosperm crumbles into flour along with seed coat matter during decortication. In view of this, conventional methods of cereal decortication are not applicable for the millet. Hence, the millet was hydrothermally processed to harden its endosperm which enables it to withstand the impact during the decortication.

The millet (2Kg) was steeped in excess water at room temperature for 8 h and the steeped millet was steamed at ambient pressure for 20 min, after which, the

steamed material was dried in a mechanical cross flow drier maintained at 40±2^{0}C till the moisture content dropped to 12±1%.

The hydrothermally processed and dried material (HTM) was graded into kernels of size '+1405μ', '-1405μ+1003μ' and '–1003μ' using a cereal sifter-cum-grader fitted with screens of 1405μ (12 mesh BSS) and 1003μ (16 mesh BSS) openings. The fraction of '-1405μ+1003μ' which formed about 85% of the material was used for the decortication studies. The graded millet was moistened with 6% additional water, tempered for about 5 - 10 min and decorticated in a horizontal carborundum twin disc mill (SABKO, Rajkot, India). The clearance between the discs was maintained at 1.35±0.05 mm (slightly lesser than the diameter of the grain) by suitably adjusting the lever of the mill to obtain 15 ± 1% degree of decortication. The milling fractions namely, the head grains or the decorticated millet (DM), grits (brokens) and the seed coat matter (SCM-HTM) were collected separately, equilibrated and weighed (Shobana and Malleshi 2007). Various unit operations involved in the preparation of decorticated millet are presented in Figure 14. The SCM-HTM thus prepared was used for isolation and characterization of polyphenols and the HTM and DM was used for determination of some of its functional properties including cooking characteristics and also the *in vivo* carbohydrate digestibility.

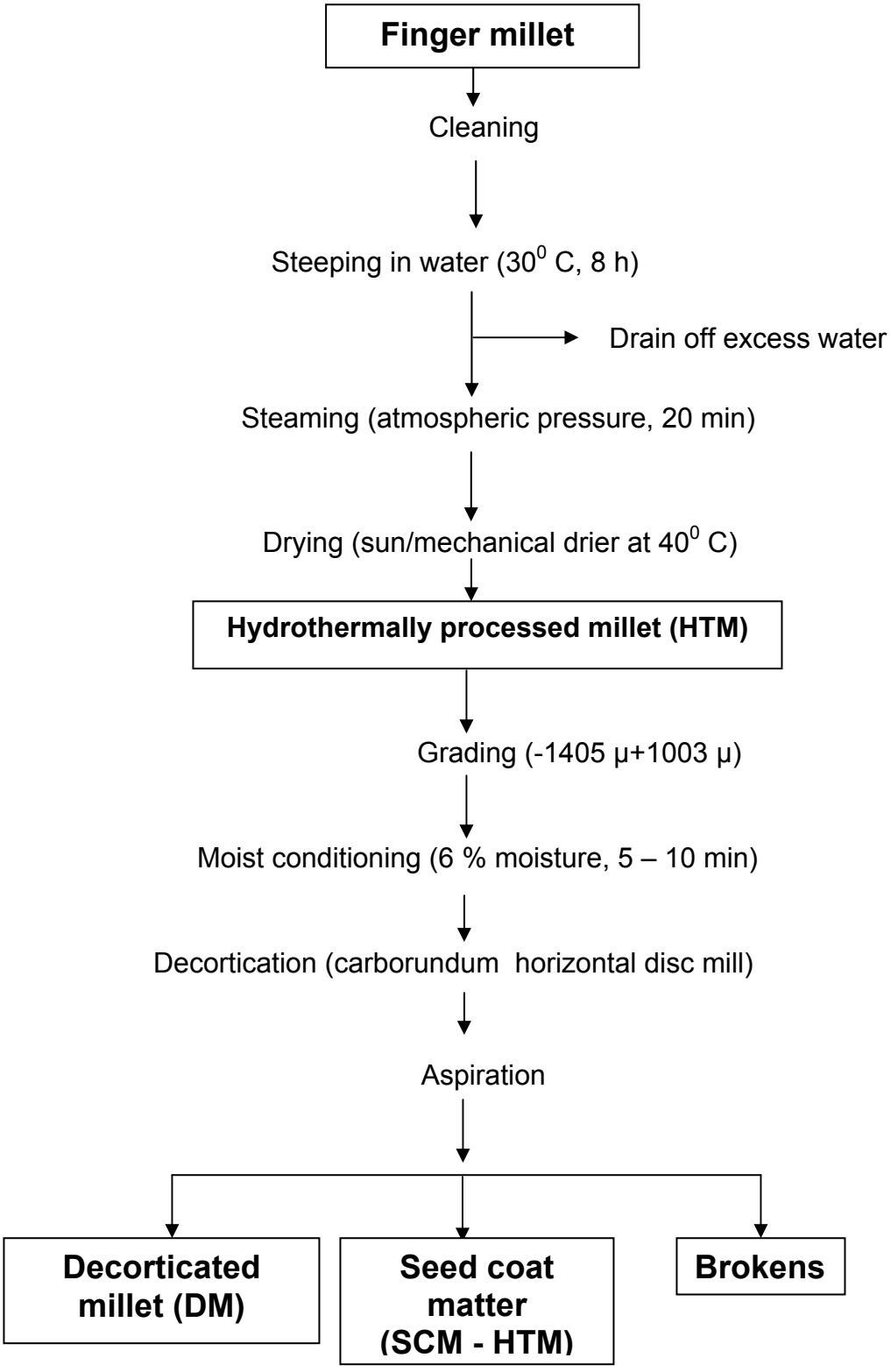


Figure 14. Flow diagram for preparation of decorticated millet (DM) and its seed coat matter (SCM-HTM)

Microscopy: The HTM and DM kernels were processed for histochemical localization of polyphenols and phytate as described earlier in the Chapter. The HTM and DM kernels as such and their cross sections were examined under scanning electron microscopy to observe the changes caused by hydrothermal processing and decortication.

The HTM, DM and SCM-HTM were used to determine;

a. Phytochemicals such as total polyphenols, tannins, flavonoids and phytate,
b. Isolation and characterization of polyphenols, and
c. Antioxidant properties (TAA, RSA and ICA)

following the methods described in part A of this Chapter.

Physico-chemical properties of decorticated millet: Some of the physico-chemical characteristics, functional properties (1000 kernel weight, volume, hardness, color, solubility and swelling power, dough characteristics), and nutrient composition of the HTM and the DM were determined as detailed below;

Physical characteristics: The samples were equilibrated to about 12% moisture content and one thousand kernels were counted in a Numegral grain counter (Tecator Co. Hoganas, Sweden) and their weight was recorded whereas, the volume of the 1000 kernels was determined by toluene displacement method. Based on the mass and volume, the apparent grain density was calculated.

The size of the kernels in all the directions was measured using digital Vernier calipers. The hardness was measured in Kiya hardness tester (Kiya Seisakusho Ltd., Tokyo, Japan), by placing the Individual kernels facing the dorsal end on the platform of the tester and crushing by operating the screw-knob and recording the force required to crush the grains, an average value for 20 grains was recorded.

The apparent color was measured in a color measuring system (Labscan, Reston, Virginia) against standard (100% reflectance) and, the difference in reflectance values between the standard and the sample (ΔE) as well as the L* a* b* values were also recorded.

The HTM and the DM were pulverized in Udy cyclone mill (UD Corporation, Boulder, USA) fitted with 0.5 mm screen and the whole meals were used for further studies.

Nutrient composition: The pulverized samples and the seed coat matter were analyzed for protein, fat and total as well as acid-insoluble ash, calcium, phosphorus, dietary fibre and starch following the standard procedures as described earlier.

Functional properties of the decorticated millet (DM)

Swelling and solubility characteristics: The solubility and swelling behaviour of the flour samples was determined according to the method of Leach et al (1959). The flour sample (500 mg) was taken in a graduated centrifuge tube and mixed with about 20 ml of distilled water and heated for 30 min in a water bath maintained at 30^0 C and 80^0 C with occasional stirring and centrifuged at 5000×g for 10 min. The supernatant was decanted into a petri plate and dried at 105^0 C to constant weight and the weight of the dry solids were determined. Parallely, the residue in the tubes was weighed and the swelling power or water absorption index (WAI) was calculated.

Pasting characteristics: The native and DM samples (2 g) were mixed with 18 ml to obtain 10% (w/v) slurry and, the same was subjected to a controlled heating (8 min), cooking (6 min) and cooling (9 min) cycle under constant shear in a rapid visco-analyser (Newport Scientific, Warriewood, Australia) to determine the pasting characteristics (AACC, 2000).

Dough characteristics: The flour from the DM (50 g on 14% moisture basis) was mixed with 35 ml of cold water (30^oC) and the dough characteristics in terms of water absorption, mixing profiles, dough development time, water uptake and stability were recorded in a Brabender farinograph (AACC 2000). But, in the case of native flour, the farinograms were recorded after mixing the flour with cold water (30^oC) as well as hot water (90^0C) separately.

Cooking time: About 10 g of DM kernels were dropped in 100 ml boiling water in a beaker and heating was continued. A few grains were withdrawn from the beaker, initially at 1 min interval upto 3 min and at every 30 sec subsequently and pressed between two glass slides. The spread ability as well as the translucency of the spread was observed by passing a light beam from the bottom, and the time taken for attaining constant diameter of the spread with full translucency was taken as cooking time.

RESULTS AND DISCUSSION

The deglummed millet used for the studies was of mild brick red color and contained about 6.3 % protein, 1.5 % fat, 72 % starch and 19.6 % dietary fibre and 2.2% minerals. The 1000 kernel weight and volume of the millet were 2.9 g and 2.1 ml respectively.

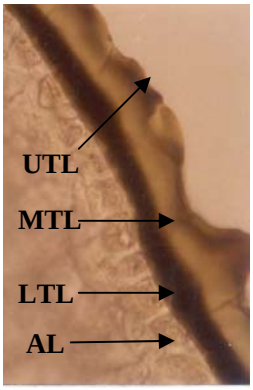
Light microscopy

The microscopic examination of the millet sections revealed the presence of multilayered testa, unilayered aleurone layer with rectangular cells, germ with scutellar epithelial cells, and starch granules compacted in endosperm cell walls.

The millet sections were viewed as such using a bright field microscope to discriminate the different layers of the seed coat prior to staining with specific dyes. The seed coat was multilayered and appeared reddish brown due to the presence of anthocyanins. McDonough et al 1986 who studied the structural details of the millet kernel in detail have also reported the presence of multilayered testa (five layer), beneath which the unilayered aleurone layer comprising of rectangular cells linking between the endosperm and seed coat exists. The sections stained with $FeCl_3$ developed characteristic dark green color revealing the presence of polyphenols in the different anatomical parts of the millet. However, the intensity of the dark green color varied among the different anatomical regions indicating that polyphenols are not distributed uniformly through out the kernel. The intensity of the color was higher in the outermost and lower testa layers, moderate in the middle testa layers (Figure 15a). The aleurone, germ (Figure 15b) and also the endosperm cell walls (Figure 15c) were also stained indicating the presence of polyphenols. In the case of sorghum also, it is reported that polyphenols are mainly concentrated in the testa layer and endosperm cell walls (Dykes and Rooney 2006).

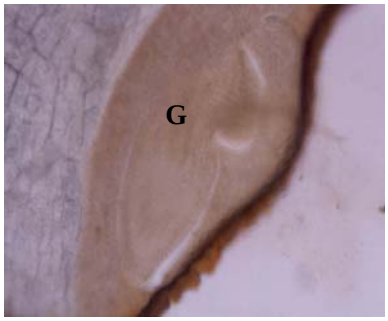
Many plant tissues contain a variety of substances which are naturally fluorescent (autofluorescent) under appropriate excitation wavelengths and the fluorescing tissues may be identified microscopically based on the color of the fluorescence. Polyphenols are the most common autofluorescent compounds in cereal grains and generally occur in cell walls of many tissues. The presence of polyphenols in the millet endosperm cell walls are depicted in (Figure 15d).

a



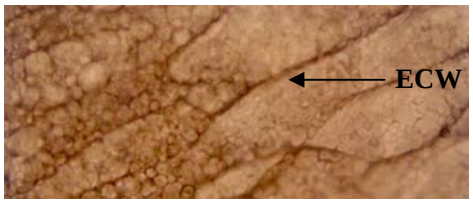
The concentration of polyphenols in the seed coat shown by the intensely stained region of the uppermost testa layer (UTL) and lower testa layers (LTL). MTL-Middle testa layer

b



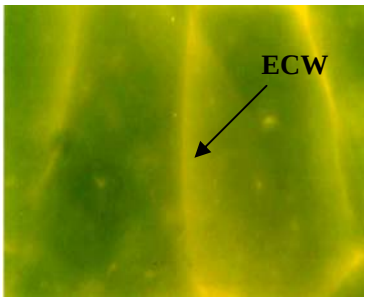
Intensely stained germ (G) showing the presence of polyphenols in the scutellar cells

c



Presence of polyphenols in endosperm cell walls (ECW)

d



Autofluorescent polyphenols in the endosperm cell wall (ECW), Ex: 520, Em: 540nm

Figure 15. Histochemical localization of the polyphenols in the millet kernel sections by staining with $FeCl_3$ and also by autofluorescence

The histochemical examination also revealed the presence of phytate in the aleurone (Figure 16a), scutellum and germ (Figure 16b), by the intense fluorescence observed by staining with acriflavin-HCl. Cereal phytin is a deposit of myo-inositol hexaphosphate and associated cations usually containing calcium, magnesium and potassium. It occurs in the aleurone and scutellum of all cereals, and is the primary mineral reserve in the grain. In the earlier studies, the presence of phytin has been shown in the grain by polarizing optics or bright field stains such as Toluidine Blue O. These methods have been useful, but both had their own limitations, however, Acriflavine-HCl provides improved specificity for high resolution fluorescent detection of phytin crystals at longer wavelengths in higher contrast which can be detected as globoids (Fulcher 1982). The cells of aleurone and scutellar region are known to be principle storage organelles and the cells of the aleurone contain numerous droplets of lipid and aleurone bodies, the later also store protein and phytate. It has been reported that, phytin bodies are the primary mineral reserve in the germ. The scutellum, a major storage tissue, is similar to aluerone tissue in many ways and store large amounts of protein, lipids and phytin bodies. In the present study, the distribution of phytate in the millet kernel was clearly localized (Figures 16a, 16b).

Alizarin red S has been used as both bright field reagent and as a fluorochrome, and is known to stain calcium associated with phytin bodies (Krishnan et al 2001). Calcium associated phytate reserves stained deep red indicating its abundant presence in the aleurone cells (Figure 16c), and scutellar region of the germ (Figure 16d). The study clearly shows that, the millet aluerone layer and germ is a rich source of calcium associated phytate reserves.

Scanning electron microscopy

The scanning electron photomicrographs of the millet kernel and the SCM fraction are shown in Figures 17, 18.

The millet kernels have an undulated surface containing typical mounds (Figure 17a). The germ appears to be embedded in a shallow depression in the kernel (Figure 17b) and probably because of this, the germ is not easily detached during decortication which is described subsequently.

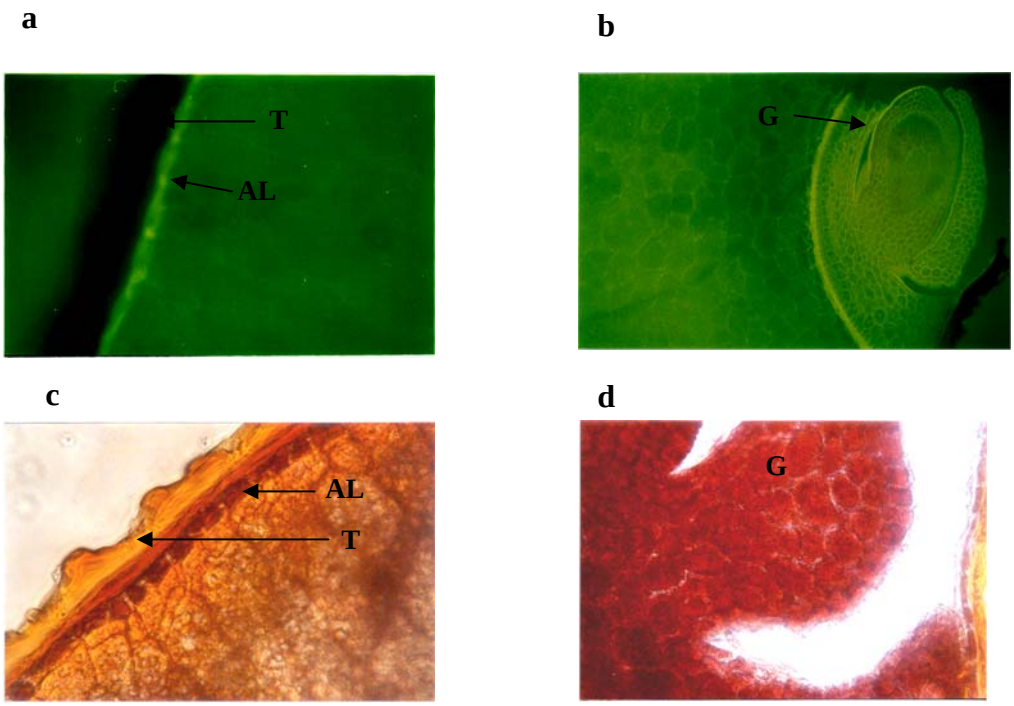


Figure 16. Histochemical localization of the phytate (stained with acriflavine HCl) and calcium associated phytate reserves (stained with Alizarin red S) in the millet kernel sections

a. Section showing the presence of phytate reserves with intense fluorescence in the aleurone layer (AL). T-testa layer (Ex: 520, Em: 540nm)
b. Section of germ (G) showing the presence of phytate with intense fluorescence (Ex: 520, Em: 540nm)
c. Section depicting the presence of calcium associated phytate reserves in the rectangular cells of the aleurone layer (AL). T-Testa
d. Section of germ (G) depicting the calcium associated phytate deposits in the scutellar epithelium

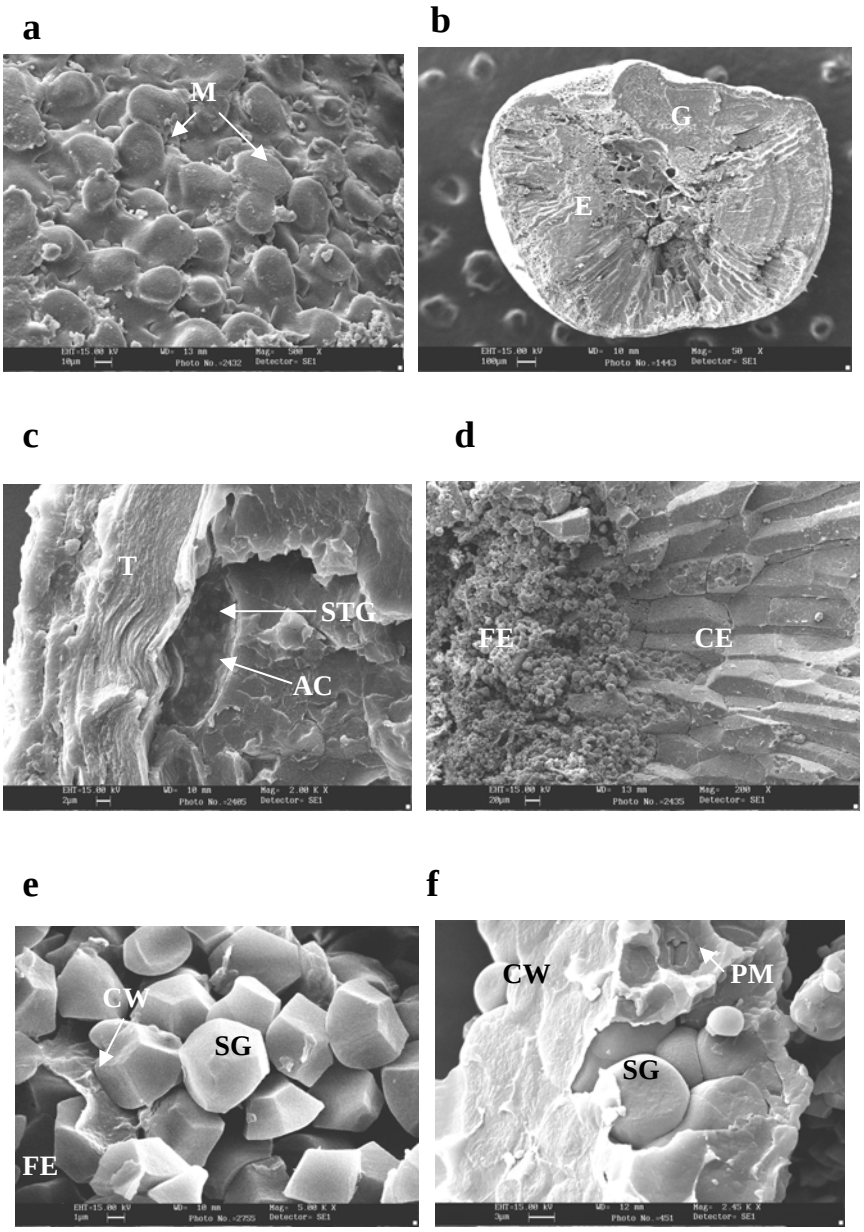


Figure 17. Scanning electron photomicrographs of native finger millet kernel

a. Surface mounds (M) on the surface - (500X), b. Cross section depicting the endosperm (E) and germ (G) - (50X), c. Cross section showing the testa (T) and aleurone cell (AC) with storage granules (STG) - 2.00 KX, d. Corneous (CE) and floury endosperm (FE) - 500X, e. Starch granules (SG) in the floury endosperm (FE), CW - cell wall (5.00 KX), f. An endosperm cell showing the cell wall (CW), starch granules and protein matrix (PM) - 2.45 KX

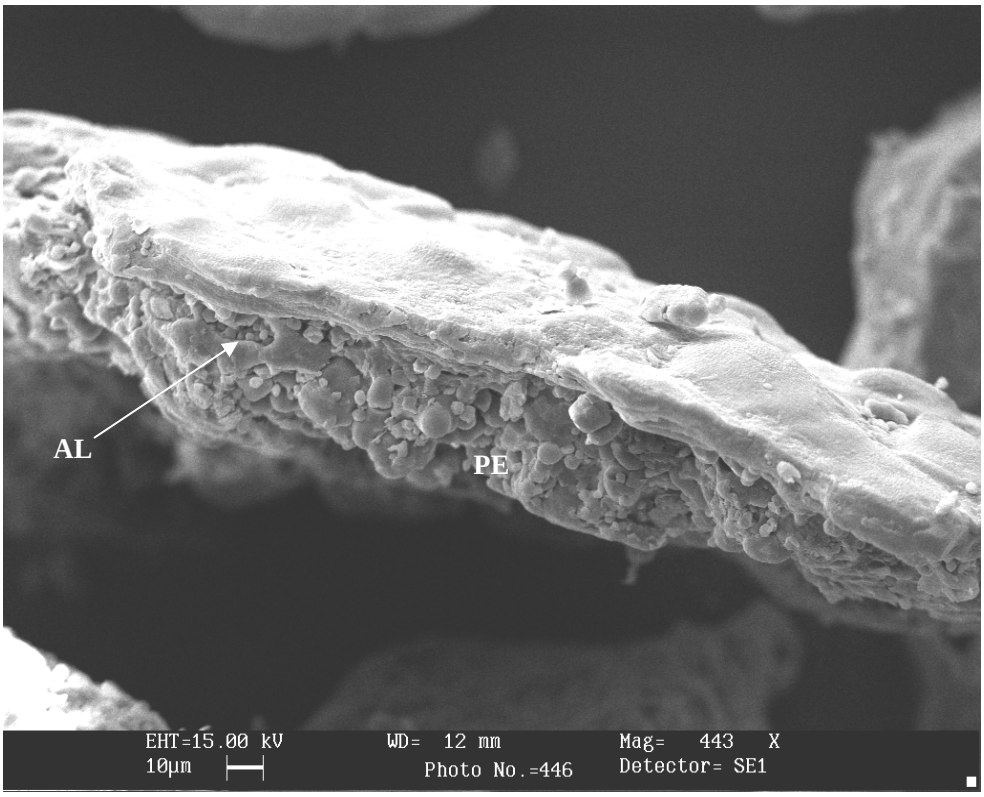


Figure 18. Scanning electron photomicrograph of finger millet seed coat matter (SCM) showing the presence of aleurone layer (AL) and peripheral endosperm (PE) - 443X

The testa is multi-layered with 15.6 ± 2 μ thickness, but the aleurone is unilayered with rectangular cells of about (20 × 8 μ dimensions) filled with spherical storage granules (2 ± 1μ) containing lipid, protein and phytate (Figure 17c). Figure 17d, depicts the cellular organization of the starch granules in the corneous and floury endosperm. The starch granules are of near hexagonal shape and are loosely packed in the floury endosperm region (Figure 17e), whereas, the granules are compacted inside the cells in the corneous endosperm region (Figure 17f) and the cell walls appear like a papery thin diaphragm, and cell organization is of hexagonal type and the protein matrix are distinctly visible (Figure 17f).

Figure 18 shows that, some portion of the aleurone layer as well as the peripheral endosperm remains adhered to the testa layers of the SCM fraction prepared by sequential milling and sieving methodology and this may be the possible reason for the higher phytate as well as polyphenol content of the SCM. Moreover, the presence of the peripheral endosperm might be the possible reason for higher yield (22%) of the SCM.

Yield and composition of the milling fractions

Separation of the seed coat following the conventional methods of cereal pearling or milling are not all applicable to the millet, because during milling, the entire kernel including the seed coat fragments into the finer particles. This is due to highly fragile nature of the millet endosperm and also due to the rigid attachment of the testa or seed coat with the endosperm. Hence, to prepare flour largely free from seed coat, incipient moist conditioning, grinding and sieving is usually suggested (Malleshi and Desikachar 1981).

The yield and composition of the refined flour (RF) and seed coat matter (SCM) are given in Table 11. The yield of the endosperm rich fraction (RF) was 78% and that of SCM was 22%. The RF was significantly whiter (Δ E 17.2) than the whole meal (WM) and SCM. The whiteness indicates the least contamination of the pulverized seed coat matter. The RF is mainly derived from the endosperm matter which may contain finer particles of aleurone and testa. This is clear by the lower levels of protein (3.6 %), fat (0.9 %), ash (1.1 %) and dietary fibre (6.8 %) content of RF. Generally, low level of ash in wheat flour reflects low levels of seed coat or bran content (Hatcher and Kruger 1997) and this may hold good for millet flour also. On the other hand, the SCM is highly colored (Δ E 53.6) and contained considerably higher proportion of protein, fat, ash and dietary fibre compared to the WM. The major nutrients in the RF are significantly lower than the WM, whereas, that present in the SCM are significantly higher than the WM. With respect to the contents of calcium and other minerals, including the major phytochemicals, a similar trend as that of major nutrient was observed.

On the other hand, the SCM contained about 18.6% starch, and nearly double the quantity of dietary fibre (47.7%) as well as the ash (5.6%) compared to that of WM (Table 11). This shows that, the SCM is largely the seed coat constituents and whatever starch it contains is mainly due to the peripheral endosperm portion adhering to the specks of the seed coat (Figure 18). These observations with respect to the nutrient composition are in agreement with the earlier studies on the nutrient contents of the millet and its milling fractions (Kurien et al 1959).

Table 11. Physico-chemical characteristics and phytochemical contents of refined flour (RF) and seed coat matter (SCM) of the millet

Parameter	WM	RF	SCM
Yield (%)	100	76 ± 2	22 ± 2
Color			
L*	66.1 ± 0.0	73.1 ± 0.4	56.0 ± 0.6
a*	2.1 ± 0.0	1.5 ± 0.1	6.3 ± 0.3
b*	9.1 ± 0.0	9.8 ± 0.1	5.5 ± 0.3
ΔE	26.2 ± 0.0	17.2 ± 0.7	53.6 ± 0.5
Moisture (%)	9.8 ± 1.0	11.0 ± 1.5	11.3 ± 1.3
Protein (g%)	8.7 ± 0.5	3.6 ± 0.5	13.6 ± 1.3
Fat (g%)	1.5 ± 0.3	0.9 ± 0.2	3.2 ± 0.2
Ash (g%)	2.2 ± 0.1	1.1 ± 0.2	5.6 ± 0.2
Starch (g%)	72.0 ± 1.0	87.0 ± 2.0	18.6 ± 1.0
Dietary fibre (g%)			
Insoluble	16.1 ± 0.5	5.2 ± 0.6	43.8 ± 0.5
Soluble	3.5 ± 0.1	1.6 ± 0.1	3.9 ± 0.2
Total	19.6 ± 1.2	6.8 ± 0.9	47.7 ± 2.0
Calcium (mg%)	321 ± 10	163 ± 7	830 ± 15
Phosphorus (mg%)	201 ± 6	106 ± 7	526 ± 5
Iron (mg%)	3.3 ± 0.2	0.33 ± 0.3	5.9 ± 0.2
Zinc (mg%)	1.60 ± 0.3	1.32 ± 0.2	2.84 ± 0.3
Copper (mg%)	0.94 ± 0.1	1.2 ± 0.5	2.73 ± 0.2
Potassium (mg%)	472 ± 7	469 ± 9	502 ± 9
Total Polyphenols[a] (mg%)	2300 ± 9	1480 ± 3	11200 ± 7
Tannins[b] (mg%)	840 ± 9	243 ± 2	2305 ± 7
Flavonoids[c] (mg%)	136 ± 7	106 ± 5	209 ± 7
Phytate (mg%)	217 ± 6	69 ± 4	685 ± 9

WM - whole meal, RF - refined flour, SCM - seed coat matter

* values are average±SD of three independent determinations

[a]gallic acid equivalents
[b]catechin equivalents
[c]catechin equivalents

The data presented in Table 11, reveals that, it is possible to prepare the millet flour with high starch and low fibre contents following the protocol developed. The RF, being mainly starchy in nature, may find its application for special foods such as custards, because of the highly desirable gelling properties of millet starch besides, it may also be suitable for bakery products and noodles. The SCM being a good source of dietary fibre may find use in high fiber foods.

Phytochemicals

Phenolics, tannins, phytates and flavonoids: The polyphenol content of the WM (2.3%), RF (1.48%) and the SCM (11.2%) indicates that, the SCM contains around 70% of the kernel polyphenols (Figure 15a, Table 11) which was evidenced in microscopic studies also. Similarly, phytate content of the SCM (685 mg%) is almost three times higher than the WM (217 mg%), and three fold higher than RF (69 mg%). Hence, the seed coat fraction along with the aleurone layer may serve as a good source of polyphenols and phytate as evidenced by their distribution in microscopic studies (Figure 15, 16). In the millet kernels, the lower most seed coat (testa) layer is completely fused with the aleurone layer and while milling, the seed coat tends to separate along with the aleurone (Figures 17c, 18) and this may be the possible reason for increased levels of phytate in the SCM. Fractionation of the millet by sequential milling - sieving methodology, enabled preparation of the fraction rich in polyphenols and phytate contents (SCM) as well as the fraction of the millet largely free from these phytochemicals (RF) which are known to bind the minerals and reduce their bioavailability.

In most of the cereals, the concentration of phytochemicals including polyphenols and phytate are reported to be concentrated in the SCM (Naczk and Shahidi 2006, Oberleas 1983). This also holds good with the millet since the analytical data (Table 11) clearly showed that the polyphenols and phytate content of the SCM is much higher than the WM as well as the RF. The millet SCM contains good amount of protein in addition being a rich source of calcium, dietary fibre and health beneficial phytochemicals such as polyphenols including flavonoids, tannins, phytate, hence, the SCM may serve as a functional ingredient in functional foods as they may impart several health benefits to the consumers.

Fractionation and identification of phenolics: The fractionation of the polyphenols extracted by acidic methanol solvents system from WM, RF and SCM through reverse phase HPLC indicated the presence of several phenolic acids. The constituent phenolic acids are gallic, protocatechuic, gentisic, caffeic, vanillic and ferulic acid (Figure 19). The HPLC chromatogram indicated the presence of several other peaks and hence, efforts were made to identify them by direct infusion electrospray ionization mass spectrometry (ESI-MS). Since ESI-MS has been extensively used for the identification of phenolic compounds from legumes, wine extracts, yerba mate and green tea (Moller et al 2005, Markowicz et al 2007), it was used for the identification of the millet phenolics also. The ESI-MS finger prints of polyphenol extracts of WM, RF and SCM shown in Figure 20, indicate the presence of numerous phenolic compounds with different molecular weights ranging from 100-2000. Phenolic compounds identified based on their m/z values from ESI-MS finger print are given in Table 12. The presence of trans-cinnamic acid (Molecular weight 148), and also that of a few phenolics identified by HPLC were confirmed from the mass spectrum. Structure of some of the common simple phenols and phenolic acids present in the cereals are given in Figure 21.

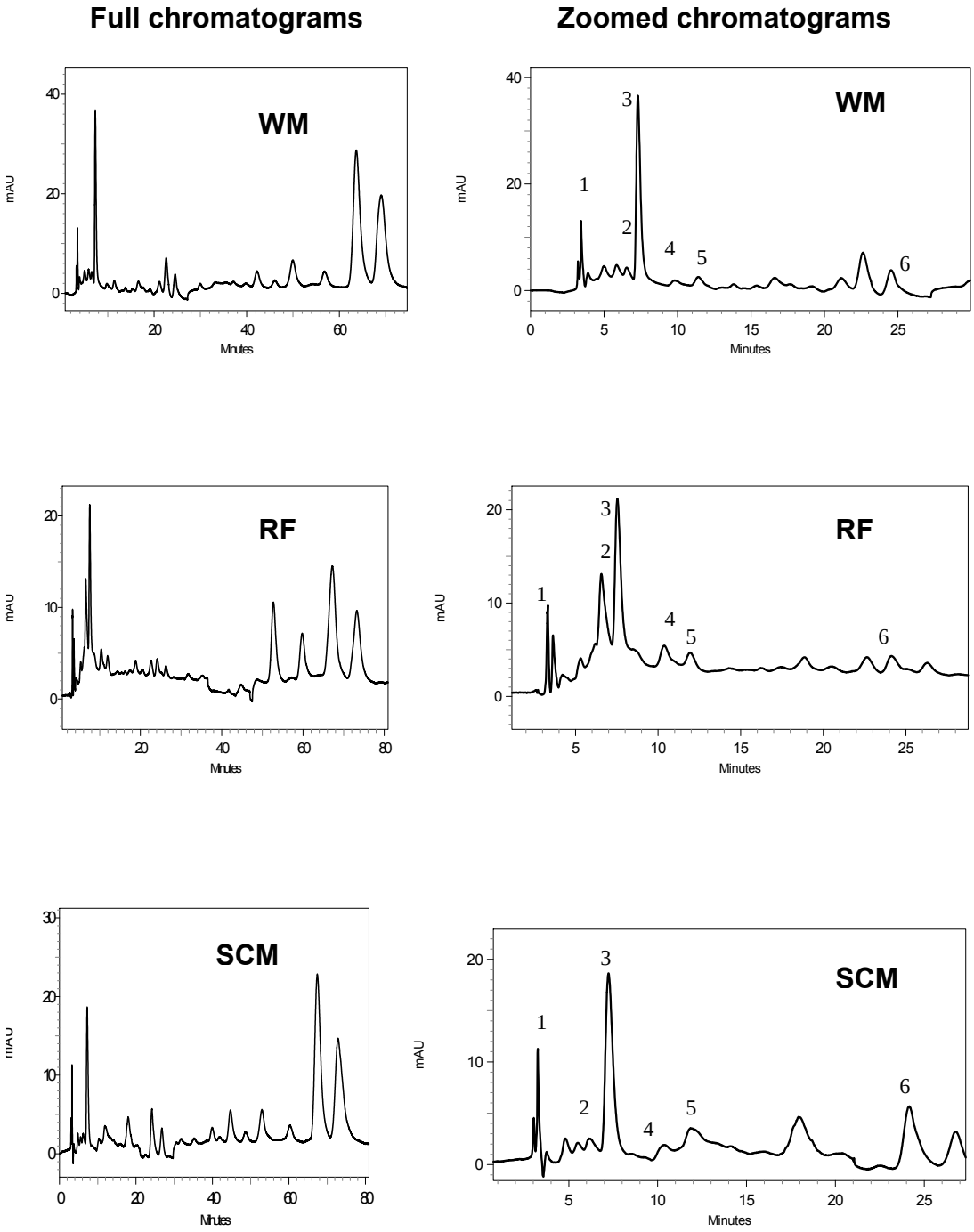


Figure 19. HPLC Chromatograms (full and zoomed) of polyphenols from acidic methanol extracts of whole meal (WM), refined flour (RF) and seed coat matter (SCM) at 320 nm

In zoomed chromatograms, Peak No. 1. gallic acid, 2. protocatechuic acid, 3. gentisic acid, 4. caffeic acid, 5. vanillic acid, 6. ferulic acid

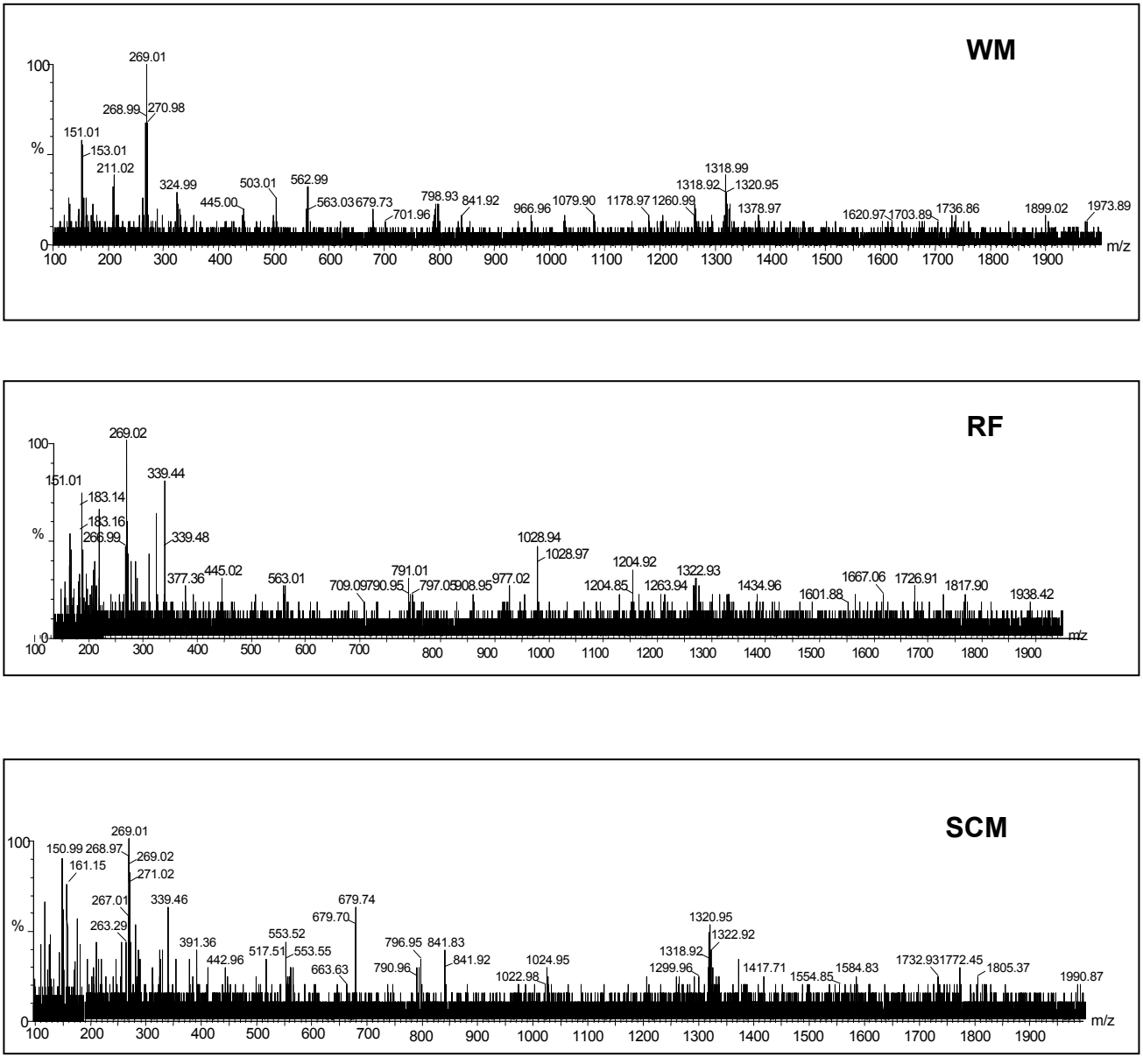
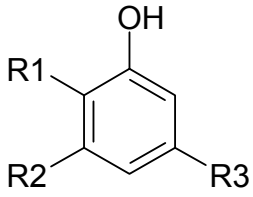


Figure 20. ESI-MS spectra (full spectra) of polyphenols from acidic methanol extracts of the millet whole meal (WM), refined flour (RF) and seed coat matter (SCM)

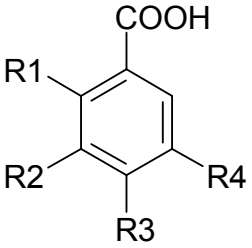
Table 12. Phenolic compounds identified from the zoomed ESI-MS spectra of acidic methanol extracts of whole meal (WM), refined flour (RF) and seed coat matter (SCM)

Phenolic compound	$[M-H]^-$ (m/z)*	Sample		
		WM	RF	SCM
Gallic acid	169.1	+	+	+
Gentisic acid	153.1	+	+	+
Protocatechuic acid	153.1	+	+	+
Syringic acid	197.6	+	-	+
Transcinnamic acid	147.1	+	+	+
Caffeic acid	179.2	+	+	+
Ferulic acid	193.3	+	+	+
P-hydroxy benzoic acid	137.1	+	+	+
4-*o*-methyl gallic acid	183.2	+	-	+
Kaempferol	285.2	+	+	+
Naringenin	271.0	+	+	+
Phloroglucinol	125.1	+	+	+
Apigenin	269.0	+	+	+
(+)-Catechin/ (-)-Epicatechin	289.3	+	+	+
Luteolin glycoside	447.0	+	+	+
Trans feruloyl-malic acid	308.2	-	-	+
Dimer of prodelphinidin (2GC)	609.7	+	-	+
Diadzein	253.4	+	+	+
Catechin gallates	440.9	+	+	+
Trimers of catechin	865.6	-	-	+
Tetramers of catechin	1153.9	-	-	+



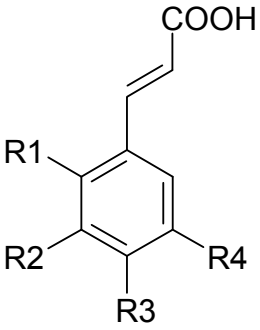
Phenol: $R_1=R_2=R_3=H$
Catechol:$R_1=OH$, $R_2=R_3=H$
Resorcinol: $R_2=$ OH, $R_1=R_3=H$
Phloroglucinol: $R_2=R_3=OH$, $R_1=H$

Simple phenols



Gallic: $R_1=H$, $R_2=R_3=R_4=OH$
Gentisic:$R_1=R_4=OH$, $R_2=R_3=OH$
Salicylic: $R_1=$ OH, $R_2=R_3=R_4=H$
p- (OH)- benzoic: $R_1=R_2=R_4=H$, $R_3=OH$
protocatechuic: $R_1=R_4=H$, $R_2=R_3=OH$
Syringic: $R_1=H$, $R_2=R_4=OCH_3$, $R_3=OH$
Vanillic: $R_1=R_4=H$, $R_3=OH$, $R_2=OCH_3$

Hydroxy benzoic acids



p- coumaric: $R_1=R_2=R_4=H$, $R_3=OH$
Caffeic:$R_1=R_4=H$, $R_2=R_3=OH$
Ferulic: $R_1=R_2=H$, $R_4=OCH_3$, $R_3=OH$
Sinapic:$R_1=H$, $R_2=R_4=OCH_3$, $R_3=OH$

Hydroxy cinnamic acids

Figure 21. Structure of some common phenolic acids in cereals

The mass spectra indicated that, the millet phenolics are complex in nature and contained phenolic compounds belonging to different classes ranging from simple phenol (phloroglucinol), phenolic acids including benzoic acid (gallic, gentisic, protocatechuic, syringic, *p*-hydroxy benzoic acid) and cinnamic acid derivatives (transcinnamic, caffeic, ferulic acid), 4-*0*-methyl gallic acid, flavones (apigenin,

luteolin glycoside), flavanones (naringenin), flavonols (kaempferol), trans-feruloyl-malic acid, flavanols [(+)-catechin/(-)-epicatechin, dimer of prodelphinidin (2GC)], Isoflavones (diadzein), catechin gallates, trimers and tetramers of catechin. The chemical structures of some of the above mentioned phenolic compounds are given in Figure 22. Significant differences among the polyphenol profile of the RF and SCM were not observed and the possible reason could be due to the contamination of the seed coat matter in the RF. Dissection of seeds by hand have been widely practiced for effective separation of SCM and the endosperm components in many of the cereals compared to milling-sieving methods, which are crude ways for the separation of botanical components of cereals. Moreover, micro quantities of phenolic compounds contributed by the SCM in the RF are detected in the mass spectra. Presence of simple phenols, phenolic acids, anthocyanins, flavan-4-ols, flavones, flavanones, flavonols, dihydroflavonols, proanthocyanidin monomers, dimers, polymers have been reported in several cereals (Dykes and Rooney 2007). The characterization of polyphenols extracted from the millet SCM indicates the presence of very large number of constituent phenolics. The number of phenolics identified in the present study (19 Nos.) is probably the highest number reported for the millet so far to the best of our knowledge. The phenolic compounds identified in the millet are in general known to possess proven health benefits with respect to antioxidant, anticancer, anti-diabetic properties and some of the health benefits derived by the millet consumers may be partly attributed to the presence of these nutraceuticals.

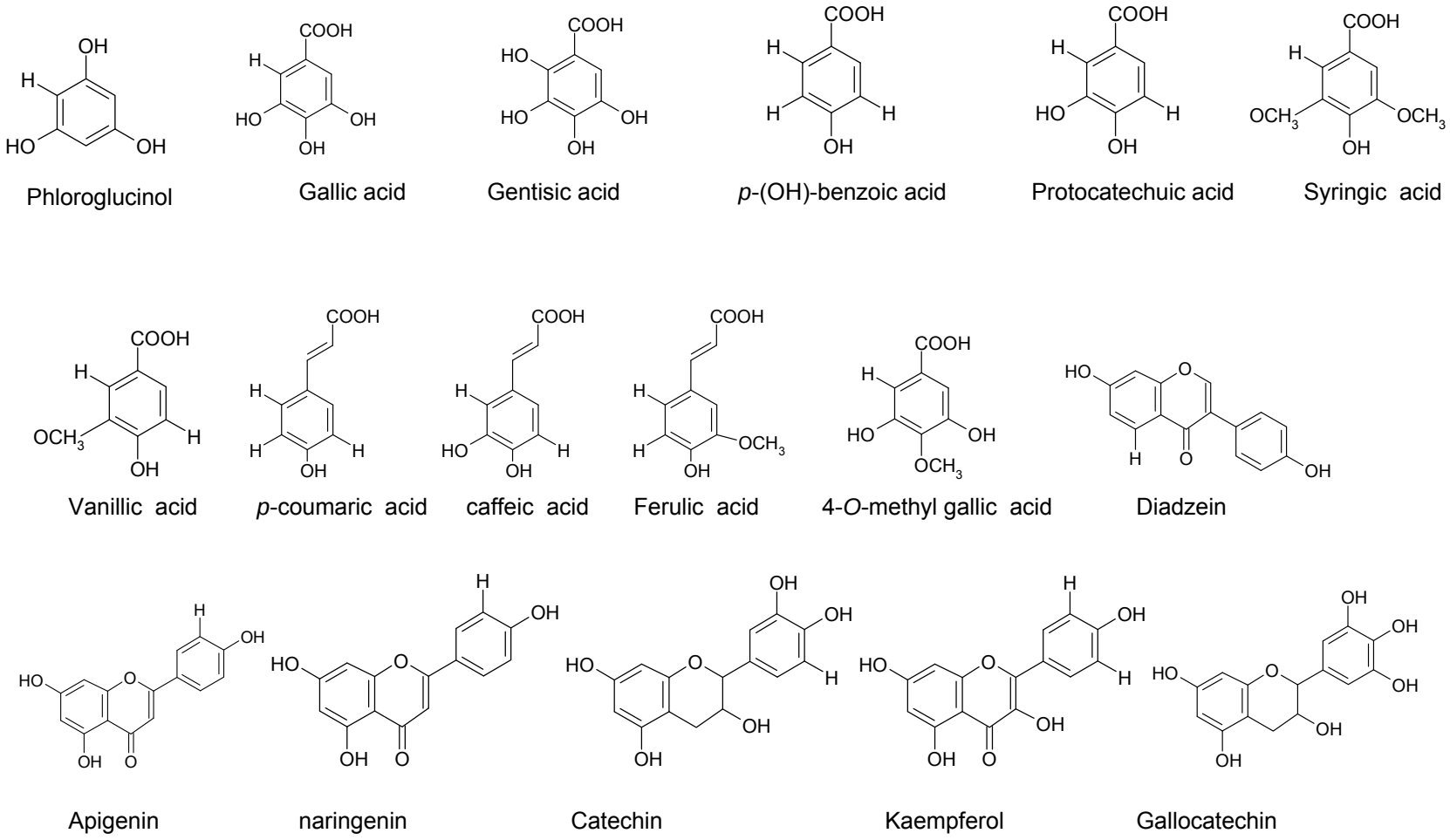


Figure 22. Structures of phenolic compounds identified in finger millet seed coat polyphenol extract

Antioxidant properties: The SCM exhibited higher TAA and RSA compared to the RF as well as the WM. The TAA of the SCM was nearly 2.4 times higher than WM (207, 86 mM ascorbic acid eq TAA/g flour for SCM and WM respectively) and 3.6 fold higher compared to RF (Table 13). Although, the RF contains very low proportion of polyphenols still it exhibited moderate TAA and RSA. The TAA and RSA of the RF may be mainly due to the polyphenols derived from the endosperm cell walls (Figures 15c,15d).

The DPPH scavenging ability directly correlated with the polyphenol, tannin and flavonoid contents (R^2= 0.92, R^2=0.90, R^2= 0.85) of the WM, RF and the SCM.

The methanolic extracts of the millet exhibited a slow kinetic DPPH radical scavenging behaviour compared to the rapid kinetic behaviour of ascorbic acid (Figure 23). Similar observations were reported by Yu et al (2002) for three hard winter wheat varieties. Moreover, Brand Williams et al (1995) reported higher DPPH radical scavenging ability for polyphenols compared to monophenols and they also reported highest radical scavenging ability for gallic acid (a triphenol) followed by gentisic, caffeic, protocatechuic and ferulic acids. The HPLC chromatograms (Figure 19) of the acidic methanolic extracts of the millet samples revealed the presence of gallic, protocatechuic, gentisic, vanillic, caffeic and ferulic acids. These phenolic acids are known to exhibit slow kinetic behaviour. Hence, the radical scavenging properties of the millet may be attributed to the synergistic action of these constituent phenolics.

Sripriya et al (1996) investigated the antioxidant properties of polyphenols extracted with methanol which was able to quench about 77% of hydroxyl radicals. However, they did not report the constituent phenolics of the methanolic extract. In the current study, HPLC profile and the mass spectrum (data not presented) indicated the presence of several phenolic compounds including gallic, gentisic, protocatechuic, syringic and transcinnamic acid in the methanolic extracts. Hegde and Chandra (2005a) reported higher DPPH scavenging ability for the kodo millet compared to great millet, finger millet, little millet, foxtail millet and barnyard millet. Zielinski and Kozlowska (2000) studied the antioxidant activity and total phenolics in selected cereal grains and their different morphological fractions and reported higher

antioxidant and phenolic contents in the bran layers which was true in the present study also.

The study clearly indicates that, the polyphenols and phytate contents of the seed coat play an important role in the radical scavenging ability of the millet. Hence, the millet seed coat, which is a by-product of millet refining, malting and decortication industries, could be a highly valuable ingredient for functional foods.

Table 13. Antioxidant properties of the millet refined flour (RF) and seed coat matter (SCM)*

Sample	TAA	RSA	ICA
WM	86 ± 3	67 ± 3	64 ± 7
RF	58 ± 5	50 ± 4	48 ± 4
SCM	207 ± 6	84 ± 3	79 ± 3

WM - whole meal, RF- endosperm rich fraction/refined flour, SCM - seed coat matter

* values are average ± SD of three independent determinations

TAA: Total antioxidant activity (mM ascorbic acid equivalent TAA per g flour)

RSA: Radical scavenging ability [% radical scavenged per 10 mg flour (t=20 min)]

ICA: Iron chelating ability (mM EDTA equivalent ICA per g flour)

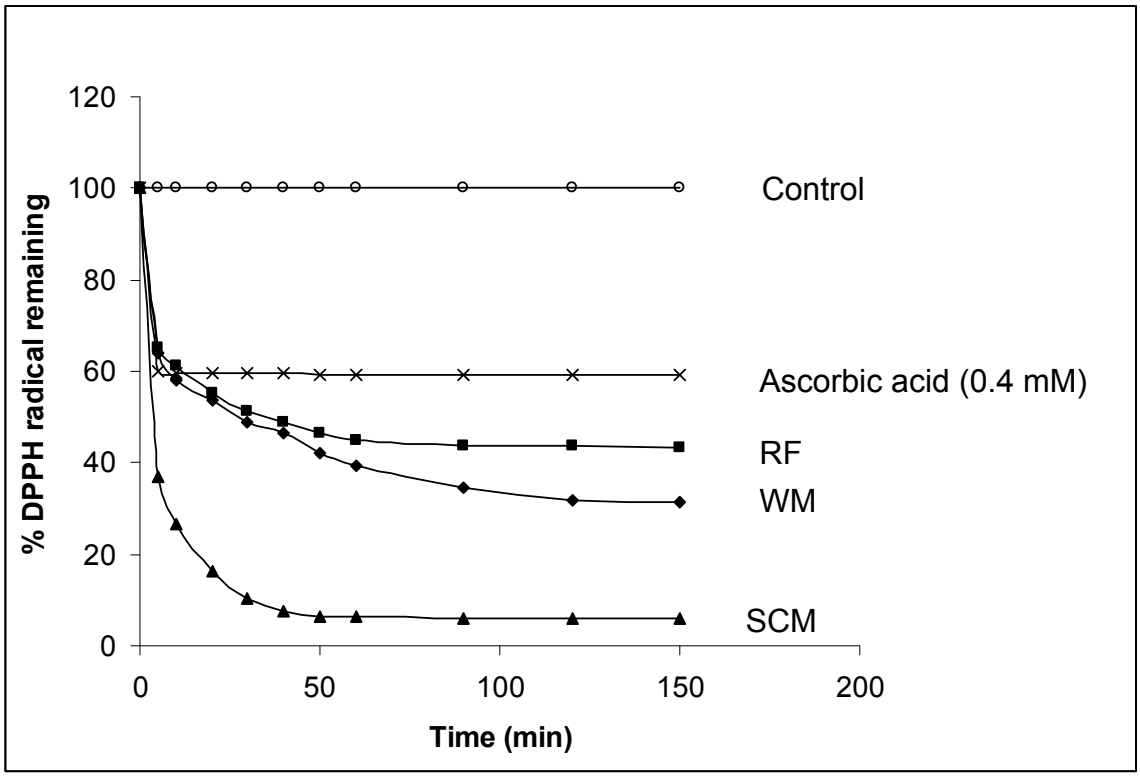


Figure 23. DPPH radical scavenging activity of the millet whole meal (WM), refined flour (RF), and seed coat matter (SCM)

Hydrothermal processing and decortication

Hydrothermal processing and drying of the millet caused changes in the physico-chemical and functional properties of the millet. Among the physical features, darkening of the color and slight distortion in the shape were prominent (Figure 24). Hydrothermal processing caused significant textural and physico-chemical modifications in the hydrothermally processed millet (HTM). While, darkening or the browning could be due to polymerization of phenolics and pigments such as anthocyanins, the undulations on the surface could be due to the desorption of water and also due to the cementing of the endosperm components during drying the kernels. The decorticated millet (DM) was of cream color and devoid of seed coat material and the seed coat matter prepared by decortication of HTM (SCM-HTM) was of dark brown color (Figure 24).

It was observed that, the material dried to 12±2% moisture content at about 40^{o}C was slightly mellowable and less friable and could withstand the impact during milling whereas, the material dried at temperatures higher than 50^{0}C was highly brittle and breaks into grits during decortication. Accordingly, the steamed millet dried in a cross flow drier maintained at 40^{0}C was more suitable for decortication. About 5 Kg batch of the steamed millet was also sun dried and it was noted that the morphology of the sun-dried material was comparable to the mechanically dried (40^{0}C) sample. In the case of rice also, it has been reported that, drying the steamed rice at temperatures higher than 60^{0}C induces the cracks and causes large-scale breakage during milling (Henderson 1954).

The preliminary investigations on the decortication of the HTM dried at different temperatures to varying moisture levels, indicated that, both drying conditions as well as the moisture content of the dried material influence decortication characteristics. Rapid drying of steamed kernels by exposing them to temperatures higher than 50^{0}C causes steep moisture gradient between the interior and surface portions in the kernel till major quantum of moisture is removed and as a result, slight peripheral expansion and contraction at the interior of the grain occur. This manifests in compressive stress at the surface and tensile stress at the centre leading to development of cracks in the grains (Kunze 2001).

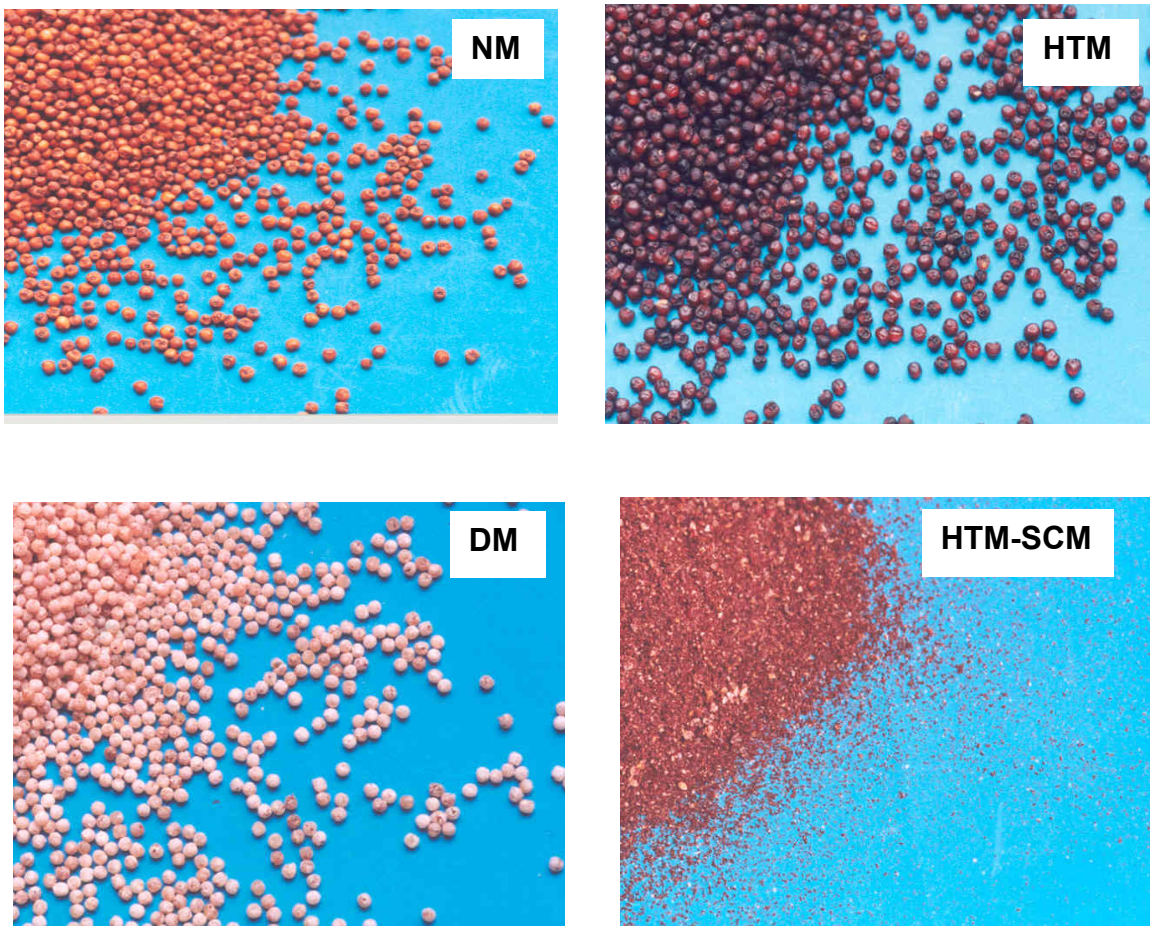


Figure 24. Effect of hydrothermal processing on the morphological features of native millet (NM), hydrothermally processed millet (HTM), decorticated millet (DM) and seed coat matter from hydrothermally processed millet (SCM-HTM)

On the other hand, drying at about 40^0C or lower than that, causes slow movement of moisture within the kernel and it does not cause extensive fissuring. Dehusking and debranning experiments of the HTM in Engleburg huller, McGill miller, horizontal cone polisher, barley pearler and similar cereal milling machinery working on the principles of abrasion or friction were not fruitful. On the other hand, decortication in horizontal disc mill wherein both the discs are embedded with carborundum (about 20 grade) was found effective. This could be due to the peeling of the seed coat while, the grains spin between two emery coated discs. Further, it was noticed that, moistening the surface of the HTM by spraying with 6$\pm$0.5% additional water and tempering for a short time (5-10 min) was highly advantageous with respect to decortication of the millet. Incipient moistening not only softens the seed coat but also renders it leathery and soft and gets scrapped between the twin carborundum discs of the mill. While, less than 4% additional water was insufficient to moisten the seed coat effectively, addition of more than 8% moisture not only caused stickiness and lump formation among the grains, but also caused elongation of the kernels to some extent during milling. Size grading the millet to near uniformity improved the decortication characteristics considerably and reduced the breakage of the kernels. The milling fractions consisted of the decorticated head grains, grits and the seed coat. The degree of decortication (% of the kernel removed mainly in the form of seed coat during milling) was 15±3% and the yield of head grains and grits or the brokens were 82±5% and 3±2% respectively (Table 14). The millet was decorticated to 15$\pm$3% degree of decortication because, lesser degree of decortication yielded grains with some portion of testa layer remaining with the millet kernels and affected the appearance, cooking as well as its eating qualities. Milling the millet to higher than 15$\pm$3% degree of decortications resulted in removal of endosperm portion also. Since, the germ portion of the millet is located in a slightly depressed portion of the kernel surrounded by a characteristic ridges (McDonough et al 1986), it remained intact with the decorticated grains even at 15% degree of decortication. The decorticated material was of near spherical shape and cream colored with glossy and translucent look (Figure 24) and could be cooked similar to rice.

Table 14. Influence of incipient moist conditioning on the yield of decorticated millet (DM)

Parameter	Decortication without moist conditioning	Decortication after moist conditioning
Steaming time (min)	20 ± 0.5	20 ± 0.5
Steaming temperature (^{o}C)	98 ± 1	98 ± 1
Drying temperature (^{o}C)	40±2	40±2
Additional moisture (%)	-	6.0
Yield (g%)		
Decorticated head grains	45 ± 5	82 ± 5
Brokens (grits)	35 ± 3	3 ± 2
Seed coat matter	20 ± 2	15 ± 3

Yield values are average ± standard deviation of three independent milling experiments

The seed coat material prepared by hydrothermal processing was comparatively darker than the seed coat matter prepared by sequential milling-sieving methodology (Figure 24).

The scanning electron microscopy of the HTM and the DM grains indicated that, the DM was nearly spherical compared to the irregular shape of HTM (Figures 25a and b). The DM was completely devoid of the mounds typically present in the seed coat of the native and HTM grains (Figure 17a, Figures 25b, c and d) and this may be due to the removal of testa during decortication. The hydrothermal processing increased the intactness of testa and aleurone with the endosperm (Figure 25e). This is in contrast to other cereal grains, wherein, the hydrothermal treatment normally loosen the endosperm and the seed coat and in addition, the starch granules lost their granular structure, probably due to complete gelatinization caused by hydrothermal processing (Figure 25f). The aleurone layer also on hydrothermal processing, undergoes compression and complete loss of its typical rectangular shape (Figure 25g). Moreover, the aleurone layer is removed during decortication. Further, hydrothermal processing caused a significant reduction in the thickness of the testa or seed coat layer (15.6±2 μ for native millet and 8±4 μ for the HTM respectively). This reduction may be due to the loss of vacuolar spaces that may be present in between the testa layers in the native millet during hydrothermal processing. Major portions of the germ were retained even after hydrothermal processing and decortication (Figures 25g and h).

Unlike other cereal grains, the germ in the millet is located in the groove of the millet and probably its intactness with endosperm increases with hydrothermal processing and hence it is not normally detached after decortication.

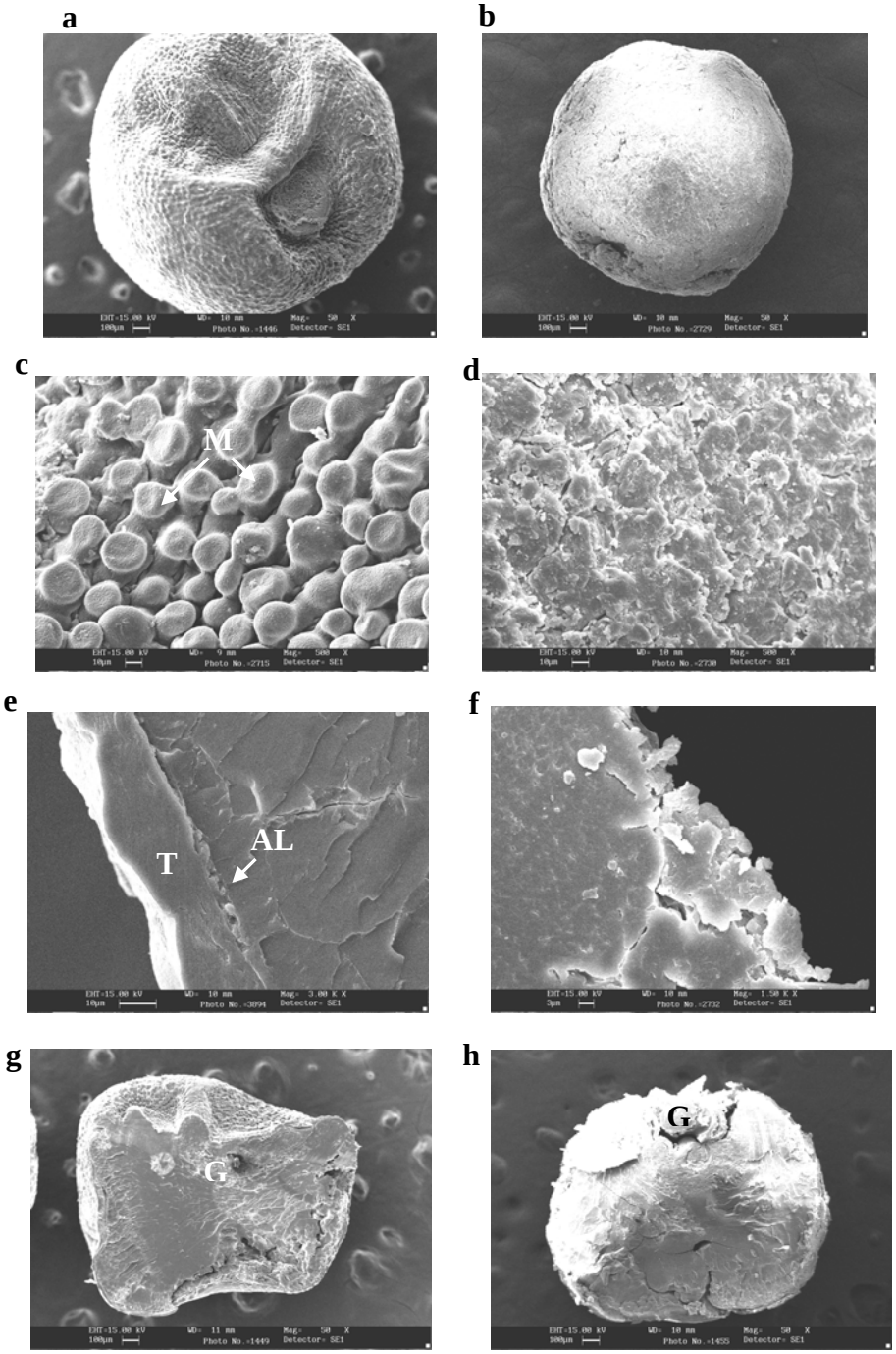


Figure 25. Scanning electron photomicrographs of hydrothermally processed and decorticated millet

a. Hydrothermally processed millet (HTM) (50X), b. Decorticated millet (DM) (50X), c. Surface mounds (M) on the seed coat of HTM (500X), d. DM surface - complete loss of seed coat (500X), e. Fused seed coat and aleurone of the HTM (3.00 KX), f. DM (complete loss of seed coat). g. Cross section of HTM (50X) showing germ (G), h. Cross section of DM (50X) showing germ (G)

The status of the polyphenols and phytate in the HTM and DM was examined by both chemical and histochemical methods. Microscopically it was observed that, the polyphenols concentration continues to be in the peripheral layers and germ in the case of HTM. It was interesting to note that, the inward migration of phenolics from the testa and aleurone layer into the peripheral endosperm occurred during hydrothermal treatment (Figure 26a). This could explain the possible reason for the pale brown color of the decorticated millet. However, the presence of polyphenols in the germ were noticed in both HTM and DM (Figure 26b).

Partial degradation of calcium associated phytate reserves of the aleurone layer (Figure 27a) was noticed, but retention of the phytate reserves in the germ of both the HTM and DM (Figure 27b) were also observed. Degradation of phytate during hydrothermal processing has also been reported in cereals such as wheat, rye, barley and oats (Fredlund et al 1997). From this study, it can be observed that, even after decortication, the decorticated millet contained some portion of the seed coat polyphenols in addition to the endosperm phenolics. Hence, the nutraceutical value of the millet to some extent is retained even after decortication.

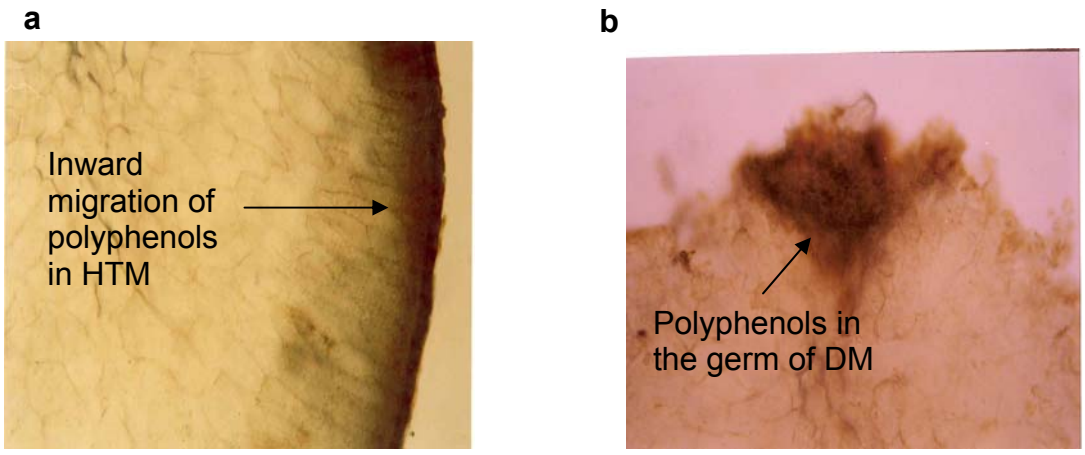


Figure 26. Histochemical localization of polyphenols ($FeCl_3$ staining) in hydrothermally processed (HTM) and decorticated millet (DM) sections

a. Inward migration of polyphenols (dark green coloured) from testa and aleurone towards peripheral endosperm in the hydrothermally processed millet (HTM), b. Polyphenols in the germ of the decorticated millet (DM)

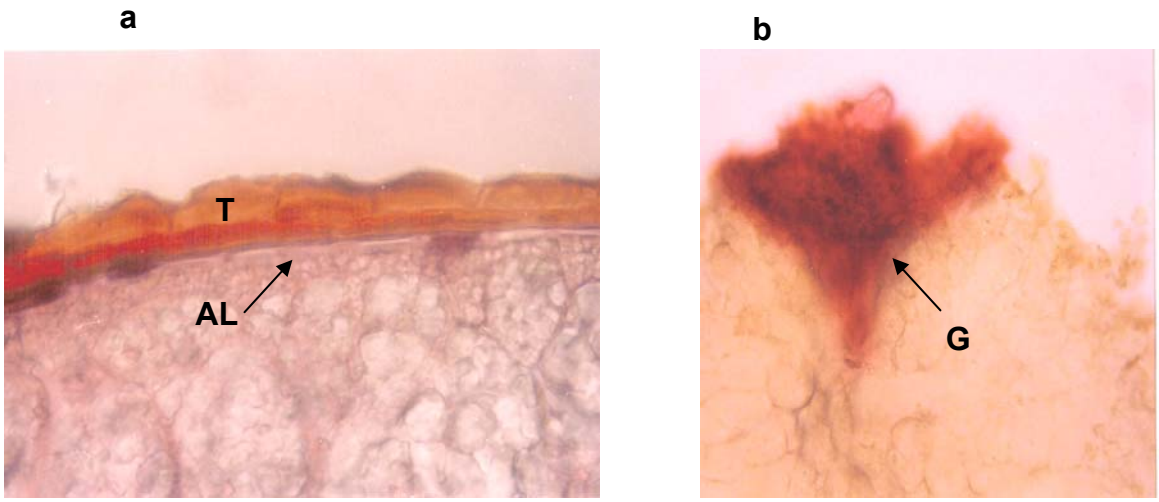


Figure 27. Histochemical localization of calcium associated phytate reserves (Alizarin red S staining) in the hydrothermally processed (HTM) and decorticated millet (DM)

a. Partially degraded calcium phytate reserves in the aleurone layer of hydrothermally processed millet (HTM). AL- aleurone, T-testa
b. Retention of calcium associated phytate reserves in the germ (G) of decorticated millet (DM).

Phytochemicals

Hydrothermal processing of the millet caused a 40% decrease in the total polyphenol content, whereas, decortication of the same caused a 65% decrease in the total polyphenol content (Table15). The decrease in the total polyphenol content of the HTM could be attributed to the loss of water soluble phenolics during soaking and also due to decrease in the extractability of polyphenols caused by hydrothermal processing. As processing at higher temperatures are known to cause degradation of phenolic compounds and also decrease the extractability by the formation of complexes with macromolecules or maillard products and in addition, loss of phenolic compounds during soaking of rice in water prior to parboiling has been reported elsewhere (Ramalingam and Antoniraj 1996). The changes in the phenolic content of the cereals during processing has been studied by several workers and the loss of phenolics on soaking and cooking has been established (Towo et al 2003). Moreover during extrusion cooking (a kind of hydrothermal processing) of cereals such as wheat, barley, rye and oats, Zielinski et al (2001) reported an increase in the phenolic acid content and indicated that, hydrothermal processing of cereals may liberate phenolic acids and their derivatives from the cell walls. However, Haslam (1981) reported that, during hydrothermal processing, phenolics form complexes with proteins and carbohydrates through hydrogen bonds and hydrophobic interactions whereas, proanthocyanidins form covalent bonds with proteins leading to the decrease in the total assayable polyphenol content. The seed coat (testa) represents the bulk of polyphenols in the millet (Mcdonough et al 1986, Figure 15a) as well as in other cereals (Naczk and Shahidi 2006). The loss of polyphenols in the case of decorticated millet is due to the removal of the polyphenol rich tissues of the millet, namely the testa and aleurone layers.

Ramachandra et al (1977) also observed that, dehulling of the high tannin finger millet varieties reduced their tannin contents by 80% whereas, our reports (Shobana and Malleshi 2007) indicate that, hydrothermal treatment and decortication of finger millet decreases polyphenol content by 74.7%. Towo et al (2003), reported 19, 21 and 41% loss in total phenolic content of the millet on soaking in water, germination and cooking respectively.

Table 15. Phytochemical contents of hydrothermally processed (HTM), decorticated millet (DM) and seed coat matter (SCM-HTM)

Sample	Phytochemicals (mg%)*			
	Total Polyphenols	Tannins	Flavonoids	Phytate
NM	2300 ± 9	840 ± 9	136 ± 7	217 ± 6
HTM	1380 ± 7	233 ± 5	68 ± 4	153 ± 4
DM	807 ± 9	115 ± 9	63 ± 3	75 ± 5
SCM-HTM	7900 ± 10	1119±9	173±6	534±7

NM - native millet, HTM - hydrothermally processed millet, DM - decorticated millet, SCM - HTM - seed coat matter prepared by decortication of HTM

* values are average ± SD of three independent determinations

Polyphenols (gallic acid equivalents)
Tannins (catechin equivalents)
Flavonoids (catechin equivalents)

The same authors also reported a decrease in total phenolics, catechols and resorcinol contents of the millet on soaking in $NaHCO_3$, magadi-soda and lactic acid solutions and also during germination and cooking. Beta et al (2005) and Pathirana et al (2006) indicated that, pearling of wheat caused a significant reduction in the phenolic content and antioxidant activity. Fermentation and dehulling of pearl millet is known to cause a significant reduction in total polyphenol content (El Hag et al 2002).

Hydrothermal processing and decortication of the millet caused an 73% and 86% reduction in the tannin contents respectively (Table 15). Chibber et al (1978) in their studies indicated that, dehulling of sorghum resulted in a 98% reduction in tannin content whereas, Ramachandra et al (1977) reported 80% reduction of tannin contents on dehulling of the millet. Processing or treatment techniques including soaking in different media (Beta et al 2000), cooking (Lyimo et al 1992), germination (Mibithi-Mwikya et al 2000) and fermentation (Usha and Chandra 1998) have been shown to reduce tannin levels in grains to various extent.

The flavanoid content of the millet was around 126 mg% which reduced to 68 and 62.5 mg% on hydrothermal processing and decortication respectively (Table 15). Hilu et al (1978) reported the presence of flavonoids such as orientin, isoorientin, vitexin, saponarin, violanthin, leucin, tricin in the millet. Decortication is known to reduce the flavonoid content in pearl millet (Akingbala 1991). The author attributed the decrease to the removal of pericarp tissue, which contains greatest concentration of flavonoids in the kernel. Hence, this holds good for the millet also as most of the phenolics are concentrated in the peripheral tissue.

Hydrothermal processing and decortication caused a 30 and 65% reduction in the phytate contents of the millet. Fermentation, dehulling, germination, cooking methods have also been known to reduce phytate contents in grains (El Hag et al 2002, Mibithi-Mwikya et al 2000, Duhan et al 2002). Hence, the lower levels of the phytate contents in the decorticated millet (Table 15) could be attributed to the thermal degradation of phytate during hydrothermal processing and also due to the removal of aleurone layer during decortication.

Fractionation and identification of polyphenols: Polyphenols extracted by acidic methanol solvents system from HTM, DM, SCM-HTM on fractionation through

reverse phase HPLC indicated the presence of phenolic acids such as gallic, protocatechuic, gentisic, caffeic, vanillic and ferulic acids along with some unidentified peaks (Figure 28). The ESI-MS finger prints of HTM, DM, SCM-HTM polyphenol extracts shown in Figure 29, indicate the presence of numerous phenolic compounds with molecular weights ranging from 100-2000 similar to native millet out of which only 19 phenolic compounds could only be identified. Phenolic compounds identified based on their m/z values from the zoomed ESI-MS finger prints are given in Table 16.

The mass spectra indicated the presence of simple phenol (phloroglucinol), phenolic acids including benzoic acid (gallic, gentisic, protocatechuic, syringic, p-hydroxy benzoic acid) and cinnamic acid derivatives (transcinnamic, caffeic, ferulic acid), 4-*0*-methyl gallic acid, flavones (apigenin, luteolin glycoside), flavanones (naringenin), flavonols (kaempferol), trans-feruloyl-malic acid, flavanols [(+)-catechin/(-)-epicatechin, dimer of prodelphinidin (2GC)], Isoflavones (diadzein), catechin gallates, trimers and tetramers of catechin similar to that of native millet whole meal (WM) and SCM. The study clearly indicates that, hydrothermal processing and decortication did not cause a drastic change in the polyphenol composition except with the loss of few phenolics (Table 16). Even though the RF, DM were devoid of seed coat matter, their polyphenol composition were almost similar to that of WM and HTM (Tables 12 and 16). The phenolic compounds present in the DM may be partly attributed to the inward penetration of phenolic compounds from the periphery (seed coat and aleurone) to the endosperm regions during hydrothermal processing (Figure 26a).

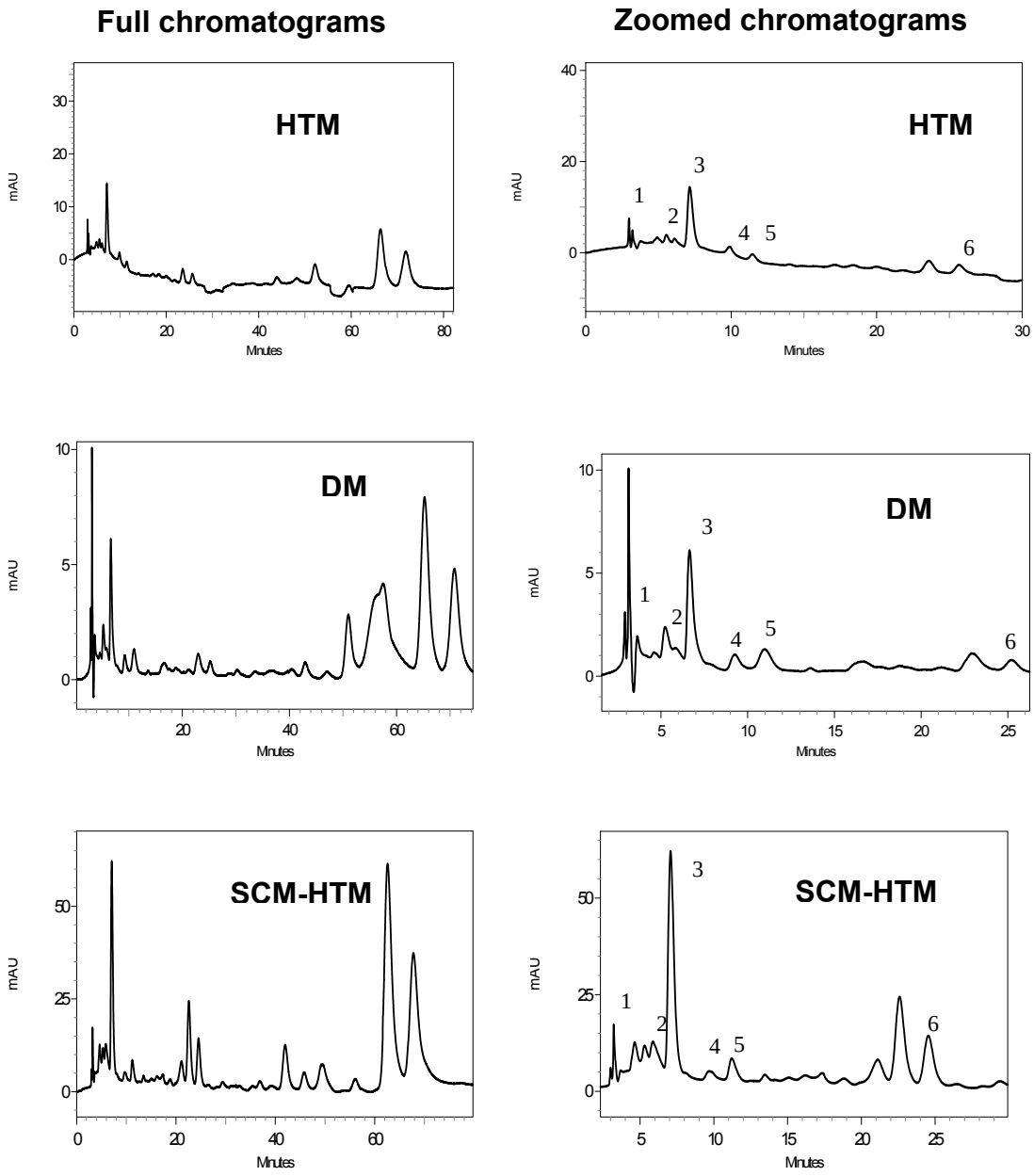


Figure 28. HPLC chromatograms (full and zoomed) of polyphenols from acidic methanol extracts of hydrothermally processed millet (HTM), decorticated millet (DM) and seed coat matter (SCM-HTM) at 320 nm

In zoomed chromatograms, Peak No. 1. gallic acid, 2. protocatechuic acid, 3. gentisic acid, 4. caffeic acid, 5. vanillic acid, 6. ferulic acid

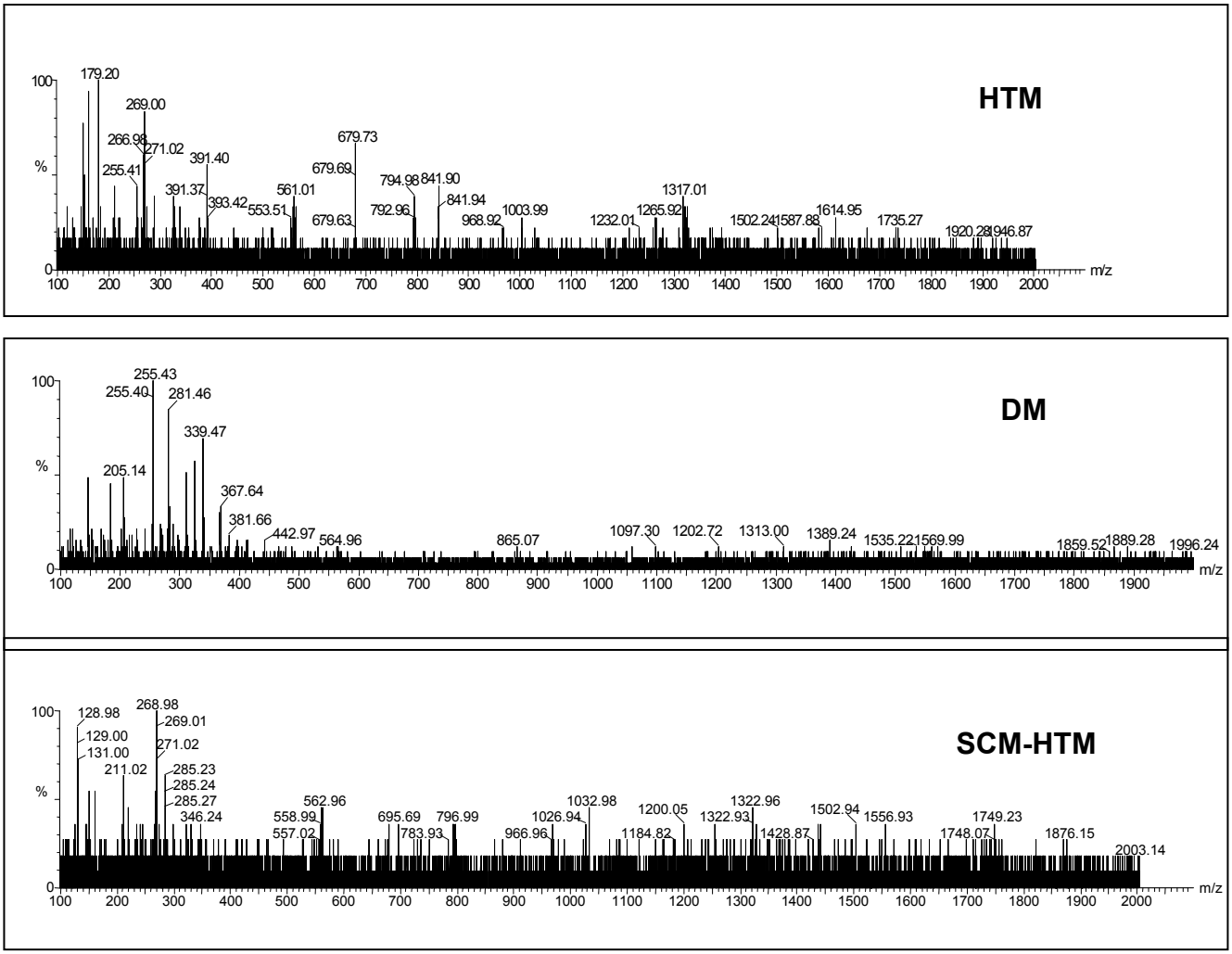


Figure 29. ESI-MS spectra (full spectra) of polyphenols from acidic methanol extracts of hydrothermally processed (HTM), decorticated millet (DM) and seed coat matter (SCM-HTM)

Table 16. Phenolic compounds identified from the zoomed ESI-MS spectra of acidic methanol extracts of hydrothermally processed millet (HTM), decorticated millet (DM) and seed coat matter (SCM-HTM)

Phenolic compound	$[M-H]^-$ (m/z)*	Sample		
		HTM	DM	SCM - HTM
Gallic acid	169.1	+	+	+
Gentisic acid	153.1	+	+	+
Protocatechuic acid	153.1	+	+	+
Syringic acid	197.6	+	-	+
Transcinnamic acid	147.1	+	+	+
Caffeic acid	179.2	+	+	+
Ferulic acid	193.3	+	+	+
P-hydroxy benzoic acid	137.1	+	+	+
4-*o*-methyl gallic acid	183.2	+	+	+
Kaempferol	285.2	+	+	+
Naringenin	271.0	+	+	+
Phloroglucinol	125.1	+	+	+
Apigenin	269.0	+	+	+
(+)-Catechin/ (-)-Epicatechin	289.3	+	+	+
Luteolin glycoside	447.0	+	+	+
Trans feruloyl-malic acid	308.2	-	-	-
Dimer of prodelphinidin (2GC)	609.7	-	-	-
Diadzein	253.4	+	+	-
Catechin gallates	440.9	-	+	-
Trimers of catechin	865.6	+	+	+
Tetramers of catechin	1153.9	-	-	+

Antioxidant properties: Hydrothermal processing and decortication of the millet brought down the TAA (total antioxidant activity) by 47% and 66% respectively. This reduction in the TAA HTM may be due to the complexing of the phenolic compounds with other macromolecules such as carbohydrates etc., and hence rendering them less active. The SCM-HTM exhibited higher TAA and RSA ($DPPH^{\cdot}$ scavenging ability) compared to the HTM and DM (Table 17). Hydrothermal processing and decortication caused 47% (46 mM ascorbic acid eq TAA/g flour), 66% (29 mM ascorbic acid eq TAA/g flour) decrease in the TAA of the native millet whole meal or WM (86 mM ascorbic acid eq TAA/g flour) respectively. The kinetics of $DPPH^{\cdot}$ scavenging ability of HTM, DM and SCM-HTM are presented in Figure 30, which clearly indicates that SCM-HTM exhibited potent radical scavenging ability compared to the HTM and DM and the possible reason for higher RSA of SCM-HTM may be attributed to its higher polyphenol contents.

The SCM-HTM exhibited highest ICA (iron chelating ability) compared to HTM and DM (Table 17). This characteristic feature of the SCM-HTM may be advantageous with respect to its utilization in the antioxidant rich food supplements as iron chelators are inhibitors of Fenton reactions (iron catalysed oxidation reactions). Phytate and tannins are potential metal chelators, hence the higher ICA of the SCM may be due to higher levels of phytates and tannins in the SCM (Table 15).

Table 17. Antioxidant properties of the hydrothermally processed millet (HTM), decorticated millet (DM) and seed coat matter (SCM-HTM)*

Sample	TAA	RSA	ICA
HTM	46 ± 2	36 ± 5	50 ± 4
DM	29 ± 3	30 ± 6	41 ± 5
SCM-HTM	179 ± 9	70 ± 4	69 ± 3

*values are average ± SD of three independent determinations

TAA: Total antioxidant activity (mM ascorbic acid equivalent TAA per g flour)

RSA: Radical scavenging ability [% radical scavenged per 10 mg flour (t=20 min)]

ICA: Iron chelating ability (mM EDTA equivalent ICA per g flour)

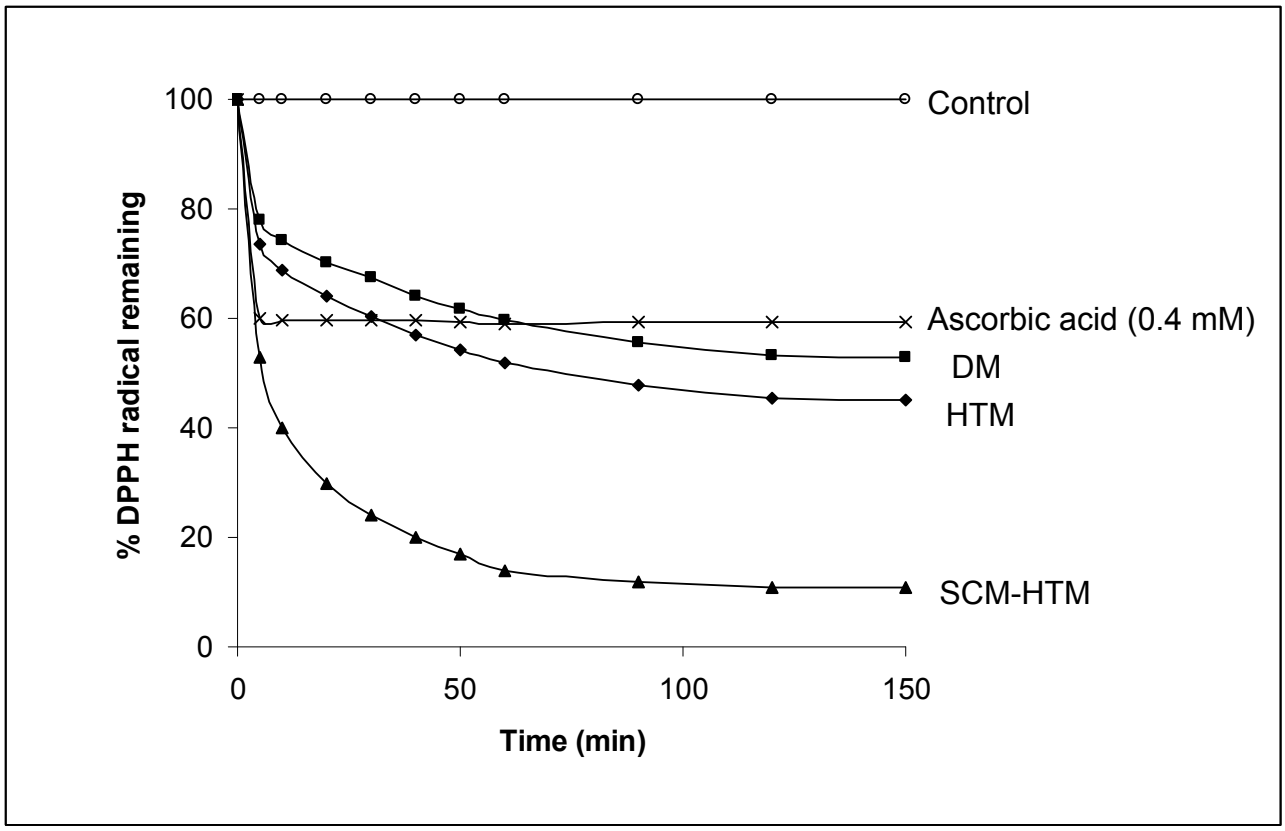


Figure 30. DPPH radical scavenging ability of hydrothermally processed millet (HTM), decorticated millet (DM) and seed coat matter (SCM-HTM)

Physico-chemical characteristics of hydrothermally processed (HTM) and decorticated millet (HTM)

The hydrothermal treatment increased the hardness of the millet from 1.1 to 7.1 kg/cm^2, measured in terms of breaking or crushing strength of the kernels. The bulk density of the DM was slightly higher than the native millet (NM), probably due to removal of the seed coat, and also due to the reduced porosity in the kernel (Table 18). In the case of rice also, similar observations have been reported (Pillaiyar and Mohandoss 1981).

Protein, fat, calcium and phosphorus contents of the DM were 6.3, 0.9, 0.18 and 0.10% and were lower by 22, 40, 43 and 48% respectively than the NM (Table 18), whereas the seed coat matter prepared by hydrothermal processing (SCM-HTM) was darker (Figure 24) and contained 18.1 ± 2.0 starch, 10.1 ± 0.4% protein, 3.5 ± 0.4 % fat, 4.9 ± 0.1 minerals and 50.2 ± 1.0 % dietary fibre (Table 19). The reduction in some of the nutrients could be mainly due to separation of the seed coat, as it has been reported that, the seed coat contains about 28% of protein, 49% calcium and 14% phosphorus (Kurien et al 1959). Similar observations have been made by Subrahmanyan et al (1938), on parboiling of rice and by Serna-Saldivar et al (1994) on sorghum. Decortication reduced the dietary fibre content of the millet by about 33.2% but at the same time, the proportion of soluble fibre content increased considerably. The increase in the soluble fibre content of the product has special nutritional significance because of its physiological advantages in terms of hypoglycemic and hypocholesterolemic characteristics. Formation of the resistant starch also contributes towards dietary fibre content and complements the health benefits of the millet. The loss of nutrients including the dietary fibre could be regulated by judicious milling. McDonough et al (1986) reported that testa layer of the millet is highly pigmented and contributes for the bulk of polyphenols and tannin content of the millet, and hence, significant reduction in the polyphenol contents occurs on decorticating the millet. Similarly, phytate phosphorus is located largely in the scutellum and to a smaller extent in the aleurone cells (Wada and Maeda 1980, Pore and Magar 1979) and in view of that, reduction in phytate phosphorus occurs on decortication of the millet.

Table 18. Physicochemical characteristics of the native (NM), hydrothermally processed (HTM) and decorticated millet (DM) (dry weight basis)*

Parameter	NM	HTM	DM
Appearance	Spherical	Slightly undulated	Spherical and opaque
Color (∆E)	Brown (68.9)	Dark brown (75.1)	Light cream (42.8)
Hardness (kg/cm^2)	1.3 ± 0.5	7.3 ± 0.4	7.0 ± 0.6
1000 kernel wt (g)	2.76 ± 0.4	2.94 ± 0.5	2.2 ± 0.5
1000 kernel volume (ml)	2.1 ± 0.1	2.0 ± 0.2	1.5 ± 0.2
Apparent density (×10^3 Kg/m^3)	1.312 ± 0.02	1.335 ± 0.03	1.469 ± 0.03
Protein (g%)	8.7 ± 0.7	8.3 ± 0.5	6.5 ± 0.6
Fat (g%)	1.4 ± 0.2	1.3 ± 0.3	0.8 ± 0.2
Starch (g%)	71.5 ± 2.0	68.5 ± 3.2	81.3 ± 3.4
Dietary fibre			
Total dietary fibre (g%)	19.1 ± 1.2	18.1 ± 1.6	10.3 ± 1.8
Soluble dietary fibre (g%)	1.8 ± 0.6	0.9 ± 0.6	2.8 ± 0.5
Insoluble dietary fibre (g%)	17.3 ± 1.0	17.2 ± 1.2	7.5 ± 1.0
Minerals (g%)	2.3 ± 0.1	1.8 ± 0.1	1.1 ± 0.2
Acid insoluble ash (g%)	0.12 ± 0.02	0.08 ± 0.01	0.07 ± 0.01
Calcium (mg%)	321± 17	311 ± 12	185 ± 15
Phosphorous (mg%)	210 ± 8	160 ± 6	111 ± 10
Equilibrium moisture content (%), at 30^{0}C	33 ± 0.5	46 ± 1.0	55 ± 0.5
Solubility (%), at 30^{0}C	3.9 ± 0.5	7.4 ± 0.3	8.5 ± 0.4
Swelling (%), at 30^{0}C	70 ± 4	165 ± 8	190 ± 7
Swelling (%), at 80^{0}C	260 ± 8	263 ± 5	270 ± 11
Cooking time (min)	16 ± 1	17 ± 1	5 ± 1

* Average ± standard deviation of three independent determinations

∆E: Color deviation from standard.

Table 19. Nutrient composition of the seed coat matter prepared by decortication of hydrothermally processed millet (SCM-HTM) *

Parameter	SCM-HTM
Yield (%)	15 ± 2
Color (% whiteness, ∆E)	Dark brown, (3.2 ± 1, 78.6)
Moisture (g%)	12 ± 1.2
Protein (g%)	10.1 ± 0.4
Fat (g%)	3.5 ± 0.4
Starch (g%)	18.1 ± 2.0
Dietary fibre	
Total dietary fibre (g%)	48.1 ± 1.2
Soluble dietary fibre (g%)	2.1 ± 0.6
Insoluble dietary fibre (g%)	50.2 ± 1.0
Minerals (g%)	4.9 ± 0.1
Calcium (mg%)	850 ± 10
Phosphorus (mg%)	510 ± 12

* Average ± standard deviation of three independent determinations

∆E : Color deviation from standard.

Reduction in some of these constituents has nutritional advantages since, these are considered as anti-nutritional factors and their reduction improves the bioavailability of minerals (Erdman 1981).

Pasting characteristics

The pasting characteristics of the NM and DM flours recorded in the rapid visco-analyzer are presented in Figure 31. The DM exhibited noticeable (7.5 RVU) cold paste viscosity, which remained more or less constant during heating, cooking and cooling phases. From the viscograms, it may be noted that gelatinization temperature (76º C), and the peak viscosity (180 RVU) of the millet are typical to cereals where as the pasting characteristics of the DM were similar to precooked starchy foods.

Dough Characteristics

The dough prepared by mixing the NM flour with cold water was of very poor strength and consistency (Figure 32a), whereas, that prepared with hot water (Figure 32b), exhibited moderate consistency and dough strength (500 BU in 1.5 min), but collapsed after 9 min of mixing. The poor dough characteristics of the NM prepared by mixing with cold water is due to very low hydration characteristics of raw starch but, addition of hot water to the millet flour and mixing immediately, partially gelatinizes the starch and as a result the adhesiveness of the flour particles increases, and this is reflected by the dough consistency during initial stages of mixing. The flour from the DM absorbed 64% cold water, formed dough of good strength and reached to near 500 BU consistency within 1 min and dropped gradually to 300 BU after mixing for 9 min (Figure 32c). The high water holding capacity of the DM may be due to the presence of the gelatinized starch and its, stability could be due to intermolecular adhesiveness of the starch. The dough characteristics of the DM exhibits characteristics of pre-gelatinized flour and also indicates its suitability for *chapathi* (unleavened pan cake) and bakery products.

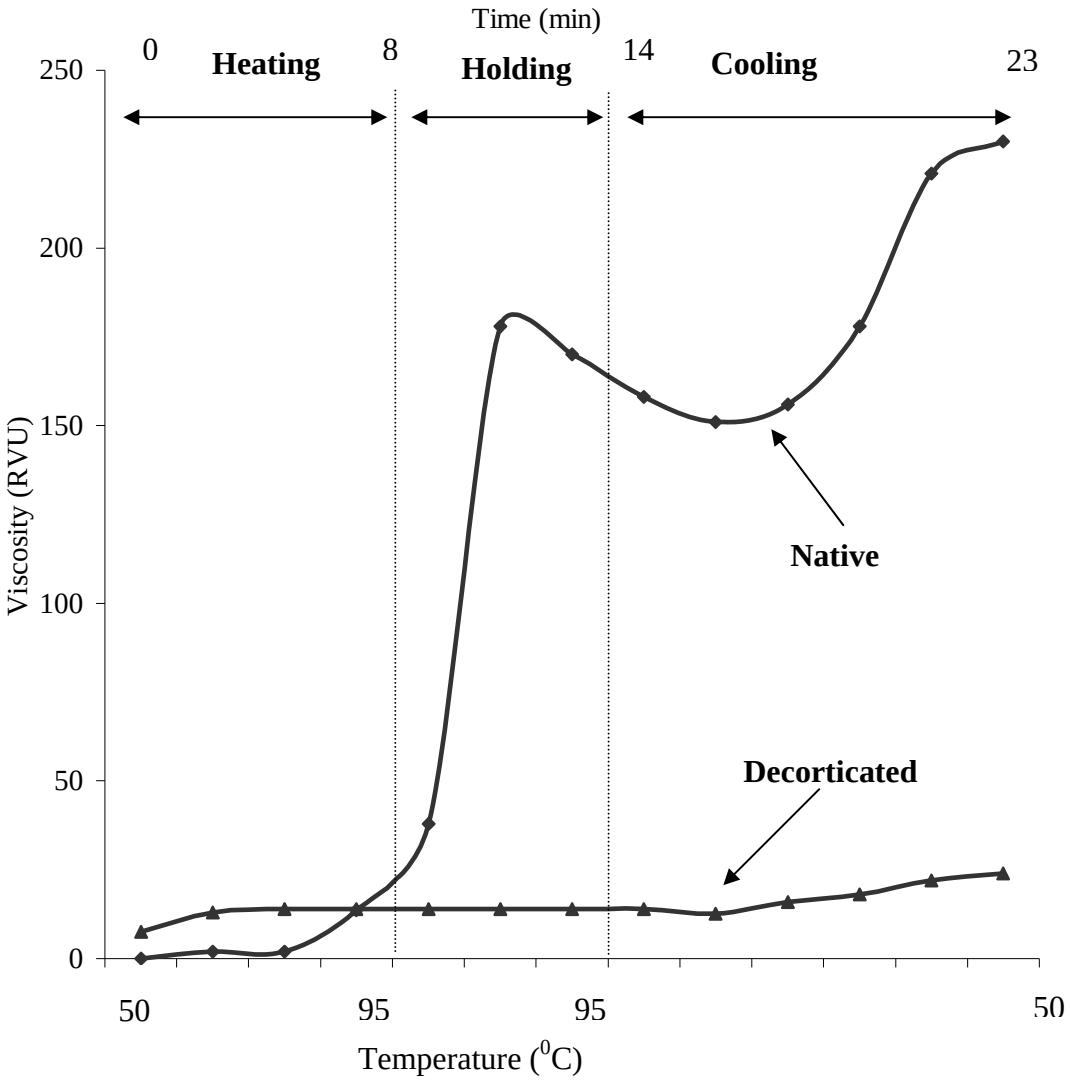


Figure 31. Pasting characteristics of native (NM) and decorticated millet (DM) determined in rapid visco-analyser at 10% (w/v) concentration

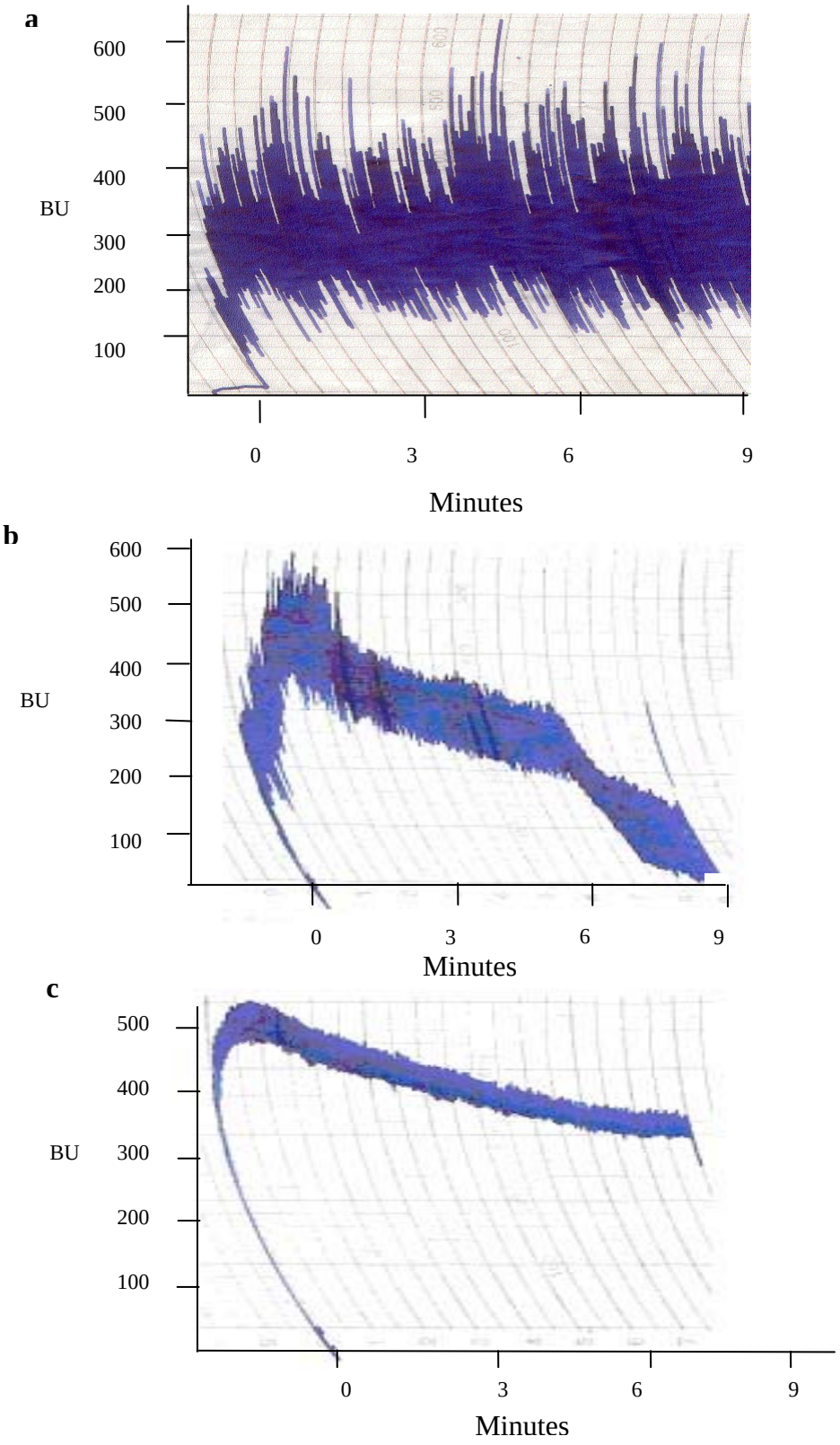


Figure 32. Farinograms of native (NM) and decorticated millet (DM)
a. NM (cold water), b. NM (hot water), c. DM (cold water)

Cooking characteristics

The DM cooks to soft textured discrete grains within 5 minutes when dropped into boiling water and the cooked millet retain the discreteness and spherical shape of the kernels (Figure 33). This enables its consumption along with *sambar* (traditional pulse based spicy gruel) similar to rice or after seasonings with spices and can also be used to prepare snacks, savory/sweet dishes. Processing of the DM for preparation of the expanded millet similar to expanded rice (Ushakumari et al 2007) and utilization of expanded millet as adjunct in confectionery have been highly promising. The DM could be size graded to semolina or flour for utilization similar to wheat and can also be used for the preparation of traditional millet products such as *mudde* (stiff porridge) *roti* (unleavened pancake) and a*mbali* (gruel). Exploratory experiments on utilization of the millet flour in bakery products, as a thickener in soups and beverages, and also in the form of flaked and puffed product have been encouraging. Hence, the product has potential as a new food material for the non-traditional millet consumers world wide.

Figure 33. Cooked decorticated finger millet (DM)

Comparative quality characteristics of the seed coat matter from the native (SCM) and hydrothermally processed millet (SCM-HTM)

The differences in the nutrient and phytochemical contents of the millet endosperm (RF, DM) and seed coat matter prepared by sequential milling and sieving method (SCM) and by decortication of hydrothermally processed millet (SCM-HTM) are indicated in Table 20. The study clearly brought out the differences in the endosperm rich matter as well as the SCM prepared by the two different methods. The RF contained lower protein (3.7 g%) and higher starch content (87 g%) compared to DM (6.5% protein and 82% starch). Considerable differences in the nutrient composition between the RF and DM could be due to the difference in the methods adopted for their preparation, namely, moist conditioning, grinding, sieving (sequential milling and sieving) in the former and the latter by decortication of hydrothermally processed millet. In the case of RF, since the starch content is higher, the protein content is lower, as most of the seed coat matter is separated out by sieving. But in the case of DM, some portions of the peripheral tissues may be attached, contributing to the higher protein content. On the other hand, the nutrient composition of the SCM and SCM-HTM were nearly comparable. However, the phytochemical contents including the polyphenol content of the SCM-HTM was lower (7.9% polyphenols) compared to the SCM (11.2 g% polyphenols). The SCM of the native millet also contained higher levels of tannins, flavonoids and phytate compared to the SCM-HTM. The lower levels of polyphenolic constituents of SCM-HTM could be due to complexing of phenolics with proteins and carbohydrates through hydrogen bonds, hydrophobic interactions during processing (Haslam 1981). Apart from this, proanthocyanidins form covalent bonds with proteins possibly decreasing the total assayable polyphenols content. Reduction in total phenolic content in the SCM-HTM could be due to the loss of phenolics during soaking in water and cooking (Towo et al 2003).

The study clearly indicates that, hydrothermal processing and decortication did not cause a drastic change in the polyphenol composition except with the loss of few phenolics (Table 21).

The seed coat matter (SCM) prepared from the native millet (NM) has several favorable properties such as light color compared to the SCM-HTM, moreover the pulverizability is much higher than SCM-HTM which facilitates its utilization in blended formulations and other food products. Since it contains higher proportion of polyphenols and dietary fibre besides minerals and also the SCM could be prepared at household to industrial level utilizing the existing cereal milling facilities, it may have better pharmaco-nutritional applications. In view of this, for further studies on understanding the role of the millet seed coat matter in the carbohydrate digestibility as well as diabetic ameliorative qualities, the SCM prepared from the NM was used.

Table 20. Comparative quality characteristics of refined flour (RF), decorticated millet (DM) and the seed coat matter prepared from native millet (SCM) and hydrothermally processed millet (SCM-HTM)

Parameter	NM	Endosperm		Seed coat matter	
		RF	D	SCM	SCM-HTM
Yield (%)	100	76 ± 2	82 ± 5	22.0 ± 2	15 ± 3
Moisture (%)	9.8 ± 1.0	11.0 ± 1.5	12.5 ± 2	11.3 ± 1.3	12 ± 1.2
Protein (g%)	8.7 ± 0.5	3.6 ± 0.5	6.5 ± 0.5	13.6 ± 1.3	10.1 ± 0.4
Fat (g%)	1.5 ± 0.3	0.9 ± 0.2	0.8 ± 0.3	3.2 ± 0.2	3.5 ± 0.4
Starch (g%)	72.0 ± 1.0	87.0 ± 2.0	81.3 ± 3.2	18.6 ± 1.0	18.1 ± 2.0
Dietary fibre					
Total Dietary fibre (g%)	19.6 ± 1.2	6.8 ± 0.9	10.2 ± 1.6	47.7 ± 2.0	50.2 ± 1.0
Soluble dietary fibre (g%)	3.5 ± 0.1	1.6 ± 0.1	2.8 ± 0.6	3.9 ± 0.2	2.1 ± 0.6
Insoluble dietary fibre (g%)	16.1 ± 0.5	5.2 ± 0.6	7.5 ± 1.2	43.8 ± 0.5	48.1 ± 1.2
Minerals (g%)	2.2 ± 0.1	1.1 ± 0.2	1.1 ± 0.2	5.6 ± 0.2	4.9 ± 0.1
Calcium (mg%)	321 ± 10	163 ± 7	185 ± 15	830 ± 15	850 ± 10
Phosphorus (mg%)	201 ± 6	106 ± 7	111 ± 10	526 ± 5	510 ± 12
Polyphenols (mg%)	2300 ± 9	1480 ± 3	807 ± 9	11200 ± 7	7900 ± 10
Tannins (mg%)	840 ± 9	243 ± 2	115 ± 9	2305 ± 7	1119 ± 9
Flavonoids (mg%)	136 ± 7	106 ± 5	63 ± 3	209 ± 7	173 ± 6
Phytate (mg%)	217 ± 6	69 ± 4	75 ± 5	685 ± 9	534 ± 7

RF- refined flour, DM - decorticated millet, SCM - seed coat matter, SCM-HTM - seed coat matter prepared from HTM

Table 21. Comparative phenolic composition of acidic methanol extracts of native millet (NM), refined flour (RF), decorticated millet (DM), seed coat matter (SCM), and seed coat matter prepared by decortication of hydrothermally processed millet (SCM-HTM)

Phenolic compound	NM	RF	DM	SCM	SCM-HTM
Gallic acid	+	+	+	+	+
Gentisic acid	+	+	+	+	+
Protocatechuic acid	+	+	+	+	+
Syringic acid	+	-	-	+	+
Trans-cinnamic acid	+	+	+	+	+
Caffeic acid	+	+	+	+	+
Ferulic acid	+	+	+	+	+
P-hydroxy benzoic acid	+	+	+	+	+
4-*o*-methyl gallic acid	+	-	+	+	+
Kaempferol	+	+	+	+	+
Naringenin	+	+	+	+	+
Phloroglucinol	+	+	+	+	+
Apigenin	+	+	+	+	+
(+)-Catechin/ (-)-Epicatechin	+	+	+	+	+
Luteolin glycoside	+	+	+	+	+
Trans feruloyl-malic acid	-	-	-	+	-
Dimer of prodelphinidin (2GC)	+	-	-	+	-
Diadzein	+	+	+	+	-
Catechin gallates	+	+	+	+	-
Trimers of catechin	-	-	+	+	+
Tetramers of catechin	-	-	-	+	+

SUMMARY & CONCLUSION

- Finger millet contains about 2.5% polyphenols and 0.25% phytate. Localization of these phytochemicals in the millet kernels by histochemical techniques was carried out as a prelude to prepare the fraction rich in these phytochemicals, and it was observed that, the polyphenols are concentrated in the testa/seed coat, aleurone layer, germ and also in the endosperm cell walls of the millet, whereas the phytates were largely noticed in the germ and the aleurone layer. Accordingly, the seed coat matter of the native millet (SCM) was prepared following a sequential milling and sieving method. The SCM thus prepared formed 22% of the seed matter and contained 11.2 ± 1% polyphenols, 2.3 ± 0.3 % tannins, 0.3 ± 0.05 % flavonoids and 0.7 ± 0.1 % phytate.

- The polyphenols of the SCM were extracted with acidic-methanol and characterized by HPLC and ESI-MS. The results revealed that, the millet polyphenols are a complex mixture of several phenolic compounds belonging to different classes namely, simple phenols, phenolic acids, flavonoids, tannins and anthocyanins. The constituent phenolics identified were gallic acid, protocatechuic acid, gentisic acid, caffeic acid, vanillic acid, syringic acid, ferulic acid, *p*- coumaric acid, trans-cinnamic acid, naringenin, kaempferol, luteolin glycoside, phloroglucinol, apigenin, (+)-catechin/ (-)-epicatechin, trans-feruloyl-malic acid, dimer of prodelphinidin (2GC), diadzein, catechin gallates, and also the trimers and tetramers of catechin.

- The SCM exhibited higher radical scavenging ability (84% RSA), total antioxidant activity (207.3 mM ascorbic acid equivalent TAA/g flour) and iron chelating ability (79.3 mM EDTA equivalent ICA/g flour).

- An innovative method of decortication of finger millet based on hydrothermal treatment was developed and the quality characteristics of the decorticated millet and its seed coat matter were studied. The decorticated millet (DM) was suitable for cooking in the form of grains similar to rice and contained about 0.8% polyphenols mainly present in the

endosperm cell walls and also due to the inward migration of polyphenols from the seed coat and aleurone layer towards the peripheral endosperm during hydrothermal processing. The seed coat matter from the hydrothermally processed millet (SCM-HTM) contained 7.9 ± 0.1% polyphenols, 1.1 ± 0.09 % tannins, 0.17 ± 0.06 % flavonoids and 0.54 ± 0.07 % phytate. The antioxidant abilities of the SCM-HTM was slightly lower compared to that of the SCM which may be due to its lower polyphenol content.

- The physico-chemical characteristics of the SCM was slightly superior to the SCM-HTM in terms of colour, pulverizability, phytochemical contents as well as antioxidant potentials. In view of these, the SCM was utilized for further studies on the nutraceutical ability and also for the animal feeding trials.

From these observations, it may be concluded that, the millet polyphenols and phytate are concentrated towards the peripheral layers of the grain namely, the seed coat and aleurone layer in addition to their concentration in the germ. It is possible to prepare polyphenols and dietary fiber rich fraction of the millet by adopting sequential milling and sieving methodology. Alternatively, by hydrothermal processing also, it is possible to prepare the seed coat matter. The decorticated millet also contained a considerable amount of polyphenols due to the migration of polyphenols from the testa and aleurone layers towards the peripheral endosperm and hence may deliver several health benefits to its consumers. The millet SCM is a rich source of phenolic compounds with excellent antioxidant properties and hence may find utilization as a functional ingredient in high fiber foods for target population.

INTRODUCTION

Current nutritional guidelines for the management of diabetes emphasize on low glycemic index diets. Whole grain foods are normally suggested to diabetics because of their complex carbohydrates and dietary fiber contents. The bran layer and cell wall components of the whole grain contains several bioactive compounds such as polyphenols which offer health benefits. In addition to the dietary fiber, whole grain foods are rich in myriad vitamins, minerals, and other compounds that alone or in combination are likely to deliver significant health benefits. Lower incidence of type II diabetes has been associated with whole grain consumption (Montonen et al 2003). Moreover, whole grain consumption is known to improve the insulin sensitivity (Pereira et al 2002). In general, the beneficial effect of soluble fiber may be mediated through the slow absorption and digestion of carbohydrates that lead to a reduced demand for insulin. On the other hand, insoluble fiber shortens intestinal transit, thereby allowing less time for the carbohydrates to be absorbed. Whereas, polyphenols may affect glycemia through different mechanisms, including the inhibition of glucose absorption in the gut or its uptake by peripheral tissues. The inhibition of intestinal glucosidases and glucose transporter by polyphenols have also been reported. Polyphenols may also exert different actions on peripheral tissues that would diminish glycemia. They include the inhibition of gluconeogenesis, adrenergic stimulation of glucose uptake, or the stimulation of insulin release by pancreatic *β*-cells. The protective role of polyphenols against diabetes has been extensively reviewed (Scalbert et al 2005).

Finger millet diets are known for their lower glycemic response (Ramanathan and Gopalan 1957, Lakshmi Kumari and Sumathi 2002) and higher satiety scores (Urooj et al 2006). From good old days, there is a strong belief that, diabetics tolerate finger millet better than rice and their blood and urine sugars levels tend to be lower with the millet diets. Finger millet in general and its seed coat matter (SCM) in specific is a rich source of dietary fiber as well as polyphenols. Hence, the millet SCM may influence the *in vivo* carbohydrate digestibility of the millet foods. However, to the best of our knowledge, reports on the role of the millet SCM on the carbohydrate digestibility of millet foods are scanty. Moreover food processing in general and

hydrothermal processing in specific is known to alter the nature of carbohydrates by inducing retrogradation and resistant starch formation. These are known to affect carbohydrate digestibility of foods to a considerable extent.

Hydrothermal processing or parboiling is one of the common methods of cereal processing technology. It is largely applied to paddy and generally the parboiled rice is reported to have lower carbohydrate digestibility compared to raw rice (Casiraghi et al 1993). Recently, the hydrothermal processing has also been adopted to finger millet mainly to prepare decorticated millet. The decorticated millet is first of its kind, and can be cooked similar to rice. The quality characteristics of the decorticated millet is almost comparable to that of parboiled rice. However, there are no reports on the influence of hydrothermally processing and decortication on the carbohydrate digestibility of millet. Hence it was felt desirable to examine the influence of the millet seed coat matter as well as the hydrothermal processing on the carbohydrate digestibility of the millet foods. Hence investigations were undertaken to; (a) study the nature of carbohydrates in the native and hydrothermally processed millet, (b) to study the *in vitro* carbohydrate digestibility characteristics of the native and hydrothermally processed millet, (c) to characterize the malto-oligosaccharides released on pepsin-pancreatin digestion of the native and hydrothermally processed millet, (d) to assess the role of millet seed coat polyphenols on the millet starch digestibility and also (e) to examine the *in vivo* carbohydrate digestibility characteristics of the millet whole meal, refined flour, hydrothermally processed millet and decorticated millet by the determination of glycemic index (GI) in normal and type II diabetic subjects.

MATERIALS AND METHODS

Chemicals and reagents: Mercuric chloride was procured from SD Fine Chemicals Pvt Ltd, Mumbai, India, whereas, maltose, maltotriose, maltotetraose, maltopentaose and pancreatin were procured from Sigma Chemical Company, St. Louis, USA. Pepsin was purchased from Hi Media Laboratories, Mumbai, India. All the chemicals used were of analytical grade. Glass (double) distilled water was used for the preparation of the reagents.

Samples: The whole meal from native millet (NM), refined flour (RF), hydrothermally processed millet (HTM) and decorticated millet (DM) were prepared following the protocols described in Chapter I.

Isolation of starch: The native millet (500g each) was steeped in excess water (containing 100 ppm mercuric chloride to prevent incipient sprouting of the grains during steeping) for 16 h at ambient conditions. The steeped material was washed several times to free from leachates and mashed with five volumes of distilled water in a waring blender. The slurry was screened through 200 mesh (BSS sieve) sieve to separate the seed coat matter, and the residual material retained over the sieve was was again wet ground, sieved and the process was repeated until it tested negative to iodine. The starchy material was centrifuged and the proteinaceous matter was scrapped off. The starchy material was again dispersed in excess water and the pH of the slurry was adjusted to 9.5 by adding 1N NaOH drop wise with constant stirring and centrifuged, after which the supernatant and the proteinaceous matter was discarded and the starchy material was dispersed in 0.1 M NaCl and toluene (10:1) solution. The contents were agitated for about 5 h in a mechanical shaker to deproteinize the starch and the protein was separated by centrifugation. Finally the starch was washed with distilled water repeatedly and dried by solvent exchange and analyzed for protein, fat and ash contents (Adkins and Greenwood 1966).

Gel permeation chromatography of the millet starch and flour samples: The isolated millet starch and also the flours of native millet (NM), hydrothermally processed millet (HTM) and decorticated millet (DM) were dispersed in aqueous DMSO (85%) to dissolve the carbohydrates and an aliquot of the clear solution containing 10 mg carbohydrate (dry weight basis) was fractionated by ascending chromatography on a sepharose CL-2B gel (Pharmacia, Sweden) column (1.7×90 cm) at a flow rate of 14 ml/h, using water containing 0.02% sodium azide as an eluant. Total carbohydrate (Dubois et al 1956) and the absorption maxima (λ max) of the iodine-polysaccharide complex of the eluants were determined (Chinnasamy and Bhattacharya 1986).

***In vitro* carbohydrate digestibility:** The millet starch isolate and the meals from NM (native millet), refined flour (RF), HTM and DM (100 mg) were mixed with 10 ml of

water containing 0.1 ml of termamyl and heated in a boiling water bath for 10 min. To the contents, 15 ml of 0.1 M glycine-HCl buffer (pH 2.0) containing 10 mg pepsin was added and incubated at 37^0C for 2 h. The contents were neutralized with 0.1 N NaOH and to that 15 ml of 0.1 M phosphate buffer (pH 6.8) containing 10 mg of pancreatin was added and incubated at 37^0C and aliquots of the hydrolysates were withdrawn at different time intervals ranging from 0-240 min, heated in boiling water bath to arrest the reaction, cooled to room temperature, filtered with Whatman No. 1 filter paper and the filtrates were analyzed for the reducing sugar content by DNS method with maltose as the reference standard. The extent of hydrolysis was calculated by the formula;

$$\% \text{ of starch hydrolyzed} = \frac{\text{Maltose equivalents released} \times 0.95}{\text{Starch content of the sample}} \times 100$$

Simultaneously, the hydrolysates were precipitated with three volumes of absolute ethanol and kept overnight at 4^0C, centrifuged at 7000 rpm to separate the undigested residual starch and the clear supernatants consisting of oligosaccharides were concentrated by vaccum evaporation and taken for the **characterization of malto-oligosaccharides** by direct infusion ESI-MS.

The mass spectra of the oligosaccharides were obtained from the mass spectrometer (Alliance, Waters 2695) using positive electro-spray ionization mode. Capillary and core voltages were maintained at 3.5 kV and 100 V respectively. The source and desolvation temperatures were maintained at 80^0C and 150^0C respectively. The flow rates of the core gas (argon) and desolvation gas (nitrogen) were 35 Lh^{-1} and 500 Lh^{-1} respectively.

Glycemic Index (GI) determination: The *in vivo* carbohydrate digestibility of the native millet (NM) flour, refined flour (RF), hydrothermally processed millet (HTM) flour in the form of dumpling or '*mudde*' and decorticated millet (DM) in the form of cooked grains were determined in terms of GI following the protocol described by Wolever et al (1991).

For the purpose, the available carbohydrate content of the millet samples were estimated adapting the method of Holm et al (1988) with minor modifications. The

defatted millet samples (100 mg) with 5 ml of water containing 0.1 ml termamyl were heated in a boiling water bath for 15 min. The contents were cooled to room temperature and 10 ml of 0.2M glycine-HCl buffer (pH 2.0) containing 10 mg pepsin was added and incubated at 37^0C for 2 h in a shaking water bath. Subsequently, the pH of the reaction mixture was adjusted to 6.9 by the addition of NaOH (0.5N) and, to that 10 ml of 0.5M phosphate buffer (pH 6.9) containing 10 mg of pancreatin was added and incubated at 37^0C for 2 h in a shaking water bath. Followed by that, the pH of the reaction mixture was adjusted to 4.8 with dilute acetic acid and 10 ml of acetate buffer (0.05M) pH 4.8 containing 10 mg amyloglucosidase was added and incubated at 55^0C for 2 h in a shaking water bath. The contents were made upto 100 ml and centrifuged. The clear supernatant was taken for the glucose estimation by glucose oxidase method.

Preparation of foods: For the GI studies, dumpling or stiff porridge (*mudde*) was identified among the various traditional millet products as it is one of the most commonly consumed millet food. Moreover, the method of preparation of dumpling is known to gelatinize the starch completely unlike other millet products namely *roti* or unleavened pancake. Dumplings were prepared from NM, RF and HTM flour whereas, the DM was cooked in the form of discrete grains similar to rice. For the preparation of dumplings, initially a thin slurry was prepared by cooking a small quantity of the flour in excess water, boiled for a few minutes, subsequently the remaining flour was added and left in the form of a heap, and heating was continued to partially steam the flour for a few minutes and then mixed well to a smooth consistency and shaped into a ball. On the other hand, the DM was cooked in adequate quantity of water as discrete grains and used for the study. Both the dumplings and cooked DM were served with *dhal sambar* (a mild spicy accompaniment prepared using cooked and mashed legume splits) as accompaniment. The diet was planned in such away that, each of the subject received food equivalent to 50g available carbohydrates, out of which 35g was contributed by the test foods (millet) and the rest (15g) was from *dhal sambar*.

Subjects: Three male and three female normal healthy subjects and four female and three male type II diabetic subjects were selected among the staff and students of the

laboratory (falling in the age group of 25 - 60 years) for the GI study, after screening (with voluntary consent) for their glucose tolerance characteristics. The body weight and height of the subjects wearing light clothing and no shoes, and the waist and hip circumference were measured. Based on these values the body mass index (kg/cm^2) and the waist-to-hip ratio were calculated. The important parameters of the body composition of the subjects, namely, the body fat content, lean body mass, body water content, basal metabolic rate (estimated) and the impedance were determined using Bodystat (Bodystat 1500; Bodystat Ltd., Douglas, UK), a gadget that works on bio-electrical impedance.

Glycemic response: The blood glucose levels of the subjects after overnight fasting were recorded following the finger-prick method (capillary blood) using a glucose sensor (Accutrend; Roche Diagnostics Corporation, Indianapolis, IN, USA), following internationally accepted protocol by a qualified technician. Soon after that, the subjects were provided with the standard food (white bread) and *dhal sambar* equivalent to 50 g available carbohydrates and were advised to consume the same within 10 min. The subjects were allowed to drink adequate quantity of water during the test period. Subsequently, the postprandial blood glucose were measured at 15, 30, 45, 60, 90, 120 and 150 min. On four different occasions, the test foods were given to the subjects with an interval of minimum of 2-3 days between each test. The blood glucose response curve was constructed and the incremental area under the blood glucose response curve (IAUC) with fasting blood glucose value as the baseline was measured (FAO/WHO 1998). The IAUC reflects the total rise in blood sugar (glucose) levels after the ingestion of the test food. The GI was calculated by the formula mentioned below;

$$\text{GI (\%)} = \frac{\text{IAUC for 50g equivalent available carbohydrate of the test food}}{\text{IAUC for 50g equivalent available carbohydrate of white bread}} \times 100$$

The glycemic load of the food is calculated by multiplying the amount of available carbohydrate contained in the specific serving size of the food by the GI value of that food which is then divided by 100 (Faster Powell et al 2002).

$$GL = \frac{GI \times \text{Carbohydrate content in serving size (g/serving)}^*}{100}$$

* 50 g in the present study

RESULTS AND DISCUSSION

The millet carbohydrates

Finger millet is a rich source of dietary carbohydrates mainly comprising of starch and non-starchy polysaccharides. Since, starch accounts for the 80% of the carbohydrates, it is the main detrimental factor for the carbohydrate digestibility of the millet foods. Hence for the *in vitro* carbohydrate digestibility of the millet samples, isolated millet starch was also utilized for the studies, so that the carbohydrate digestibility pattern of the processed millet would be compared with that of the starch isolate. The starch isolated from the native millet and also the carbohydrates from the whole meals of NM, HTM and DM solubilized in DMSO were fractionated through Sepharose CL-2B. On gel filtration, the starches were fractionated into two main fractions, one large fraction (Fraction I) eluting at void volume (generally referred as amylopectin in the literature) and another small fraction (Fraction II) towards the total volume (generally referred to as amylose). The elution profiles of the starch isolate and also the carbohydrates from the native millet meal were comparable similarly, the profiles of HTM and DM were also nearly comparable. However, the fraction I for the HTM and DM were lower as compared to that of NM. This could be due to the thermal degradation of the high molecular weight portion of the carbohydrate fraction to form intermediate lower molecular weight carbohydrates during hydrothermal treatment. The area under the fractions I and II were in the range of 75 and 25 for the native millet and the starch isolate, but in the case of HTM and DM, the area under the fractions I and II were 70 and 30 respectively. On the other hand, the differences in the profiles of the native and the HTM were mainly in the lowered area of the

fraction I and slightly higher areas under fraction II. Apart from this, the presence of a small intermediate peak between the peaks of fractions I and II is prominently present in HTM and DM. The elution profile mainly represents the nature as well as the molecular makeup and the constituents of the starchy component of the millet because, the matter used for the elution is only the solubilized carbohydrate namely the DMSO solubles (Figure 34).

The samples used in the fractionation studies were of equi-carbohydrate contents and the differences noticed in the areas under the fraction I and II in different samples is mainly due to the molecular profile of their respective carbohydrates. Lower peak for fraction I in the HTM and the DM could be mainly due to the thermal degradation of their starches during hydrothermal processing. The lower molecular weight carbohydrates formed due to degradation is normally eluted as the intermediate fraction which is predominantly present in the HTM and DM. The differences in the GPC profile of the HTM from that of NM could also be due to the formation of lipid-amylose complex during hydrothermal treatment to the millet, as reported in the case of rice (Mahanta and Bhattacharya 1989). Molecular degradation of starch has also been shown to occur on pressure parboiling of rice (Mahanta and Bhattacharya 1989) and also in the case of other thermal processing operations such as extrusion cooking and also during production of expanded (Chinnasamy and Bhattacharya 1986) as well as popped rice (Murugesan and Bhattacharya 1989). The extent of molecular degradation has been shown to be proportional to the severity of the treatment given. The study clearly showed that, the starch of the millet undergoes a slight degradation during hydrothermal processing.

***In vitro* carbohydrate digestibility**

To assess the influence of the seed coat matter on the carbohydrate digestibility of the millet, the RF and the DM (both devoid of the seed coat matter) were compared with NM and HTM along with the isolated starch. The carbohydrate digestibility of the isolated starch was highest, followed by DM, HTM, RF and NM. Among the millet starch, RF and NM, the NM exhibited the lowest carbohydrate digestibility whereas among the HTM and DM, the digestibility of the HTM was lower.

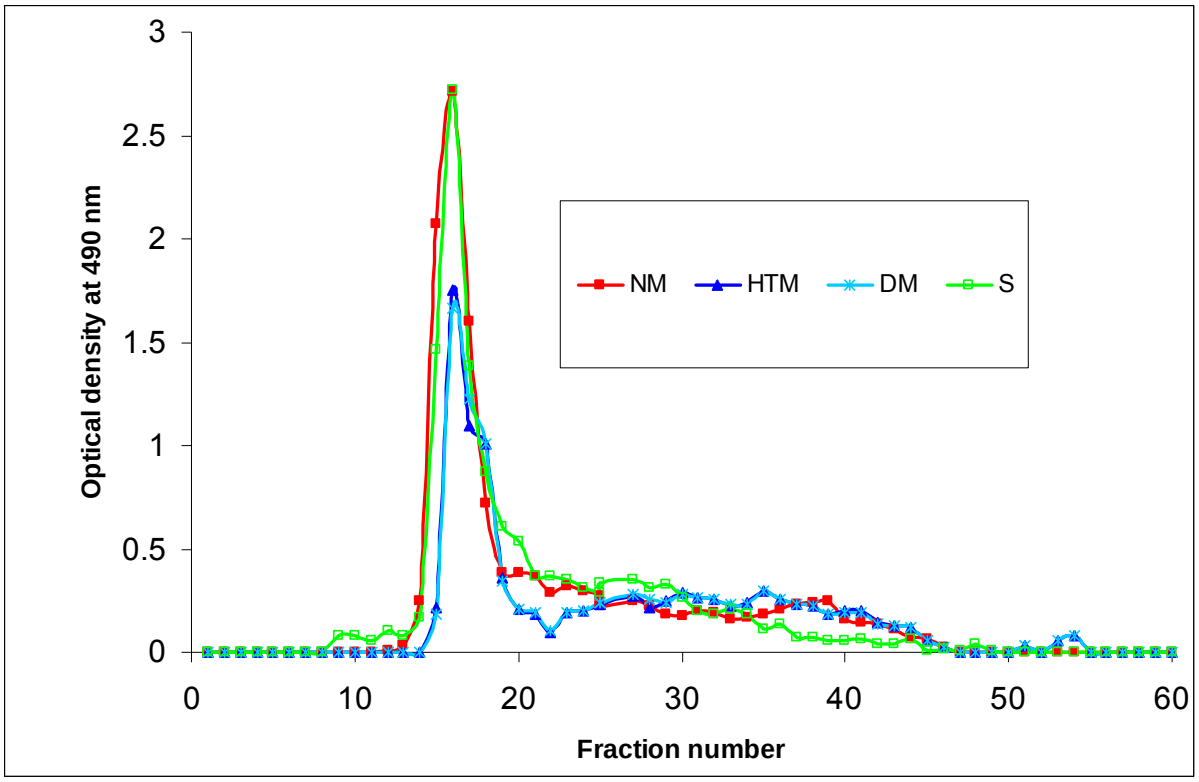


Figure 34. Elution patterns of the starch from the native (NM), hydrothermally processed (HTM), decorticated millet (DM) and the millet starch isolate (S) on sepharose CL- 2B column

The rate of digestibility of the samples presented in Figure 35 indicates that nearly 69% of the carbohydrates of the NM and 86% of the isolated starch were digested within first 45 min. Incubation for more than 100 min didnot show any appreciable increase in the maltose equivalents released and the values almost remained constant. Among the whole meals, the material freed from the seed coat matter exhibited higher degree of carbohydrate digestibility. Between the NM and RF (the counterpart of the NM without the seed coat matter), the digestibility of RF was higher (80%) compared to that of NM (76%). Similarly, in the case of HTM and DM also the digestibility of DM was higher (83%) than HTM (81%). These observations reveals that, the SCM slightly affects the carbohydrate digestibility of the millet. The higher carbohydrate digestibility values of the DM millet may be attributed to the lower phytate and polyphenol contents as, they are known to decrease carbohydrate digestibility. Hence, the study clearly indicates the vital role of the millet seed coat constituents on lowering its carbohydrate digestibility.

Characterization of malto-oligosaccharides

In continuation of the *in vitro* carbohydrate digestibility, the hydrolysates of the pepsin-pancreatin digests of the millet samples were subjected to ESI-MS analysis to identify the malto-oligosaccharides formed as a result of the enzymatic hydrolysis. The mass spectrum of pepsin-pancreatin digested samples (Figure 36) showed ions with m/z values 365.3 (maltose: fragmentation 180×2= 360-18=342+23=365), 527.41 (maltotriose: fragmentation, 180×3=540-36=504+23=527), 689.4 (maltotetraoase: fragmentation, 180×4=720-54=666+23=689) and 851 (maltopentaose: fragmentation, 180×5= 900-72=828+23=851). The sodium adduct $[Na]^+$ (23) detected in all the samples is used for the calculation. Under the experimental conditions, the mass spectrum of the digests of the isolated millet starch, NM and RF showed the presence of maltose (G2), maltotriose (G3), maltotetraose (G4) and maltopentaose (G5), whereas, only maltose, maltotriose and maltotetraose were observed in HTM and DM digests (Table 22). The absence of G5 in the hydrothermally processed millet samples (HTM and DM) is conspicuous. The HTM and DM carbohydrates may have increased susceptibility for the enzymatic digestion due to their particle size (finely pulverized samples were used for the study) in synergy with pre-gelatinization

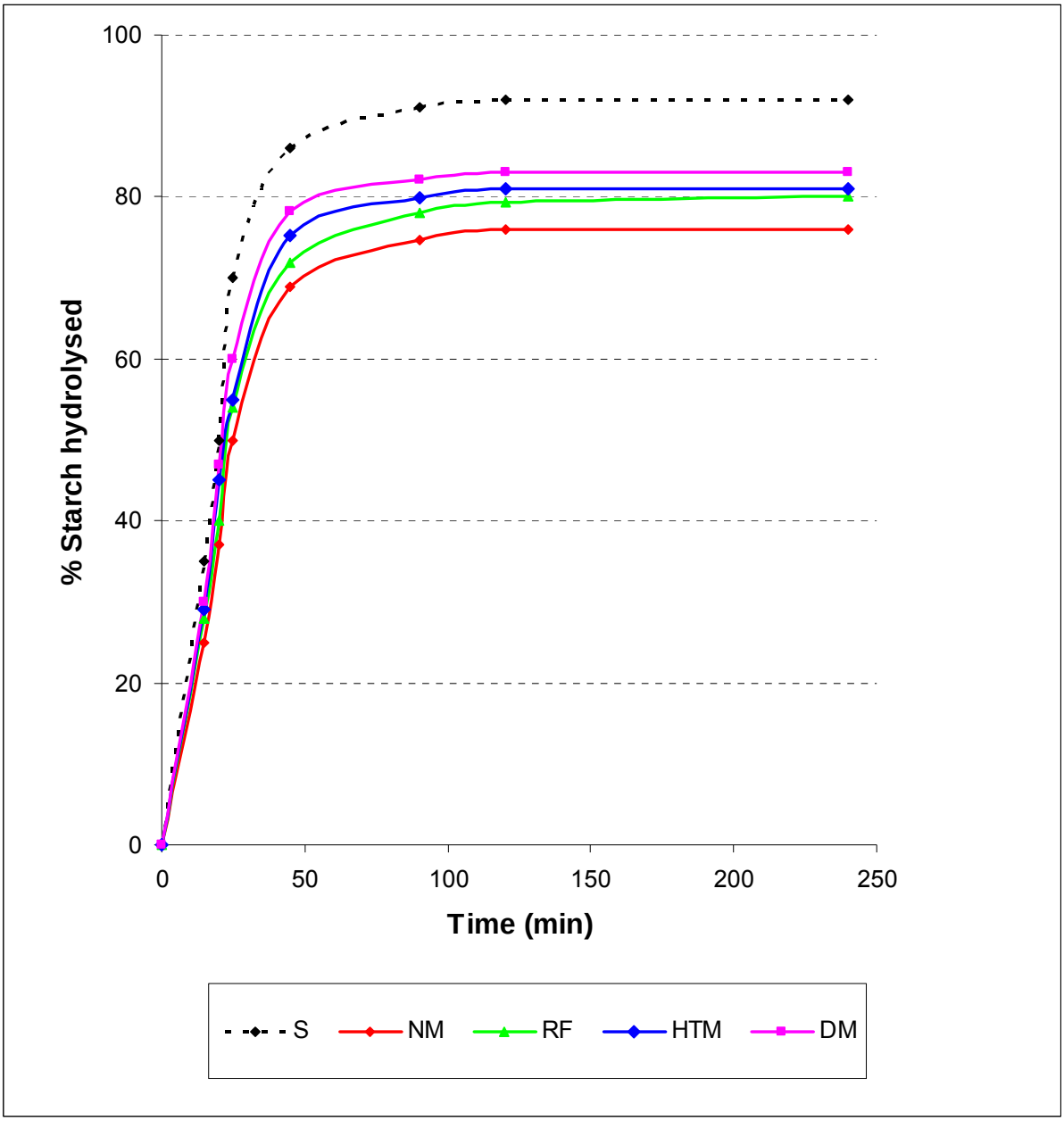


Figure 35. ***In vitro*** **carbohydrate digestibility of the native millet (NM), refined flour (RF), hydrothermally processed millet (HTM), decorticated millet (DM) and the millet starch isolate (S)**

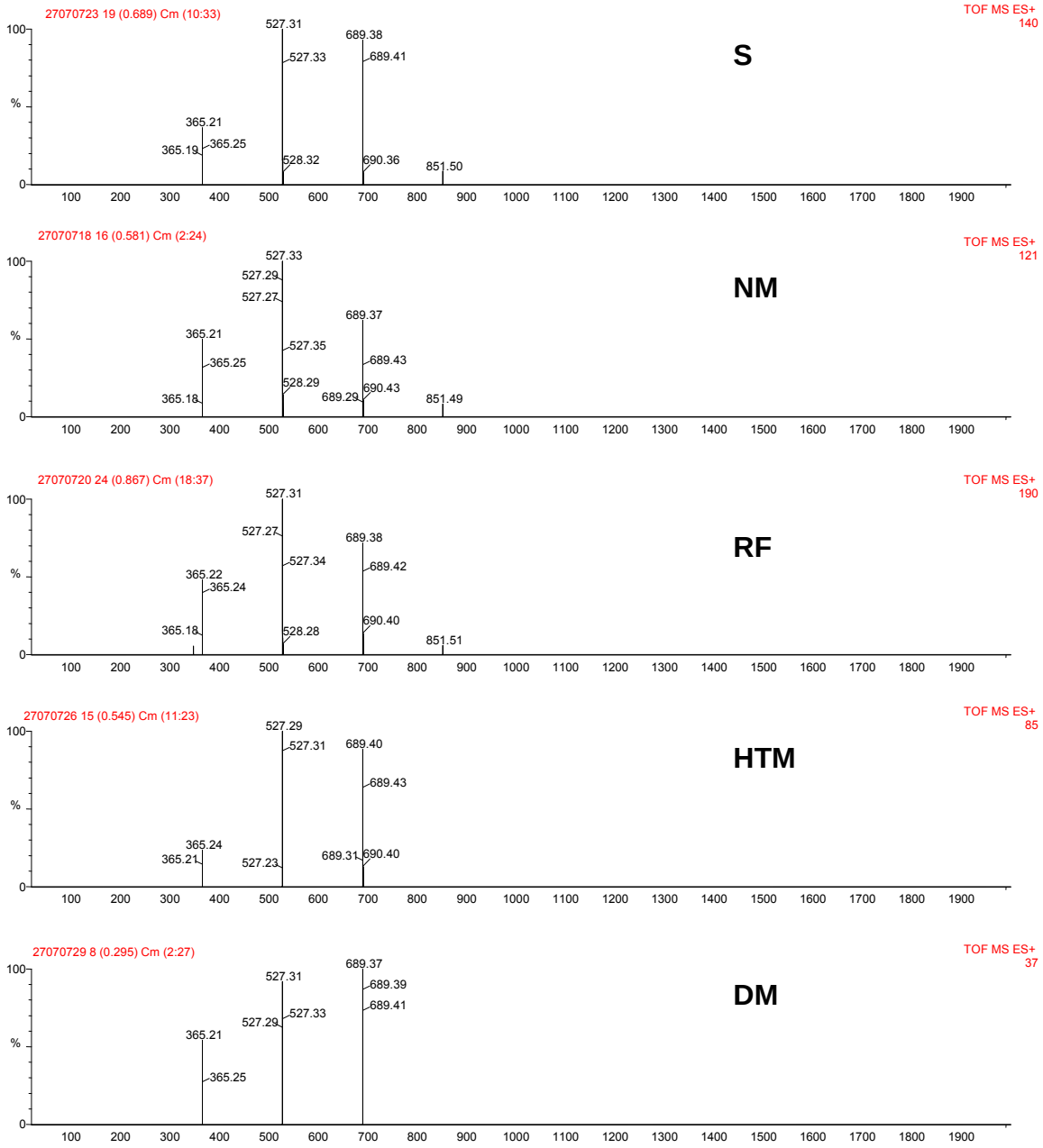


Figure 36. ESI-MS spectra of malto-oligosaccharides released on pepsin-pancreatin digestion of the millet starch isolate (S), native millet (NM), refined flour (RF), hydrothermally processed millet (HTM) and decorticated millet (DM) flour

Table 22. Malto-oligoaccharides identified from the ESI-MS spectra of oligosaccharides obtained by pepsin–pancreatin digestion of the millet starch isolate (S), native whole meal (NM), refined flour (RF), hydrothermally processed millet (HTM) and decorticated millet (DM)

Sample (pepsin-pancreatin digest)	m/z	Malto-oligosaccharides detected
S	365, 527, 689, 851	Maltose, maltotriose, maltotetraose, maltopentaose
NM	365, 527, 689, 851	Maltose, Maltotriose, Maltotetraose, Maltopentaose
RF	365, 527, 689, 851	Maltose, Maltotriose, Maltotetraose, Maltopentaose
HTM	365, 527, 689	Maltose, Maltotriose, Maltotetraose
DM	365, 527, 689	Maltose, Maltotriose, Maltotetraose

of their starches during hydrothermal processing. The study also reveals that, pepsin-pancreatin digestion (similar to the *in vivo* digestion of carbohydrates in the GI tract) of the millet samples released oligosaccharides of varying degree of polymerization (G2-G5, DP≤5).

Influence of the millet seed coat polyphenols on the millet starch digestibility

In order to understand the role of the millet seed coat polyphenols (MSCP) on the carbohydrate digestibility of the millet starch, the isolated millet starch was subjected to pancreatic amylase digestion in the presence of different levels of MSCP. The results indicated that, the MSCP decreased the release of maltose equivalents (Figure 37). The maltose equivalents decreased from 0.34 µmoles/min to 0.06 µmoles/min in the presence of 43 µg of the polyphenols and the decrease was proportional to the concentration of the polyphenols used in the reaction mixture as the presence of 9, 17, 26, 34 and 43 µg of the polyphenols released 0.26, 0.22, 0.16, 0.11 and 0.06 µmoles/min of the maltose equivalents.

Hence, the study clearly indicated that, the millet polyphenols in isolation or the millet SCM, the rich source of polyphenols may influence the carbohydrate digestibility of the millet foods. This gives an indication that, the SCM or the isolated polyphenols may be used to develop slow digestible carbohydrate foods or alternately the polyphenol isolate may be used as ‘starch blockers’. However, detailed investigations on the use of millet polyphenols as an aid to slow down the digestibility of carbohydrate rich foods may be required mainly to find out their efficacy, threshold limits and safety issues.

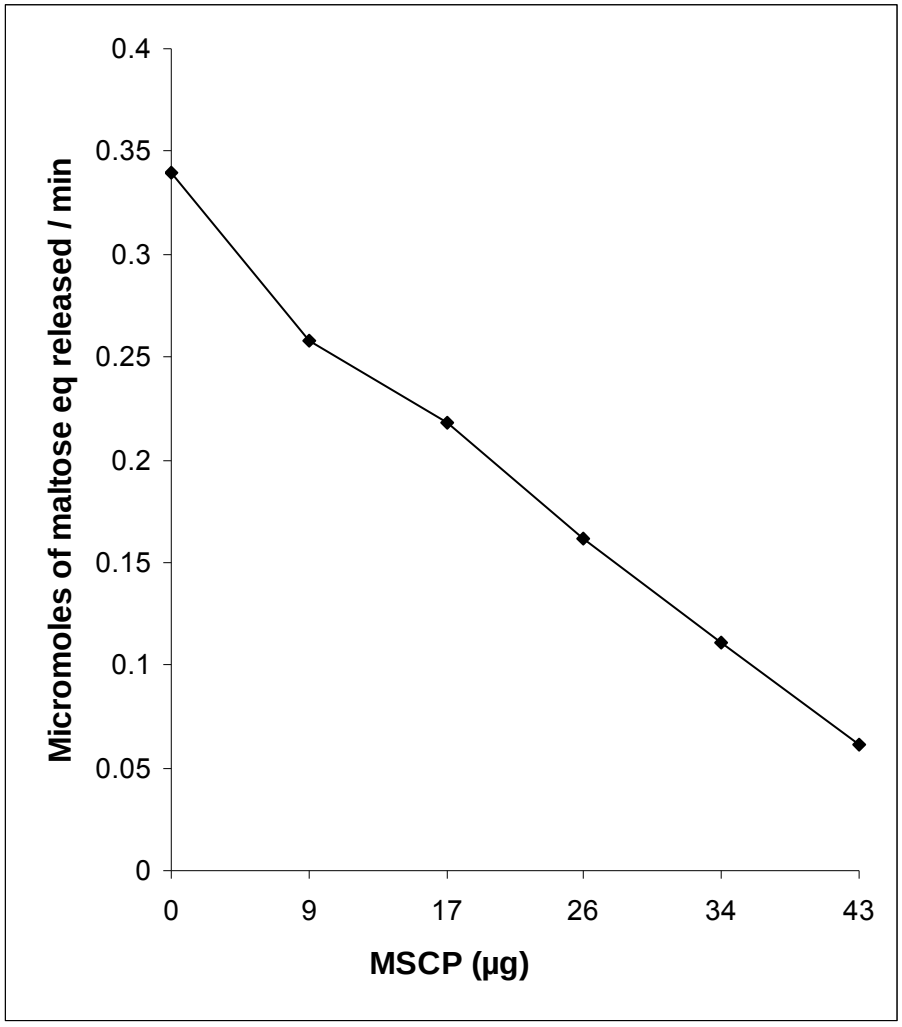


Figure 37. Maltose equivalents released on the pancreatic amylase digestion of the millet starch in the presence of different levels of the millet seed coat polyphenols (MSCP)

***In vivo* carbohydrate digestibility**

The data on anthropometric parameters (Tables 23 and 24) and the body composition of the subjects reveal that, except for one female subject, all were of normal physique matching with their age. Three of the normal subjects (one male and two female) had higher body fat content and one of the female subjects had higher BMI (30.3). However, the glucose tolerance characteristics of that subject was normal and the glycemic response pattern for the test and standard foods were also normal. In the case of type II diabetic subjects, both the male and the female subjects had higher body fat content, whereas, the females had higher BMI when compared to the male subjects (Tables 24).

The proximate composition of the NM, RF, HTM and DM used for the carbohydrate digestibility studies indicated in Table 20 reveal that, the millet products differ considerably in their protein and carbohydrate contents. The total available carbohydrate content of NM, HTM, RF and DM were 70.7, 71.8, 81.3 and 80.8 % respectively (Table 25). The reason for the higher level of available carbohydrates in the RF and DM is mainly due to removal of seed coat matter during their preparation.

The blood glucose response curves of the normal subjects are presented in Figure 38. In the case of normal subjects, the digestibility of the food was rapid among the younger male subjects compared to older male as well as females subjects. Even though two of the male subjects were over 40 years of age, their glycemic response to the test meal was comparable with those observed in the case of younger subjects. The blood glucose response curves also reveal that, the digestibility of white bread (control) and the refined flour are almost comparable except for the slight shift in the peaks of RF to about 60 and 90 min in the normal and diabetic subjects respectively (Figure 38). The digestion of RF appears to be rapid when compared to NM and DM. The peak blood glucose level (124 mg/dl) for the white bread was highest compared to other foods and the order of digestibility was HTM>RF>DM>NM for the normal subjects. Even though, the peak blood glucose value for the DM was higher compared to the other foods, the incremental area under the curve (IAUC) was lower for DM compared to RF and HTM.

Table 23. Body composition of the normal subjects participated in the glycemic response study

Parameter	N_1	N_2	N_3	N_4	N_5	N_6
Age (years)	34	42	34	25	56	26
Sex	F	F	F	M	M	M
Weight (kg)	53	64	70	68	73	59
Height (cm)	148	165	154	182	171	183
Waist to hip ratio	0.8	0.9	0.9	0.7	0.97	0.7
Body fat content (Kg)	19.1	21.3	29.5	9.7	19.7	7.1
Fat (%)	36.04	33.3	42.1	14.3	27	12
Lean body mass (Kg)	33.85	42.7	40.5	58.3	53.3	51.9
Lean (%)	63.95	66.7	57.9	85.7	73	88
Body water content (l)	25.65	31	29.8	39.1	39.1	37.5
Water (%)	48.5	48.4	42.6	57.5	53.6	63.6
Basal metabolic rate (Kcal) suggested	1241	1372	1961	1800	2232	1600
Body mass index	24.5	23.5	30.3	20.5	25	17.6
Impedance	708	653	627	626	571	641

Table 24. Body composition of the type II diabetic subjects participated in the glycemic response study

Parameter	D_1	D_2	D_3	D_4	D_5	D_6
Age (years)	48	41	46	60	56	49
Sex	F	F	F	F	M	M
Weight (kg)	70	69	70	65	62	70
Height (cm)	156	150	146	147	158	165
Waist to hip ratio	1.0	0.9	0.9	0.8	1.0	1.2
Body fat content (Kg)	28.2	30.6	32.8	35	17.2	19.1
Fat (%)	40.3	44.3	46.9	54	27.7	26.2
Lean body mass (Kg)	41.8	38.4	37.2	29.9	44.8	53.9
Lean (%)	59.7	55.7	53.1	46	72.3	73.8
Body water content (l)	31.9	29.3	29.6	26.8	35.3	39.2
Water (%)	45.6	42.5	42.3	41.2	56.9	53.7
Basal metabolic rate (Kcal) suggested	1352	1789	1251	1527	1929	1609
Body mass index	28.8	30.7	32.8	30.1	24.8	26.8
Impedance	574	626	585	707	550	530

Table 25. Available carbohydrate contents of the native millet (NM), refined flour (RF), hydrothermally processed millet (HTM), decorticated millet (DM) and white bread (WB)

Sample	Available carbohydrates (g%)
NM	70.7 ± 1
RF	81.3 ± 2
HTM	71.8 ± 1
DM	80.8 ± 1
WB	70.8 ± 1

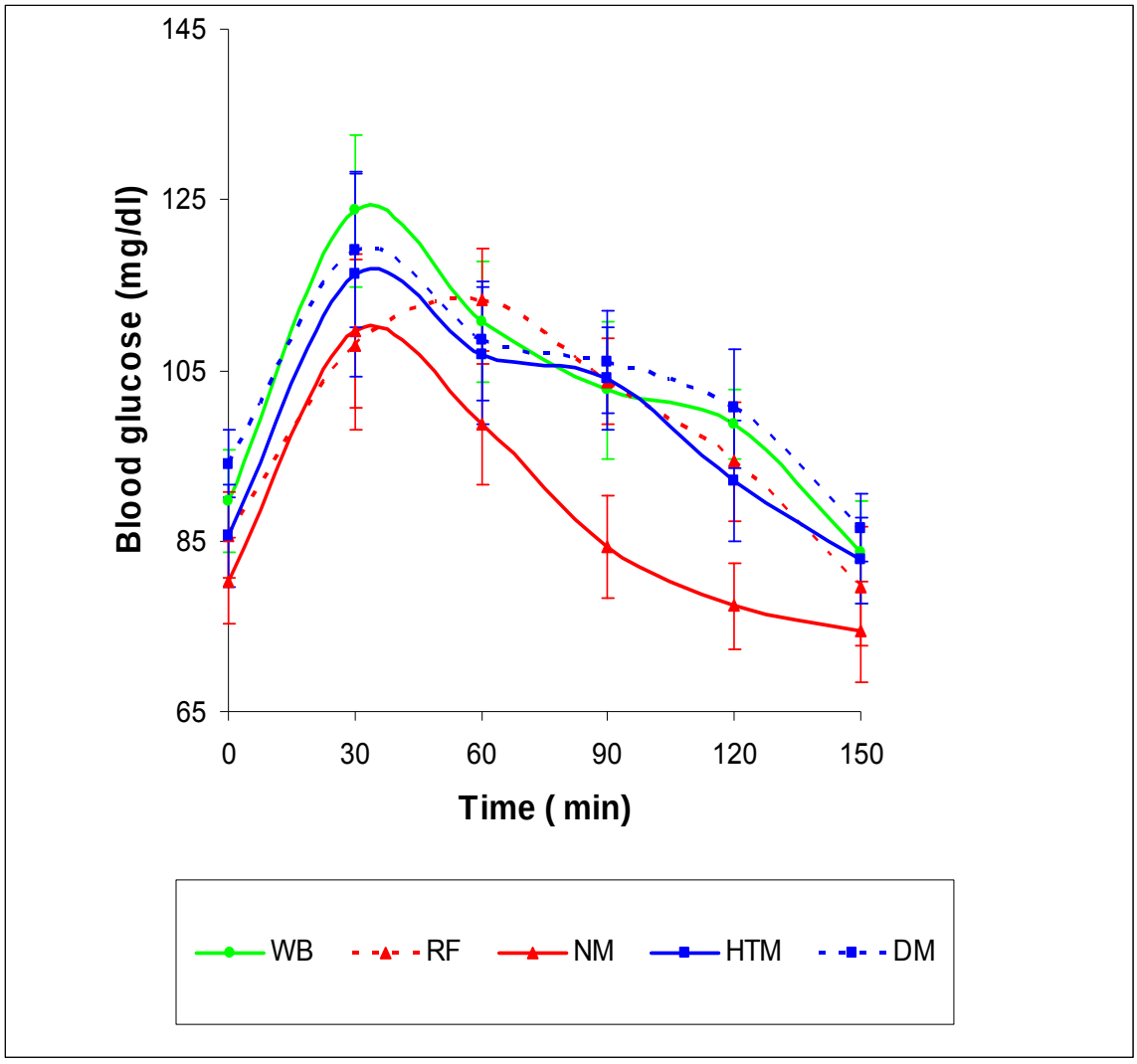


Figure 38. Glycemic response to the native millet (NM), refined flour (RF), hydrothermally processed millet (HTM), decorticated millet and standard food (white bread, WB) in normal subjects

The rapid digestibility of white bread (WB) compared with the other test foods may be attributed to its lower dietary fiber content and also due to the fermentation it undergoes during its preparation as fermentation is known to increase the carbohydrate digestibility. The blood glucose response curves of the normal subjects showed highest peak for WB (123 mg/dl), followed by DM (119 mg/dl), HTM (116 mg/dl), RF (113mg/dl) and NM (110 mg/dl). Slightly higher blood glucose peak values for the HTM and DM compared to NM and RF may be due to the presence of pre-gelatinised starch. Even though the peak values for the DM was higher compared to HTM, the area under the curve for the DM was lower compared to HTM, RF and WB, probably due to its ingestion in the form of discrete grains. The GI values for the test foods from the millet namely, NM, HTM, RF and DM in the normal were 69, 101, 100 and 81 respectively (Table 26). In the case of type II diabetic subjects also a similar trend for GI was observed for all the foods. However, the DM exhibited the lowest peak blood glucose (211 mg/dl) followed by NM (213 mg/dl), HTM (219 mg/dl), WB (230 mg/dl) and RF (236 mg/dl). Even though the peak blood glucose level for the HTM was lower compared to the WB and the RF, the area under the curve for HTM was higher (Figure 39). The GI values for NM, HTM, RF and DM in the type II diabetic subjects were 73, 107, 104 and 78 respectively (Table 26). Urooj et al (2006) have also reported a GI value of 68, 80 for the millet dumplings in the normal and type II diabetic subjects respectively. These values are nearly comparable to the GI values observed for the NM dumplings in the present study. Similarly, the GL for the NM, HTM, RF and DM were also nearly comparable in the normal (35, 51, 50 and 41 for NM, HTM, RF and DM respectively) and type II diabetic subjects (37, 54, 52 and 39 for NM, HTM, RF and DM respectively). The GI values were in the order of NM<DM<RF<HTM in both normal and type II diabetic subjects.

The finer particle size, method of preparation, the lower dietary fiber, polyphenols and phytate contents of the RF may account for its higher GI value. The NM dumplings exhibited the lowest GI in both the normal and NIDDM subjects (69 and 73 in normal and NIDDM subjects respectively). The lower GI values for the NM dumplings may probably due to its complex carbohydrates and the seed coat matter constituents such as dietary fiber, polyphenols and phytate contents.

Table 26. Glycemic index (GI) and glycemic load (GL) values for the native millet (NM), hydrothermally processed millet (HTM), refined flour (RF) and decorticated millet (DM) in normal and type II diabetic subjects

Diet	Glycemic Index		Glycemic load	
	Normal (n=6)	Type II diabetic subjects (n=6)	Normal (n=6)	Type II diabetic subjects (n=6)
NM dumpling	69±3	73±4	35±3	37±2
HTM dumpling	101±3	107±2	51±2	54±4
RF dumpling	100±3	104±4	50±2	52±3
Cooked DM	81±2	78±3	41±3	39±4

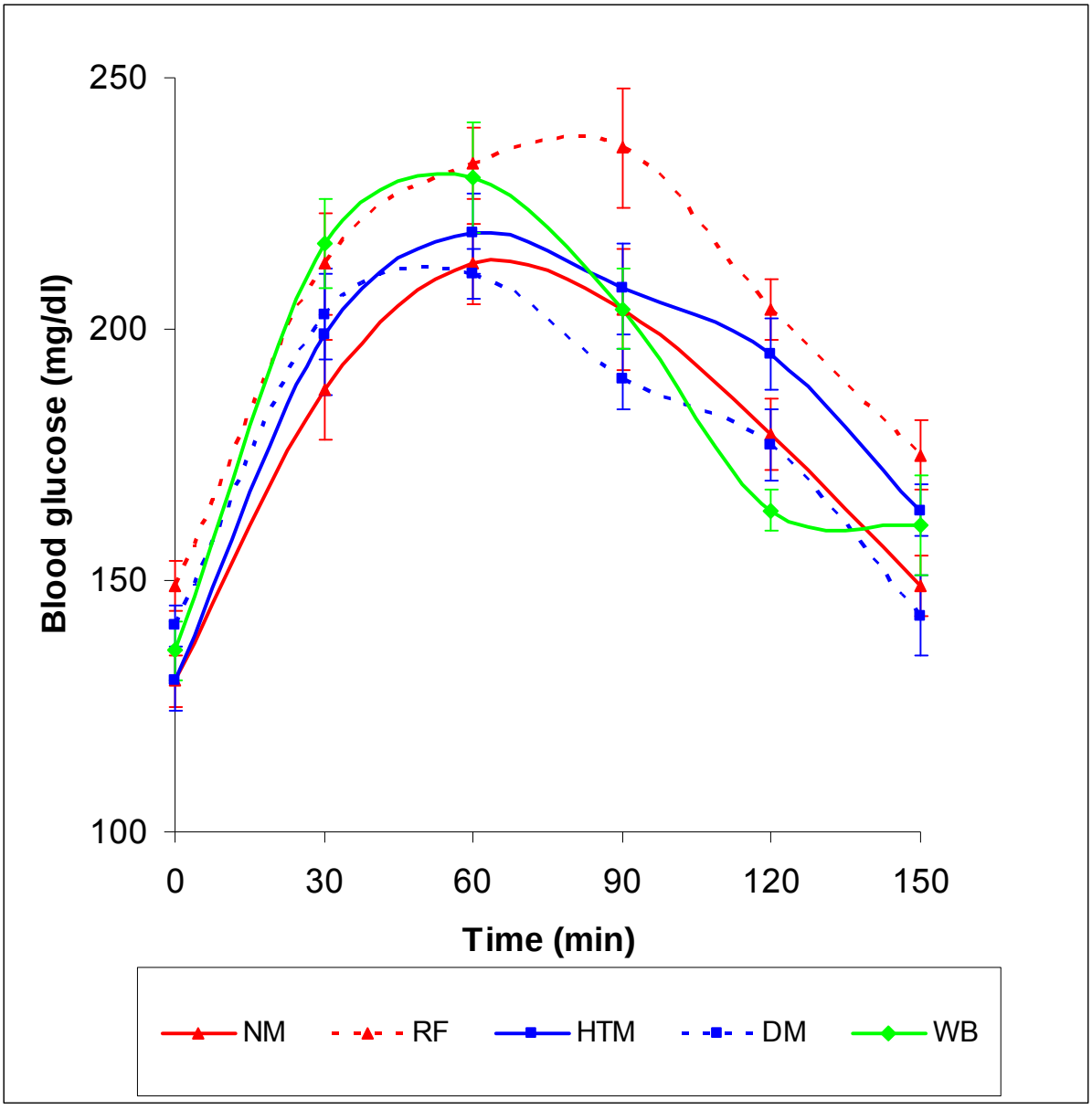


Figure 39. Glycemic response to the native millet (NM), refined flour (RF), hydrothermally processed millet (HTM), decorticated millet and standard food (white bread, WB) in type II diabetic subjects

The role of polyphenols and phytic acid in blood glucose attenuation has been studied well. Phytic acid is known to reduce the blood glucose response to foods (Thompson et al 1987) as it is a known inhibitor of amylase. Three mechanisms have been proposed by which phytate could inhibit amylases and modulate carbohydrate digestibility. Amylases are calcium dependent enzymes and their activity could be reduced by limiting the availability of calcium due to its binding to phytate, or by binding of phytate to the enzyme bound calcium. Alternatively, phytate could bind to starch by hydrogen bonding, or phytate could bind to the protein portion of a starch-protein complex, and may reduce the ability of starch to reach the active site of amylase (Thompson 1986). Moreover, it has been reported that, the polyphenol content in the diet especially the large polymeric or the condensed tannins may probably bind with carbohydrate hydrolysing enzymes and reduce their activity leading to reduced glycemic response (Thompson et al 1983). This is very much possible as the millet is a rich source of polymeric tannins (Table 21).

The Influence of dietary fibre in reducing the glycemic response has been reported by several workers. Kimmel et al (2000) and also Nelson et al (1998) reported decreased postprandial blood glucose and also an improved glycemic control in dogs (with naturally acquired diabetes mellitus) maintained on the diet enriched with insoluble fiber. Seki et al (2005) in their study reported that, insoluble dietary fiber is the major constituent responsible for lowering the postprandial blood glucose concentration in the pre-germinated brown rice. Since the millet seed coat is rich source of insoluble dietary fiber, polyphenols and phytate, the lower glycemic response of the whole meal millet products is possible, however the method of food preparation and also the form in which the food is ingested is also known to influence the glycemic response of the food product.

In the case of rice also, it has been observed that, the product prepared from flour showed significantly higher glycemic and insulinemic response compared to the rice consumed in the form of grains in normal (O'Dea et al 1980) and diabetic subjects (Collier and O'Dea 1982). O'Dea et al (1981) studied the digestibility of four cooked forms of rice including white rice, unpolished (brown) rice, ground white rice and ground brown rice and observed that the rate of digestibility of the ground brown

and white rice was more rapid compared to the intermediate and lower digestibility rate for whole white rice and whole brown rice respectively. The postprandial glucose and insulin responses were greater after ingestion of ground rice than rice grains. Grinding increases the surface area of the starch and hence ground rice is more accessible to the hydrolytic enzymes than the starch in the unground rice samples. Wong and O'Dea (1983) demonstrated that grinding the lentils before cooking increased the starch hydrolysis rate 5 fold, while blending them after cooking gave an intermediate rate. However, the same effect was not observed in case of ground cooked lentils. Grinding raw foods and then cooking them would destroy the seed coat, possibly allowing faster and greater swelling of the starch granules. This increases the degree of starch gelatinization leading to increased rate of starch digestion and enhanced glycemic response. Thus, in the present study, the flour based preparations showed higher glycemic response compared to the food ingested in the form of grains. Urooj and Puttaraj (1999) examined the digestibility index (DI) of some Indian conventional food preparations by *in vitro* methods and reported the DI in the order of *chapathi*<*poori*<semolina *idli*<*idli*<*pongal*<ragi *roti*<rice flakes. The authors also reported a higher degree of starch granular rupture and folding in *dosai, idli, pongal,* rice flakes, ragi *roti* samples and intermediate changes in *chapathi, poori*, semolina *idli and uppittu*. Thus preparation methods and ingredients too play a vital role in the carbohydrate digestibility. In a different study (Sharavathy et al 2001), reported that apart from the method of preparation, the ingredients utilized, the form of food and the accompaniments utilized along with food influence the carbohydrate digestibility.

In the case of the DM, inspite of the absence of the seed coat constituents (considerable lower levels of dietary fiber, polyphenols and phytate), the lower GI could be due to its consumption in the grain form, and the presence of retrograded starch formed during hydrothermal processing. On the other hand, the higher glycemic response for the HTM, even though it contains the seed coat and also the retrogradation of starch, may be attributed to the finer particle size and its intake as dumpling. Since, finer the particle size of food, higher the surface area, and higher

will be their susceptibility to the enzymatic digestion causing higher glycemic response (O'Dea et al 1980).

Retrogradation of starch increases the resistant starch content and decreases the carbohydrate digestibility. Parboiling is one of the good old methods of cereal processing especially in the field of paddy processing, and parboiled rice is known to be of lower GI. According to Wolever et al (1986), the GI for parboiled rice was lower (67) as compared to that of regular white rice (83). Parboiling of rice facilitates amylose to retrograde and or to form inclusion complexes with polar lipids and there by lowers its carbohydrate digestibility or GI and in addition, alters the cooking qualities of rice (Unnikrishnan and Bhattacharya 1986). Preparation of the decorticated millet involves hydrothermal processing which is nothing but parboiling, and may cause retrogradation and resistant starch formation. Mangala et al (1999) reported the resistant starch formation during processing of rice and finger millet and reported that, cooking and cooling (at 4^0C for overnight) of the rice and ragi flour suspensions led to increased levels of resistance starch formation. They also reported that, by repeating the cooking-cooling cycles it is possible to increase the resistant starch formation. On the context of above discussions, the lower GI of the DM could be due to the synergistic effect of the resistant starch content as well as its ingestion in the form of discrete grains. On the other hand, the higher GI for the dumpling prepared from the HTM could be due to the finer particle size of the flour and method of preparation (fine flour cooked in water to prepare dumpling).

There are conflicting reports on the GI of the millet foods, Ramanathan and Gopalan (1957) reported a lower peak postprandial blood glucose values and glycemic response to the ragi diet compared to raw milled rice, parboiled rice, and wheat. They also indicated lower peak postprandial blood glucose values for the millet starch isolate compared to rice starch isolate in normal subjects as well as diabetic subjects. In line with the above report, Lakshmi Kumari and Sumathi (2002) reported lower GI for whole millet *roti* and *dosa* compared to wheat *roti* and rice *dosa*. They also reported that, malting increased the carbohydrate digestibility of the millet foods.

However, Chitra and Thilagabhaskaran (1989) did not observe any significant differences between wheat *phulkas* and ragi *adai* in normal, type I and type II diabetic subjects. On the other hand, Kavita and Prema (1995) reported lower glycemic response to wheat and ragi based diets compared to rice and tapioca diets in NIDDM subjects. The glycemic index to the millet dumpling and *roti* were 80 and 88 respectively in the NIDDM subjects and 68 and 80 respectively in the normal subjects (Urooj et al 2006). The authors also reported a higher degree of gelatinization and digestibility index for the millet dumpling compared to the millet based *roti* preparation.

Recently, Shobana et al (2007) formulated diabetic foods utilising decorticated millet (DM) as the cereal base and evaluated the same for its glycemic response on normal subjects and reported a GI of 93, as compared to 55 for a similar formulation based on wheat. They attributed the high GI of the decorticated millet based formulation to the higher degree of polishing for the decorticated millet and also due to their lower dietary fiber and polyphenol contents.

The *in vitro* rate of starch hydrolysis and digestibility index of the different millet based preparations were studied by Roopa et al (1998). They reported that, the DI (digestibility index) of *roti*, *dosa*, dumpling and *puttu* from the millet were higher compared to similar products prepared from cereals such as rice, wheat and jowar. Apart from the particle size of the cereal used for preparing the food product and also the kind of food, the accompaniments used along with the cereal food such as spicy and accompaniments rich in fat are known to influence the GI.

The lower GI values generally observed for the millet foods in some of the studies compared to rice and other cereals could be explained based on the information available on the nature of the millet starch and its molecular organisation besides the influence of its polyphenol, phytate and dietary fiber contents. The nature of starch present in the food also plays an important role in the glycemic response it elicits. Mohan et al (2005) studied the characteristics of the native and enzymatically hydrolysed finger millet and rice starches and reported that, the degree of crystallinity for the millet starch is higher (30.09%) compared to that of rice starch (21.69%). They further indicated that, the quantum of heat flow recorded by DSC (differential

scanning calorimetry) in terms of energy required to gelatinize the millet starch was nearly 30% higher for the millet compared to that of rice. Apart from this, they also observed that, the molecular weight of the human salivary amylase digests of the millet starch were significantly higher (>500 KDa) compared to that of rice starch digest (415 KDa). These results clearly indicate the higher degree of crystallinity and lesser susceptibility characteristics of the millet starch to the digestive enzymes. Similar observations were also reported by Singh and Ali (2006) who examined the *in vitro* starch hydrolysis of different cereal and legume starches by fungal α-amylase, and observed that, among the cereal starches such as rice, wheat, maize, sorghum and finger millet, the millet starch was the most resistant to the enzymatic hydrolysis. They attributed this to the peculiar form of the millet starch and its molecular architecture compared to other cereal starches.

From the studies, it is very clear that, apart from the complex molecular make up of the millet starch, nature of processing of the millet and also the form of food plays a vital role towards the glycemic response of the millet foods. Besides, the seed coat constituents of the millet influence the glycemic response of the millet foods. Although, the GI of the conventional millet foods range from moderate to higher values, the presence of higher levels of dietary fiber and also some of the phytochemicals namely, polyphenols and phytate which are known to inhibit the carbohydrases of the GI tract and control postprandial hyperglycemia as well as protein glycation in synergy with their antioxidant potentials for sure on long term consumption may help in the nutritional management of diabetes.

Several studies emphasize on the beneficial role of low GI foods in the nutritional management of diabetes and several other chronic diseases. Low GI foods tend to delay glucose absorption and thereby result in reduced peak insulin concentrations and overall insulin demand. Several studies have also shown improvements in glycemic control with low GI diets (Augustin et al 2002). Clinical trials with more number of subjects on differently processed millet foods may help in identifying the suitable processing methods for producing millet products with low GI. The millet products processed suitably to lower their GI in synergy with accompaniments rich in vegetables and pulses may help in prevention of diabetes

and several other chronic diseases. The study also indicates that, consumption of whole meal based millet products is desirable as the protective role of the millet seed coat matter in general and polyphenols in particular would contribute for the health benefits of the millet foods.

At present, the consumption of the millet is largely confined to the traditional millet consumers, and hence to facilitate as well as to encourage the non-millet consumers to accept the millet based food products as a part of their dietary components, it is highly desirable to process the millet in the form it is acceptable by one and all. In this direction, the semi-refined millet flour as well as decorticated millet may have good potential.

SUMMARY AND CONCLUSIONS

- The nature of starch in the native finger millet (NM), hydrothermally processed millet (HTM) and decorticated millet (DM) were studied by gel permeation chromatography and the results indicated slight thermal degradation of amylopectin during hydrothermal processing of the millet resulting in the increase of lower molecular weight amylose fraction of the HTM and DM.
- The *in vitro* carbohydrate digestibility of finger millet starch by pancreatic amylase was affected by the presence of millet seed coat polyphenols and a decrease in the amount of maltose equivalents released upon pancreatic amylase digestion of the millet starch was observed. The extent of decrease was proportionate to the concentration of the polyphenols present in the reaction mixture.
- The ESI-MS analysis of the pepsin-pancreatin hydrolysates of the millet starch isolate, native millet (NM), refined flour (RF) indicated the presence of maltose, maltotriose, maltotetraose, maltopentaose. Whereas, maltopentaose was absent in the hydrolysates of the hydrothermally processed (HTM) and the decorticated millet (DM).
- The influence of the millet seed coat matter and also the hydrothermal processing on the *in vivo* carbohydrate digestibility of the millet was studied in terms of glycemic response in normal and type II diabetic subjects. The dumpling (*mudde*) from the native millet (NM), refined flour (RF), hydrothermally processed millet (HTM) and also the decorticated millet (DM) cooked in the form of discrete grains was used for the study. The GI values of NM and RF were 69 ± 3 and 100 ± 3 in the normal subjects, and 73 ± 4 and 104 ± 4 in the type II diabetic subjects respectively. Similarly, the GI values of HTM and DM were 101 ± 3 and 81 ± 5 in the normal and, 107 ± 2 and 78 ± 6 in the type II diabetic subjects respectively. The lower GI of the NM may be due to the presence of seed coat matter constituents (higher dietary fiber content, polyphenols and phytate) whereas, the lower GI of the DM could be mainly attributed to the form of food (discrete grains compared to the flour based dumpling preparation). The higher GI of the RF may be due to the lower levels of the seed coat constituents and the

higher GI of the HTM even though it contained seed coat matter may be due to gelatinization of starch during hydrothermal processing. The study indicated that, the millet seed coat matter exerts positive influence towards lowering the GI values. In addition, the nature of food processing and also the form of food are known to influence the GI values.

The millet seed coat exerts definite influence towards lowering the carbohydrate digestibility of the millet foods as evidenced by the lower GI values for the native whole meal compared to the refined flour. However, the form of food ingested also influence the glycemic response. This was clearly observed by the higher GI values for the dumpling from the whole meal of the hydrothermally processed millet compared to the lower GI values for the decorticated millet (decorticated hydrothermally processed millet consumed in the form of grains)

INTRODUCTION

Dietary management has remained as a sheet anchor in the clinical management of diabetes. Medical therapy in synergy with the diets rich in complex carbohydrates and phytochemicals with blood glucose lowering effects especially with amylase and α-glucosidase inhibitory effect has been known to halt the progression of diabetes by controlling the postprandial hyperglycemia. One of the most common therapeutic approaches to decrease postprandial hyperglycemia is to prevent the absorption of glucose after food uptake. Complex polysaccharides are hydrolyzed by amylases to dextrins or oligosaccharides which are further hydrolyzed to glucose by intestinal α-glucosidase before being absorbed into the intestinal epithelium and enter the blood circulation. Therefore, amylase and α-glucosidase inhibitors may help in reducing the postprandial hyperglycemia by partially inhibiting the enzymatic hydrolysis of higher molecular weight carbohydrates and hence delay the absorption of glucose. Acarbose, Voglibose and Miglitol are widely used either alone or in combination with insulin secretogogues in patients with type II diabetes (Saito et al 1998). However these inhibitors are reported to cause several side effects such as liver disorders (hepatic injury and acute hepatitis), flatulence, abdominal cramping etc. In addition, some of them may increase the incidence of renal tumors. In view of this, several other safer natural amylase and α-glucosidase inhibitors have been reported from plant sources (Hiroyuki et al 2001, Matsui et al 2001).

Non-enzymatic, glucose-derived, crosslinking of proteins is a pathophysiological event and has been recognized as a causative factor in diabetic complications and age-related diseases (Ahmed 2005). In persistent hyperglycemia, the modification of proteins by glucose is initiated by non-enzymatic interaction of carbonyl groups of glucose with amino groups of proteins to form an unstable Schiffs base, which rearranges rapidly to more stable Amadori products. The Amadori compounds undergo a series of slow reactions involving rearrangement, oxidation and dehydration to form stable, heterogeneous adducts known as advanced glycosylation end products (AGEs) that remain tightly bound to the protein. The post-Amadori products include, highly reactive α-dicarbonyls such as Amadori dione, 3-deoxyglucosone, glyoxal,

and methyl glyoxal (Ahmed 2005). AGE-formation from Amadori product occurs slowly over a period of months and years and they remain tightly bound to proteins and form intra- and intermolecular crosslinks with adjacent proteins. AGE-crosslinking causes flexible proteins to become rigid, consequently, the cells, tissues, and blood vessels become stiff and increasingly dysfunctional. In diabetic patients, the rate of AGE accumulation and the extent of protein crosslinking are accelerated due to exposure to highly elevated concentrations of glucose (Vasan et al 2003). Numerous studies have established the role of AGEs in the development of renal, ophthalmological, neurological, and cardiovascular complications in diabetes and aging (Ahmed 2005). Oxidation reactions can also contribute to AGE formation (Baynes 1991). Amadori products undergo autoxidation (glycoxidation) to form AGEs. In the presence of transition metals and molecular oxygen, the Amadori products are converted to protein dicarbonyl compounds via a protein enediol generating the superoxide radical (Hunt et al 1993). The protein dicarbonyl compounds can participate in AGE formation and are referred to as glycoxidation products. Once formed, the superoxide radicals can be converted to the highly reactive hydroxyl radical ($OH^{\cdot}$) via the Fenton reaction (Ahmed 2005).

It has been proposed that, oxidative stress plays an important role in the tissue damage that is associated with aging and various diseases such as diabetes (Baynes 1991) and cancer. The source of this undesirable oxidation is not known, but may be related to an increase in the concentration of transition metals, and or substances prone to oxidization and generating the reactive oxygen species, such as monosaccharides. For instance, the plasma concentration of copper (Harman 1965) and iron increase with age and in diabetes, these metals readily catalyze the reactions related to the formation of H_2O_2 and free radicals such as the superoxide anion ($O_2^{\cdot -}$) and hydroxyl radical ($OH^{\cdot}$). The $OH^{\cdot}$ is the most powerful oxidizing species among several reactive-oxygen radicals, and is able to oxidize most organic compounds such as proteins (Gutteridge and Wilkins 1983, Cooper et al 1985, Hunt et al 1988a, Hunt et al 1988b). Proteins exposed to glucose are cleaved, undergoes conformational change and develop fluorescent adducts and these changes are presumed to result from the covalent attachment of glucose to amino groups.

The fragmentation and the conformational changes observed are dependent upon $OH^{\cdot}$ produced by the glucose autoxidation (Hunt et al 1988a).

Potential therapeutic approaches for the AGE-derived pathologies include prevention of AGE-formation and breaking the already existing AGE–protein crosslinks. AGE-inhibitors of various structural classes have been identified and characterized (Vasan et al 2003, Ahmed 2005). The glycation inhibitor aminoguanidine (pimagedine) attenuates the development of range of diabetes vascular complications (Ahmed 2005). However, some problems of toxicity have been encountered in clinical trials with aminoguanidine in animal models and humans. Aminoguanidine is reported to have side effects in patients, which include flu-like symptoms, gastrointestinal disturbances and anaemia. Hence, search for newer and safer molecules with anti-glycation properties from plant sources have gained importance in recent days. Several herbal extracts and plant derived molecules have been tested for their anti-glycation properties (Morimitsu et al 1995). Phenolic compounds and antioxidant molecules are known to be effective free radical scavengers and inhibitors of protein glycation and AGE formation (Matsuda et al 2003).

Methanolic extracts of the millet were studied for their antioxidant potentials by Sripriya et al (1996) and inhibition of collagen glycation and crosslinking by Hegde et al (2002). Hegde et al (2005a) in their study, reported the antioxidant and anti-collagen glycation potential of millet diets in diabetic rats. Radical scavenging and anti-glycation properties were attributed to the phenolic content of the millet. However, there are no specific scientific reports on the health benefits of the millet constituents with special reference to the influence of its phenolic compounds on the carbohydrate digesting enzymes, namely pancreatic amylase and intestinal α-glucosidase (the two key enzymes involved in the regulation of glucose homeostasis). Since it is well known that the majority of the phenolics in the millet are concentrated in the seed coat, in the present study, evaluation of the inhibitory role of the millet seed coat phenolics (MSCP) on the pancreatic amylase and intestinal α-glucosidase were carried out. Enzyme kinetic studies were also performed to understand the possible mode of inhibition of these phenolic compounds. In addition, the ability of MSCP as

inhibitors of non- enzymatic protein glycation and $OH^{\cdot}$ radical mediated protein fragmentation were also tested.

The millet phenolics are known to exert antimicrobial activity (Chethan 2008, Varsha et al 2009). However, the inhibitory effect of the MSCP on the human pathogenic microflora are scanty. Hence, the antimicrobial potency of the MSCP were also tested on pathogenic microflora isolated from humans.

MATERIALS AND METHODS

Chemicals and reagents: Catechin, 2,2'-Diphenyl-1-picrylhydrazyl (DPPH) radical, *p*-nitrophenyl-alpha-D-glucopyranoside (PNP-glycoside), rat intestinal acetone powder, porcine pancreatic amylase and bovine serum albumin (BSA) were purchased from Sigma-Aldrich chemical company (St. Louis, USA). Nutrient agar was purchased from Hi Media Laboratories, Mumbai, whereas ethylene diamine tetra acetic acid (EDTA), ascorbic acid and fructose were procured from SISCO research laboratories, Mumbai, India. All the chemicals used were of analytical grade, and glass (double) distilled water was used for the preparation of the reagents.

The millet seed coat matter (SCM): The millet seed coat matter was prepared by sequential milling and sieving methodology as described in Chapter II.

Extraction of the polyphenols from the SCM: Polyphenols from the SCM was extracted as per the methodology described in Chapter II. The SCM (1g) was refluxed with acidic methanol (99 ml methanol mixed with 1 ml concentrated HCl) solvent system in a heating mantel maintained at 60^0 C for 10 min, the extract was cooled, filtered on Whatman No. 1 filter paper and the residue was re-extracted with acidic methanol and the process was repeated until the extract tested negative for polyphenols. The pooled extracts were neutralized to 2.5 pH with 0.1 N NaOH, concentrated under vacuum in a rotary flash evaporator, centrifuged and the supernatant (millet seed coat polyphenols or MSCP) was assayed for total polyphenol content and used for further studies (Chethan and Malleshi 2007b).

Assay of polyphenol content: The total polyphenol content of the phenolic extract was assayed by the method of Singleton et al (1995) as described in Chapter II.

Preparation of enzyme (α-glucosidase) extract from the rat intestinal acetone powder: Rat intestinal acetone powder (100mg) was homogenized in 3 ml of 0.9% NaCl solution according to the method of Oki et al (1999). After centrifugation at 12,000×g for 30 min, the clear supernatant was used for α-glucosidase assay.

Inhibition of intestinal α-glucosidase: The inhibitory effect of the polyphenols on the rat intestinal α-glucosidase was assayed according to the procedure described by Matsui et al (2001) with minor modifications. The enzyme (0.02 units) and aliquots of the MSCP (to obtain ≈ 30-80% inhibition) were pre-incubated for 10 min at 37^0C in 0.1 M PIPES [piperazine-1,4 – bis (2-ethane sulphonic acid)] buffer, pH 6.8. At the end of pre-incubation, 0.5 ml of 2.0 mM *p*-nitrophenyl-alpha-D-glucopyranoside (PNP-glycoside) was added to the enzyme-polyphenol mixture and incubated at 37^0C for 30 min. The reaction was terminated by adding 1 ml of 0.64% N-(1-napthyl) ethylenediamine solution (pH 10.7). Enzyme activity was quantified by measuring the *p*-nitrophenol released from PNP-glycoside at 400 nm. One unit of enzyme activity is defined as the amount of enzyme required to release one micromole of *p*-nitrophenol from PNP-glycoside per minute under assay conditions. Enzyme inhibitory reactions for all the polyphenol concentrations were carried out in three replications. Suitable solvent control for particular polyphenol concentrations was maintained.

Inhibition of pancreatic amylase: The method of Bernfeld (1955) was followed for the assay of inhibitory properties of the MSCP against pancreatic amylase with minor modifications. The amylase activity was determined using soluble starch (1 %) as a substrate in 0.1 M sodium phosphate buffer containing 1mM $CaCl_2$, pH 7.4 (final volume 2.0ml). The enzyme solution (0.33 units) and suitable aliquots of the MSCP (to obtain ≈ 30-80% inhibition) in 1 ml were pre-incubated for 10 min at 37^0C. The residual amylase activity was measured by adding 1 ml of starch substrate and incubated at 37^0C for 30 min. The reaction was terminated by the addition of 2.0 ml of dinitrosalicylic acid solution (DNS) and the resulting solution was heated in a boiling water bath for 5 min, the total volume of the solution was made up to 20 ml with water and the absorption of the solution was recorded at 540 nm. Enzyme activity was quantified by measuring the maltose equivalents released from starch. One unit of enzyme activity is defined as the amount of

enzyme required to release one µmol of maltose from starch per minute under assay conditions. Enzyme inhibitory reactions for all the polyphenol concentrations were carried out in three replications. Appropriate solvent controls were maintained.

Determination of IC_{50} for α-glucosidase and amylase: In order to determine the IC_{50} values, the enzyme activities of intestinal α-glucosidase and pancreatic amylase were determined in the presence of various concentrations of the MSCP (4-34 µg for α-glucosidase and 9-85 µg for amylase). IC_{50} is defined as the concentration of the polyphenols required to inhibit 50% of the enzyme activity.

Kinetics of enzyme inhibition: The mode of inhibition of intestinal α-glucosidase and pancreatic amylase by the MSCP was determined by analyzing Michaelis–Menton and Line-weaver Burk equations. PNP-glycoside in the concentration range of 0.5-4.0 mM and soluble starch in the range of 0.25-1.5% were used as substrates for α-glucosidase and pancreatic amylase respectively. Enzyme activities were determined in the absence or presence of different concentrations of the polyphenols. The concentrations of the MSCP used for the inhibitory kinetics of pancreatic amylase were 25.6, 34.2 and 42.7 µg/ml, whereas, 6.4, 8.5 and 10.7 µg/ml was used for intestinal α-glucosidase inhibitory kinetics. Dixon plots (Dixon 1953) were used to determine the inhibitory constants (K_i), whereas, secondary plots of [S]/V vs [I] were used to determine the dissociation constants (K_i') of the polyphenols (Bowden 1974).

Anti- protein glycation properties

Non-enzymatic glycation of protein: The conditions of amino-carbonyl reaction was adopted from the method reported by McPherson et al (1988) with slight modifications. Bovine serum albumin (BSA, 4mg/ml) and D-fructose (500mM) were dissolved in 200 mM sodium phosphate buffer (pH 7.4) containing 3 mM sodium azide. The glycation reaction mixture was incubated at 37^0C for 5 days in the presence and absence of the MSCP (20 and 45 µg) after which an equal volume of 10% (w/v) TCA was added and the precipitate was collected by centrifugation. The precipitate was washed twice with ice cold 5% TCA and resolved in 200 mM sodium phosphate buffer and one aliquot (containing 10µg protein) was used for SDS-PAGE (sodium dodecyl sulphate - polyacrylamide gel electrophoresis), and the other was used for fluorescent measurement.

Appropriate solvent controls were maintained and 15 mM aminoguanidine (AG), 5 mM catechin was used as reference standard. BSA alone and BSA with fructose served as blank and controls respectively. The glycation reaction mixtures containing 20 and 45 µg of the MSCP were used for the fluorescence measurement whereas, the glycation reaction mixture containing 45 µg MSCP was used for the SDS-PAGE.

SDS Polyacrylamide gel electrophoresis (SDS-PAGE): SDS-PAGE at pH 8.3 was carried out according to the method of Lammeli (1970).

Reagents:

a. Acrylamide (14.6g) and bis-acrylamide (0.4g) were dissolved in water (50 ml), filtered and stored in a brown bottle at 4^0C.
b. TRIS (18.5g) was dissolved in water (100ml) and the pH of the solution was adjusted to 8.8 with HCl (6N) and stored at 4^0C.
c. TRIS (3g) was dissolved in water (50 ml) and the pH of the solution was adjusted to 6.8 with HCl (6N) and stored at 4^0C.
d. Sodium dodecyl sulphate (SDS) (10g) was dissolved in water (100 ml).
e. TRIS (90.3g), glycine (1.44g) and SDS (0.1g) were dissolved in water (100ml).
f. Coomassie brilliant blue (0.2g) was dissolved in a mixture of methanol : acetic acid : water (25:15:60, v/v). The reagent was filtered and stored at room temperature.
g. Destaining solution: The solution was prepared by mixing methanol, acetic acid, water in the ratio of 25:15:60 (v/v).

Running gel (12%) was prepared by mixing solutions ‘a’ (4 ml) and ‘b’ (2.5 ml) with water (3.38 ml). The mixture was degassed and to that was added solution ‘d’ (0.1 ml), N’,N, N’,N’- tetramethyl ethylene diamine (TEMED, 20 µl) and ammonium persulphate (6.0 mg). The contents were mixed and poured between the assembled glass plates with edges sealed with agar (2%). Gels were allowed to polymerize at room temperature for 30 min.

Stacking gel (5%) was prepared by mixing solutions ‘a’ (0.83 ml) and ‘c’ (0.1 ml) containing SDS (4%), mercaptoethanol (10%) and glycerol (20%). To the above mixture bromophenol blue was added and heated in boiling water for 5 min.

The samples on cooling to room temperature were loaded in the wells immersed in solution 'e' and were run at constant voltage (50 V) for 3-4 h or until the tracking dye, bromophenol blue was just (0.5 cm) above the lower end of the gel.

The Sigma Dalton mark VII-L was reconstituted in 1.5 ml of sample buffer (0.0625 M TRIS- HCl, pH 6.75, containing 2% SDS, 5% β-mercaptoethanol, 10% glycerol and 0.001% bromophenol blue) and 5 µl (12 µg) of this was loaded onto the gel.

Staining: Gels were stained for proteins with reagent 'f' for 6 h at room temperature followed by destaining in reagent 'g'.

Analysis of BSA conformational changes: Tryptophan fluorescence quenching by glycation was determined as fluorescence spectra at excitation 295 nm and emission between 310-400 nm according to the method of Suryanarayana et al (2003). The fluorescence spectra of the glycated proteins (0.15 mg/ml in 200 mM sodium phosphate buffer, pH 7.4 containing 3 mM sodium azide) were obtained from fluorimeter.

Analysis of AGE formation: Advanced glycation end products (AGE) fluorescence was measured in the reaction mixture (0.15 mg/ml protein in 200 mM sodium phosphate buffer pH 7.4 containing 3 mM sodium azide) according to the method of Monnier and Cerami (1981). Fluorescence spectra were obtained from 400-500 nm with excitation at 370 nm in a fluorimeter.

Determination of DPPH radical scavenging ability: DPPH radical scavenging ability of the MSCP were determined by reacting the extracts (0.1 ml) with 6×10^{-5} mol/L $DPPH^{\cdot}$ solution (3.9 ml). The radical scavenging ability in terms of the decrease in absorbance was monitored at 515 nm. A control without any antioxidants was also included. $DPPH^{\cdot}$ scavenging capacity was expressed as % $DPPH^{\cdot}$ scavenged (Brand-Williams et al 1995). In order to determine the IC_{50} (concentration of the MSCP required to scavenge 50% of the DPPH radical) values, the DPPH radical scavenging abilities of the MSCP at various concentrations (0.5 - 5 µg) were determined.

Inhibitory studies on the $OH^{\cdot}$ mediated protein fragmentation: H_2O_2/metal-catalyzed oxidation system ($OH^{\cdot}$ generating system) consisted of 1 mg BSA, 0.05 mM $CuSO_4$, 2.5mM H_2O_2 in a final volume of 1.0 ml. Positive controls were maintained with 10 mM EDTA or 1 mM catechin. The reaction mixture was

incubated for 24 h at 37^0C. The polyphenols (MSCP) concentrations ranging from 2.5 - 5 µg were used in the study. After the incubation period, an aliquot of the reaction mixture containing 10 µg protein was applied to SDS polyacrylamide gel electrophoresis as described earlier in the chapter.

Antimicrobial properties: The organisms namely *Staphylococcus aureus* (from pus), *Klebsiella pneumoniae* (from urine), *Candida albicans* (from sputum), *E Coli* (from pus), *Staphylococcus aureus* (from blood) and *E Coli* (from urine) were isolated from the hospitalized patients in a local Hospital in Mysore and identified by standard methods (Murray 1995) and used for the studies. An 18 h old culture of selected bacteria/yeast was mixed with sterile physiological saline, was adjusted to 10^6 colony forming units (CFU) per ml. Whatman No. 1 sterile filter paper discs (6 mm) were impregnated with 20 µg of the MSCP. Parallely, aqueous methanolic extracts (millet seed coat refluxed with 70% methanol, filtered) were also tested for the antimicrobial properties. The antibacterial and antifungal susceptibility tests were carried out using agar diffusion methods. Petriplates were prepared by pouring 20 ml of nutrient agar (Hi Media laboratories). The inoculum was spread on the top of the solidified media and allowed to dry for 10 min. The discs were then applied and the plates were left for 30 min at room temperature to allow the diffusion of the millet seed coat polyphenol extract before their incubation for 24 h at 37^0C in an incubator for bacterial strains and 25 ^{0}C for yeast (*Candida albicans*) (Collins et al 1989). The inhibition zones formed around the discs were measured in millimeters. Each test was carried out in triplicates. Appropriate solvent controls were maintained.

RESULTS AND DISCUSSION

The millet seed coat polyphenols extract contained phenolic acids, such as gallic, protocatechuic, gentisic, caffeic, vanillic, syringic, ferulic, p-coumaric and transcinnamic acids in addition to the presence of naringenin, kaempferol, luteolin glycoside, phloroglucinol, apigenin, (+)-catechin/(-)-epicatechin, trans-feruloyl-malic acid, dimer of prodelphinidin (2GC)/(GC-C), diadzein, catechin gallates, trimers and tetramers of catechin (Chapter I). Some of these phenolic compounds have also been reported for sorghum, oats, wheat, maize and barley (Dykes and Rooney 2007), methanolic extracts of peas (Duenas et al 2004) and green tea.

Alpha-amylases are endoglucanases, which hydrolyze the internal α- 1,4 glucosidic linkages in starch. α-glucosidase is one of the glucosidases located in the brush border surface membrane of the intestinal cells, and is the key enzyme involved in the carbohydrate digestion. These enzymes have been recognized as therapeutic targets for modulation of postprandial hyperglycemia. Postprandial hyperglycemia is the earliest metabolic abnormality to occur in type 2 diabetes mellitus (Lebovitz 1998) and during which the blood glucose levels may be elevated in the presence of normal levels of fasting plasma glucose, constituting an early stage in type 2 diabetes, referred as 'postprandial diabetes' (Baron 1998). This state, not only initiates the development of micro- and macro-vascular complications, but can also contribute to a more rapid progression to symptomatic diabetes by causing glucose toxicity in muscle and pancreatic cells. Early identification of postprandial hyperglycemia and its effective control will aid in prevention of diabetic complications (Ratner 2001). The millet seed coat phenolics (MSCP) inhibited both the pancreatic amylase and α-glucosidase in a dose-dependent manner (Figures 40A and B). The IC_{50} values for α-glucosidase and the amylase were found to be 16.9 and 23.5 µg MSCP, respectively (Table 27). Plant phenolic compounds modulate the enzymatic breakdown of carbohydrates by inhibiting amylases and glucosidases (McDougall et al 2005). Various biological and health-beneficial effects of polyphenols have been reported (Middleton 1996). In the present study, the initial velocity 'v' of the hydrolytic reactions catalyzed by intestinal α-glucosidase and pancreatic amylase measured at different substrate concentrations [S] in the presence and absence of a fixed MSCP concentration [I], are indicated in Figure 41.

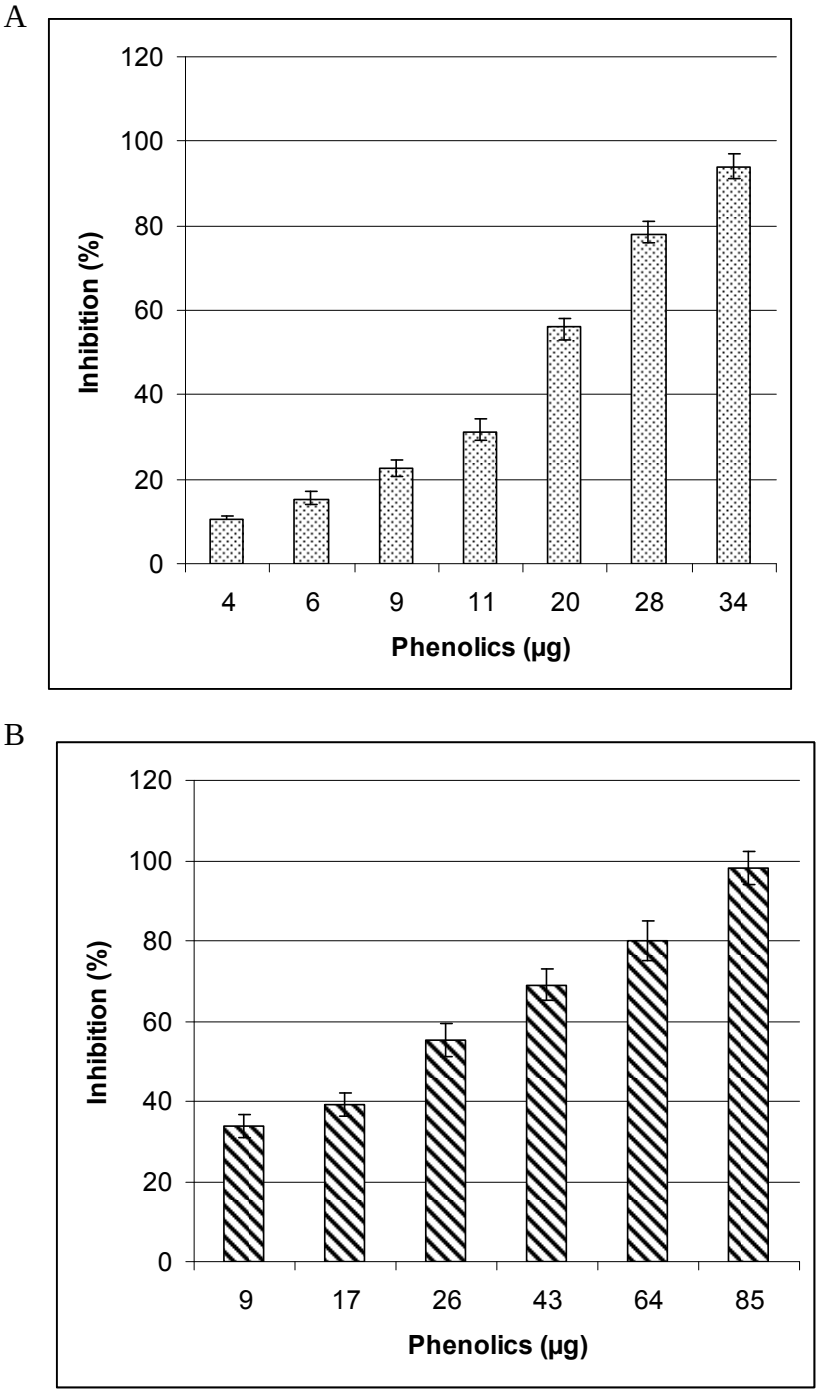


Figure 40. Inhibitory effect of different levels of the millet seed coat polyphenols on A. α-glucosidase and B. pancreatic α-amylase

Table 27. Kinetic properties of the millet seed coat polyphenols (MSCP) on intestinal α-glucosidase and pancreatic α-amylase

S.No	Parameter	α-glucosidase	Pancreatic α-amylase
1.	K_m	[a]8.0×10^{-3} M	[b]1%
2.	V_{max}	[c]83.33×10^{3}	[d]66.0×10^{-2}
3.	$V_{max'}$	[c]76.92×10^{3} (6.4µg)[e]	[d]31.3×10^{-2} (25.6µg)[e]
4.	$V_{max'}$	[c]71.42×10^{3} (8.5µg)[e]	[d]27.0×10^{-2} (34.2µg)[e]
5.	$V_{max'}$	[c]62.5×10^{3} (10.7µg)[e]	[d]21.7×10^{-2} (42.7µg)[e]
6.	IC_{50} (µg)	16.9	23.5
7.	K_i (µg)	5.0	10.0
8.	K_i'(µg)	2.5	7
9.	Mode of inhibition	Non-competitive	Non-competitive

[a] PNP glycoside

[b] Starch

[c]µmoles of *p*-nitrophenol released/min

[d] µmoles of maltose eq released/min

[e]concentration of millet seed coat phenolic compounds

The double reciprocal plots show that, straight lines were obtained with PNP-glycoside and starch substrates for intestinal α-glucosidase (Figure 41A) and pancreatic amylase (Figure 41B), respectively. Both, the slope 's' and vertical axis intercept 'i' increased with increasing concentration of the phenolic compounds. These results indicate that the binding of the phenolic compounds affected the velocity of the reaction catalyzed by α-glucosidase and amylase, proportionately to the concentration of the phenolic compounds in the reaction mixture, without affecting the Km. Non-competitive inhibition of the MSCP on the α-glucosidase-catalyzed hydrolysis of PNP-glycoside and amylase-catalyzed hydrolysis of starch can account for these results. The inhibitory kinetics of millet seed coat phenolics on the α-glucosidase catalyzed hydrolysis of PNP-glycoside (a short-chain substrate) and amylase catalyzed hydrolysis of starch (a long-chain substrate) were evaluated using Lineweaver-Burk and Dixon plots. The effect of either the inhibitor or the substrate concentration on the slope and vertical axis intercept of the corresponding plots was analyzed.

Kinetic constants for the inhibition of α-glucosidase and pancreatic amylase are listed in Table 27. α-glucosidase has a Michaelis-Menton constant (K_m) of 8×10^{-3} M for PNP-glycoside and V_{max} value of 83.33×10^{3} µmoles of *p*-nitrophenol released/min. Apparent V_{max} values in the presence of 6.4, 8.5 and 10.7 µg of millet seed coat phenolics were found to be 76.92×10^{3}, 71.42×10^{3} and 62.5×10^{3} µmoles, respectively. The K_m and V_{max} value for pancreatic amylase under the reaction conditions was found to be 1% starch and 66.0×10^{-2} µmoles of maltose eq released/min (Table 27).

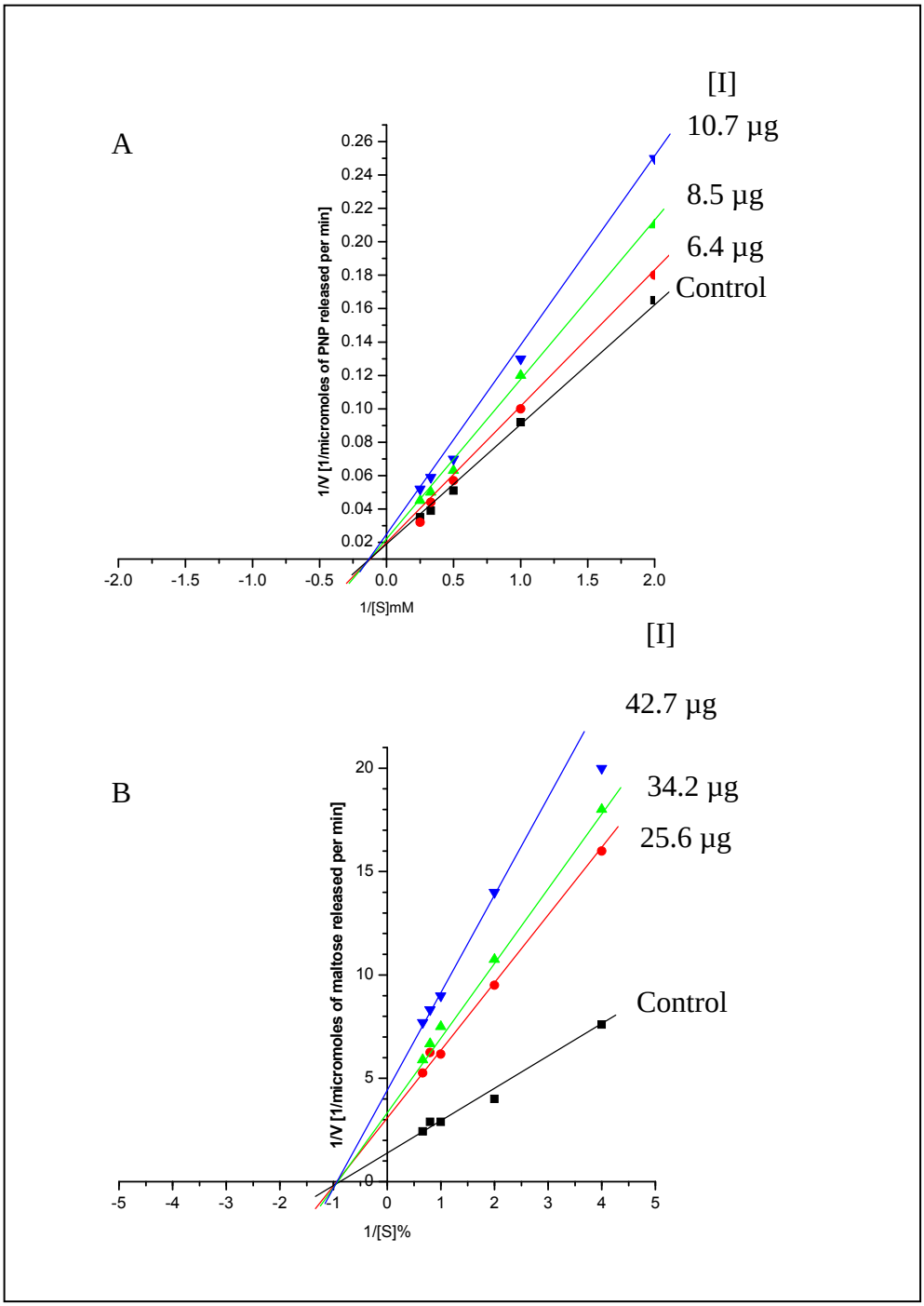


Figure 41. LB plot for determining the kinetic constants for α-glucosidase (A) and pancreatic α-amylase (B)

[I] - Inhibitor concentration

The apparent V_{max} values in the presence of 25.6, 34.2 and 42.7 µg of the MSCP were found to be 31.3×10^{-2}, 27.0×10^{-2} and 21.7×10^{-2} µmoles, respectively. The inhibitory constants (K_i) determined from the Dixon plot for α-glucosidase (Figure 42A) and pancreatic amylase (Figure 42B) were 5.0 and 10 µg of the MSCP respectively. The dissociation constant (K_i') for α-glucosidase was found to be 2.5 µg, whereas the pancreatic amylase had a K_i' of 7 µg of phenolic compounds as determined from secondary plots of [S] / V vs [I] (Bowden 1974).

The inhibition of α-glucosidase and pancreatic amylase by different classes of phenolic compounds has been studied well (Tadera et al 2006, Kim et al 2005, Shim et al 2003). α-glucosidase and pancreatic amylase were effectively inhibited by naringenin, kaempferol, luteolin, apigenin, (+)-catechin/ (-)-epicatechin, diadzein and epigallocatechin gallate (Tadera et al 2006). These flavonoids exhibited a mixed and close to non-competitive type of inhibition on yeast α-glucosidase. ESI-MS analysis indicated the presence of these phenolic compounds in the MSCP. Pine bark extract has been found to inhibit human salivary and porcine pancreatic amylases non-competitively. However, a combination of non-competitive and uncompetitive inhibition was observed in the case of α-glucosidase inhibition of PBE (pine bark extract) against yeast *S. cerevisae* α-glucosidase (Kim et al 2005).

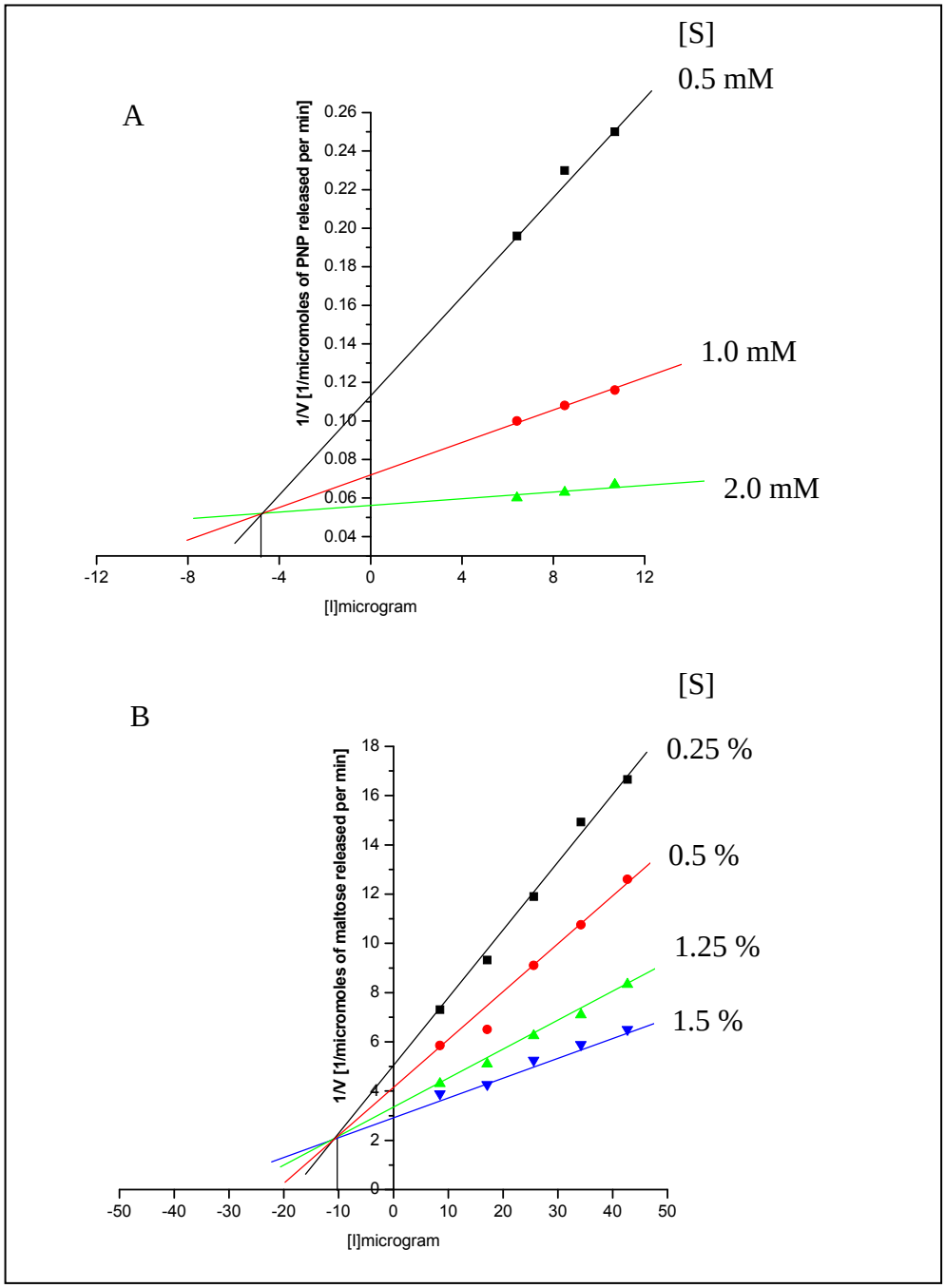


Figure 42. Dixon plot for determining the inhibitory constant (K_i) for α-glucosidase (A) and pancreatic α-amylase (B)
[S] - substrate concentration

Non-competitive nature of inhibition of α-glucosidase and porcine pancreatic amylase was reported in *Rhus chinensis* extract, a Korean herb traditionally used in the treatment of type 2 diabetes in Korea (Shim et al 2003). The MSCP exhibited an inhibitory activity similar to that of acarbose against α-amylase (Alkazaz et al 1996).

Finger millet diets have been recommended for diabetics from olden days. However, the mechanisms of action were not clearly known. The results of this investigation suggest that, the phenolic compounds present in the millet seed coat may regulate the glucose uptake from the intestinal lumen by inhibiting carbohydrate digestion and absorption, leading to normal glucose homeostasis. Further, there are extensive studies on the role of phenolic compounds in the regulation of glucose homeostasis. Phenolics from tea extract (epigallocatechin gallate, epicatechin gallate, tannins and theaflavins) have been reported to possess insulin enhancing properties (Anderson and Polansky 2002) and to regulate hepatic glucose output. Dietary phenolic acids such as chlorogenic acid, caffeic, catechin, ferulic, tannic and gallic acids have been reported to reduce glucose uptake by favoring dissipation of Na^+ electrochemical gradient, which provides the driving force for active glucose accumulation and hence glucose transport (Welsch et al 1989) and are known to contribute towards the decrease in glucose absorption and to regulate postprandial hyperglycemia. It is also possible that MSCP may regulate the glucose absorption by other mechanisms, in addition to the enzyme inhibition.

Many studies carried out over the past few years have shown that the polyphenols found in food and medicinal plants inhibit oxidative stress (Manach et al 2004, Rice-Evans et al 1996). Since the millet SCM is rich in polyphenols, especially in phenolic acids and flavonoids (Shobana et al 2009), it may inhibit the oxidative stress when taken as a component of food.

The MSCP inhibited AGE formation in a dose dependent manner. Figures 43 and 44 depicts the changes in the tryptophan and AGE fluorescence spectra of BSA after incubation with fructose, with or without co-incubation with MSCP or aminoguanidine (AG) as a standard anti-glycation agent. Tryptophan fluorescence is quenched by more than 79% after glycation with fructose.

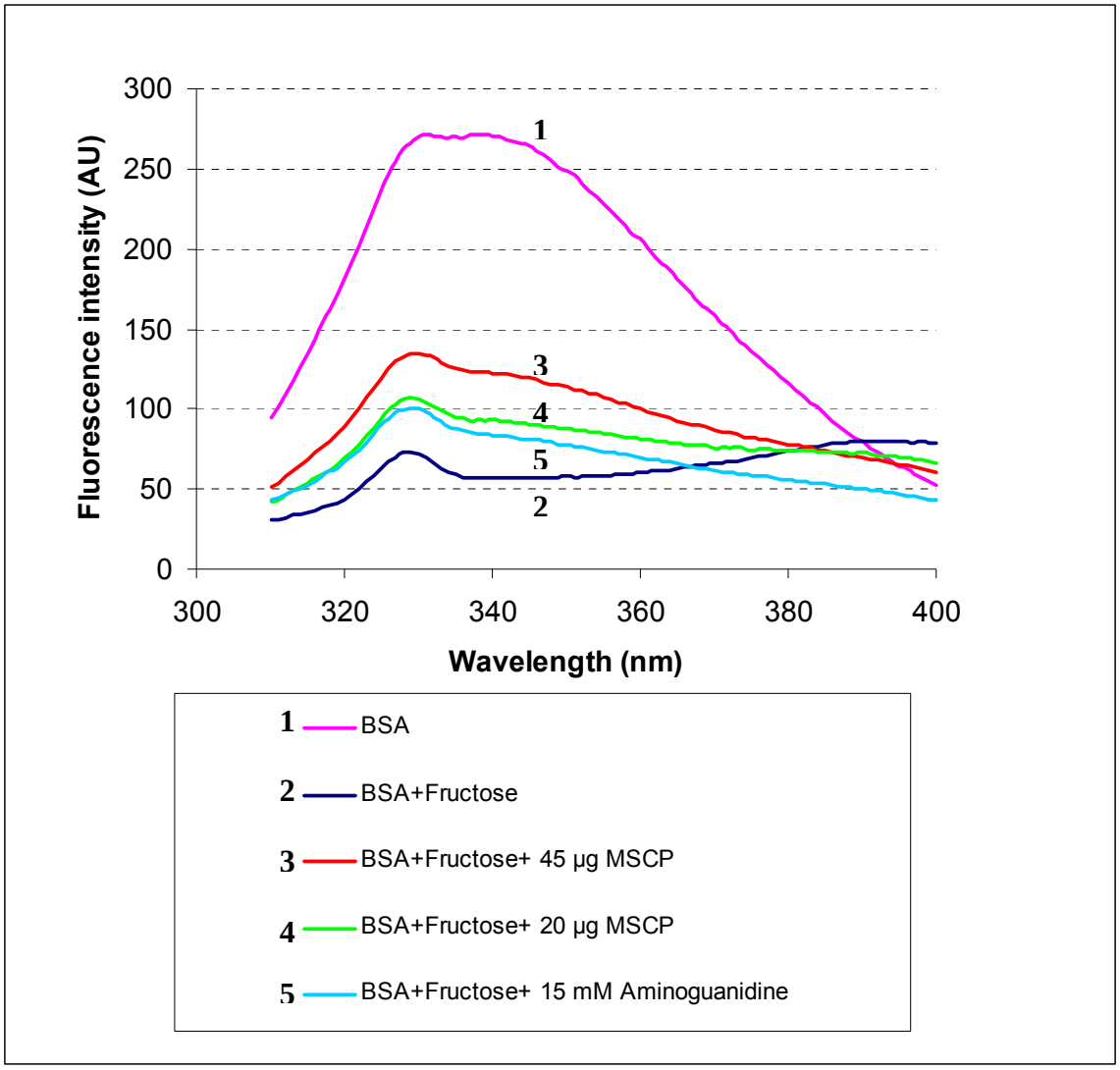


Figure 43. Tryptophan fluorescence spectra of BSA incubated in *in vitro* for 5 days at 37^{0}C in the presence of D-fructose (500 mM) and millet seed coat polyphenols (MSCP)

Positive control - 15 mM aminoguanidine.

Spectra were measured after excitation at 295 nm

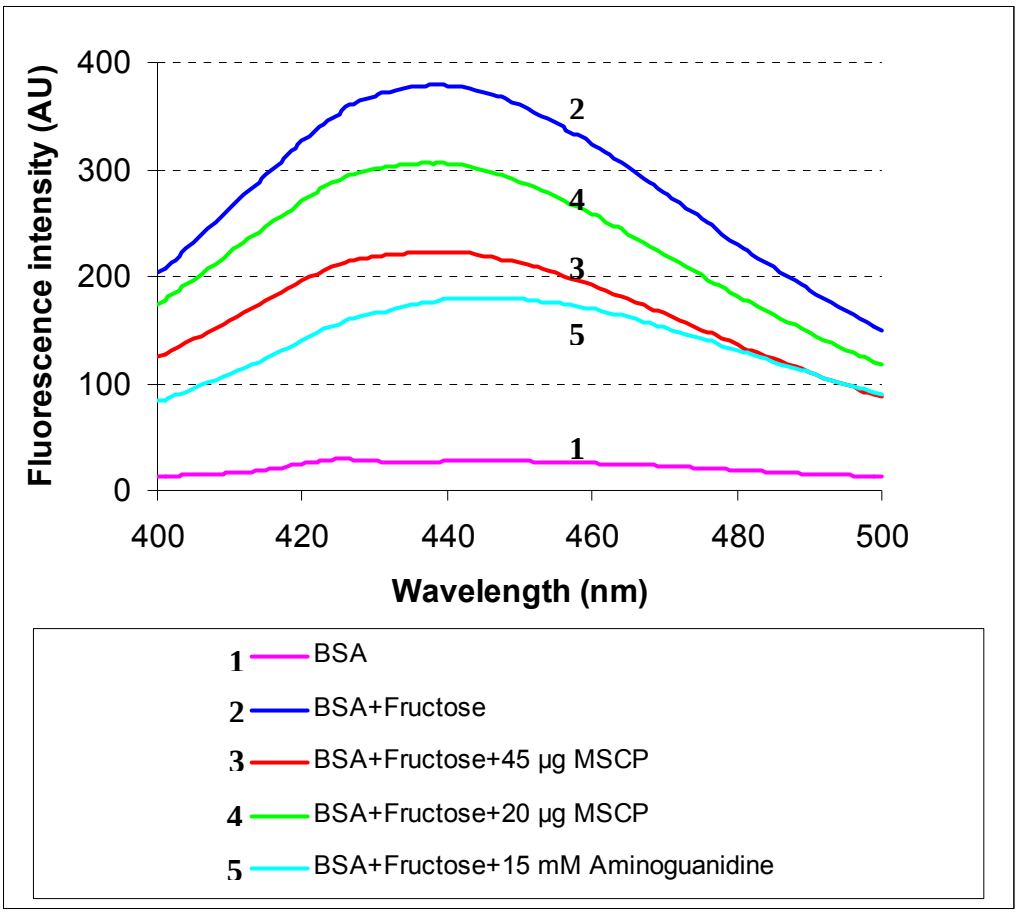


Figure 44. AGE fluorescence spectra of BSA incubated in *in vitro* for 5 days at 37^{0}C in the presence of D-fructose (500 mM) and millet seed coat polyphenols (MSCP)

Positive control - 15 mM aminoguanidine.

Spectra were measured after excitation at 370 nm

Presence of MSCP restored the 14 and 24% of tryptophan fluorescence intensity, respectively at 20 and 45 µg level and this revealed that, MSCP had a better fluorescent intensity restoration ability compared to 15 mM AG (Table 28).

The AGE fluorescence spectra of the incubated BSA samples at excitation wavelength 370 nm are depicted in Figure 44. Control BSA shows no significant signal, but when it was incubated with fructose, fluorescent products are formed, with a major peak at 438 nm. When BSA is co-incubated with fructose, and increasing concentrations of MSCP, inhibition of the AGE fluorescence was observed. AG at 15 mM was more effective in inhibiting AGE formation compared to both 20 and 45 µg MSCP. However, the concentration of the MSCP employed in the reaction mixture was very low compared to 15 mM of AG. Hence, the MSCP are potential inhibitors of both protein conformation changes (Figure 43) and also AGE formation (Figure 44). The effect of MSCP on the AGE fluorescence in the presence of fructose was tested and a dose dependent effect was observed (20, 41 and 54% decrease in AGE fluorescence (Table 28) with 20, 45 µg of MSCP and 15 mM AG respectively).

The SDS-PAGE profiles for BSA in the absence (control) or presence of 500 mM fructose co-incubated with MSCP at 20 and 45 µg concentrations (Figure 45) shows that, incubation of BSA with fructose leads to the appearance of a higher molecular weight protein band, which could be attributed to the cross-linked BSA. The MSCP at 45 µg concentrations inhibited protein cross linking resulting in low intense higher molecular weight band. No significant inhibition of protein cross-linking induced by fructose on BSA was observed with 5 mM catechin and 15 mM AG.

Table 28. *Relative tryphophan and AGE fluorescence intensities of control and glycated BSA co-incubated with millet seed coat polyphenols (MSCP) or aminoguanidine

Sample	Tryptophan fluorescence intensity at 338 nm	AGE fluorescence intensity at 438 nm
BSA	271 ± 5	27 ± 3
BSA+Fructose	57 ± 4	379 ± 7
BSA+Fructose+15 mM Aminoguanidine	84 ± 5	176 ± 6
BSA+Fructose+20 µg MSCP	94 ± 6	305 ± 7
BSA+Fructose+45 µg MSCP	123 ± 7	223 ± 6

* Values are mean ± SD of three independent determinations.

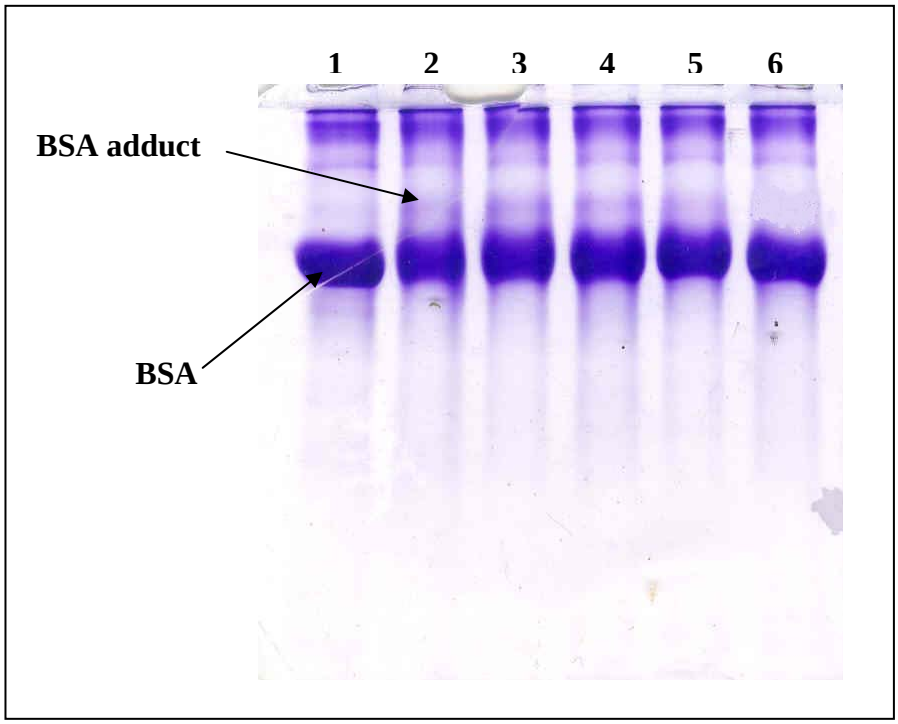


Figure 45. SDS-PAGE showing the inhibition of protein crosslinking by the millet seed coat polyphenols (MSCP)

Lane 1- BSA,

Lane 2- BSA+500 mM fructose,

Lane 3- BSA+500 mM fructose+solvent,

Lane 4- BSA+500 mM fructose+ AG (15 mM),

Lane 5 - BSA+500 mM fructose+ catechin (5 mM),

Lane 6 - BSA+500 mM fructose+ 45 µg MSCP.

The experiments on DPPH radical scavenging ability of the MSCP indicated that, the MSCP are highly potent free radical scavengers (Figure 46), which was very clear from the lower IC_{50} values (≈ 2 µg). In the other studies on evaluating the antioxidant activity of the seed coat of the millet (Shobana et al 2006) and legumes (Benninger and Hosfield 2003), it has been observed that, a direct linear relationship exists between the total phenolic contents and the total antioxidant activity. This indicates that, the phenolic compounds might be the major contributors to the radical scavenging ability of these extracts.

Glycated proteins have been shown to provide stable active sites for catalyzing the formation of free radicals through an enzyme like mechanism that mimics the characteristics of metal catalyzed oxidation systems (Degenhardt 2002). These free radical reactions lead to the formation of fluorescent and non-fluorescent AGE adducts. The superior radical scavenging properties of MSCP may significantly contribute to the protein glycation inhibitory property of the millet.

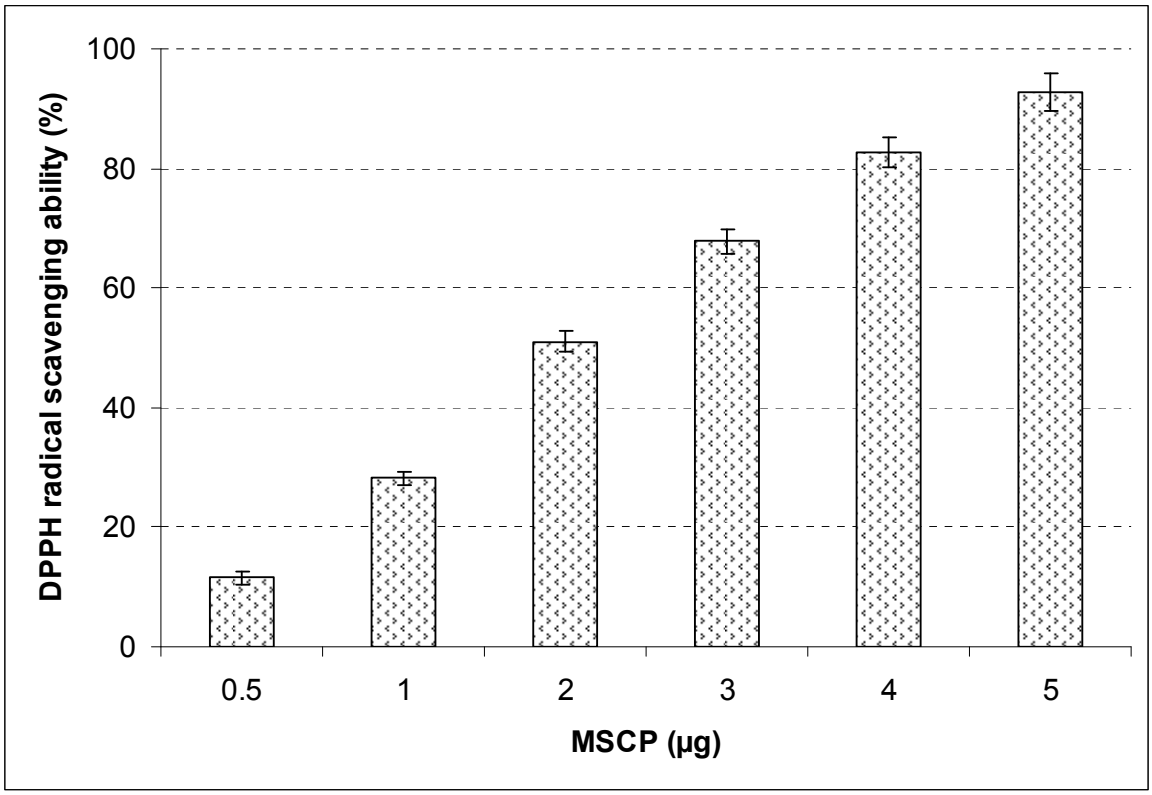


Figure 46. DPPH radical scavenging ability of the millet seed coat polyphenols (MSCP)

Carrier proteins in the blood vessel, and also the structural proteins and enzymes in the body are modified by glucose *in situ* (glycation or non enzymatic glycosylation). Diabetic patients tend to accumulate glycosyl proteins in their body tissue (Monnier and Cerami 1981) and some of the fluorescent AGEs were isolated and identified as pyrraline and pentosidine (Ahmed 2005). The AGEs are irreversibly formed and, accumulate with aging, atherosclerosis, and diabetes mellitus, and are especially associated with long lived proteins such as collagen, lens crystallins and nerve proteins. Glycated proteins accumulated *in vivo* may provide stable active sites for catalyzing the formation of free radicals, hence glycation and glycoxidation are intimately interrelated (Ahmed 2005).

In the presence of transition metals and molecular oxygen, the Amadori products are converted to protein dicarbonyl compounds via a protein enediol generating superoxide radical. The protein dicarbonyl compounds can participate in AGE formation and are referred to as glycoxidation products. Once formed, the superoxide radicals can be converted to the highly reactive $OH^{\cdot}$ via the Fenton reaction (Ahmed 2005). The $OH^{\cdot}$ is the most powerful oxidizing species among several reactive oxygen radicals and is responsible for protein fragmentation. MSCP are potent radical scavengers and hence to get further insight into the mechanism of action of MSCP as protein glycation inhibitors, the inhibitory property of MSCP on the $OH^{\cdot}$ mediated protein fragmentation was studied and the results (Figure 47) clearly indicated that, the MSCP are efficient inhibitors of $OH^{\cdot}$ mediated protein fragmentation. Lane 1 of Figure 47 shows a clear band characteristic for BSA while, lane 2, the very faint band of BSA which indicates the fragmentation of BSA in the presence of $CuSO_4$ and H_2O_2. In contrast, the other lanes show clear BSA bands similar to the control BSA when co-incubated with MSCP at different concentrations (2.5 and 5 µg) or EDTA (10 mM) or catechin (1 mM). This clearly reveals the inhibitory property of MSCP on the $OH^{\cdot}$ mediated protein fragmentation comparable to the positive controls namely catechin (1 mM) and EDTA (10 mM).

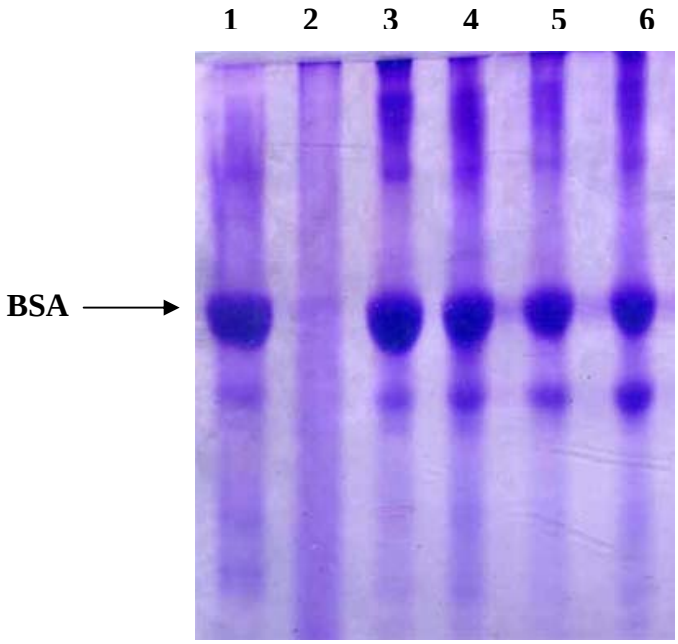


Figure 47. Inhibition of $OH^{\cdot}$ mediated protein fragmentation by the millet seed coat polyphenols (MSCP)

SDS-PAGE of BSA incubated in *in vitro* for 24h at 37^0 C in the presence of 0.05 mM $CuSO_4$, 2.5mM H_2O_2 and different levels of MSCP (2.5 and 5 µg). Positive controls EDTA (10 mM) and catechin (1 mM)

Lane 1- BSA

Lane 2- BSA+ 0.05 mM $CuSO_4$ + 2.5mM H_2O_2

Lane 3- BSA+ 0.05 mM $CuSO_4$ + 2.5mM H_2O_2 +10 mM EDTA

Lane 4- BSA+ 0.05 mM $CuSO_4$ + 2.5mM H_2O_2 +1 mM Catechin

Lane 5- BSA+ 0.05 mM $CuSO_4$ + 2.5mM H_2O_2 + 2.5 µg MSCP

Lane 6- BSA+ 0.05 mM $CuSO_4$ + 2.5mM H_2O_2 + 5 µg MSCP

Flavonoids are known to be potent inhibitors of protein glycation in addition being excellent free radical scavengers. There are various reports in the literature (Matsuda et al 2003) describing the higher antiglycation and radical scavenging properties of flavonoids such as luteolin, apigenin, kaempferol. ESI-MS finger prints of MSCP indicated the presence of these compounds in addition to catechins, phenolic acids and other classes of phenolic compounds (Shobana et al 2009). Hence the anti-glycation properties of the MSCP may be due to the synergistic effect of several phenolic compounds present.

To establish the mechanism of action of MSCP on inhibition of protein glycation, tryptophan fluorescence, AGE fluorescence studies, in addition to SDS-PAGE profiles of glycated BSA (cross linking of BSA) to determine the molecular weight changes were carried out. Tryptophan fluorescence is quenched by changes in protein structure induced by chemicals. The slight conformational changes induced by glycation and substitution of positive charges in arginine and/or lysine produce a decrease in trytophan fluorescence and this approach has been extensively used in glycation research (Nagaraj et al 2003). AG acts as a nucleophilic scavenger, preventing the first reaction in glycation consequently, it also prevents the downstream reaction in AGE formation. Similar to AG, MSCP also inhibited quenching of tryptophan fluorescence caused by glycation. The protein glycation inhibitory effect of MSCP are mainly due to the inhibition of the fructose induced protein conformational changes, formation of protein cross linking agents and also due to the antioxidant and free radical quenching property of MSCP. Thus it is possible that MSCP could regulate antiglycation properties through mechanisms that involve first phase of glycation cascade (prevention of protein conformational changes). The action of MSCP may be mediated by a direct nucleophilic scavenger action in the first phase of glycation reaction and subsequent inhibition of second phase reactions involving the formation of AGE adducts. In contrast, inhibition of free radical mediated conversion of the Amadori products to AGE (second phase of glycation reaction) were attributed to the antiglycation properties of *Ilex paraguariensis* and green tea (Lunceford and Gugliucci 2005). Moreover, MSCP are potent inhibitors of $OH^{\cdot}$ mediated protein fragmentation. This underscores the possible role of MSCP in the modulation of oxidative stress and non-enzymatic glycosylation of protein.

Polyphenols extracted in 70% methanol (aqueous methanolic extract) and also the MSCP were assayed for their antimicrobial properties on human pathogenic microflora. The study indicated the sensitivity of all the organisms tried for the assay and the MSCP produced a distinct zone of clearance (Figure 48) compared to the aqueous methanolic extracts. The zone of clearance were in the range of 10-15 mm for all the organisms for 20 µg of crude MSCP. The study indicated that, both gram positive and gram negative strains were sensitive for the millet phenolics. The fungal strain *Candida albicans* also was found sensitive to the MSCP. The study indicated the therapeutic potentiality of MSCP as antimicrobial agents. The antimicrobial properties of the MSCP may be attributed to the synergistic action of several phenolic compounds present. The sites and number of hydroxyl groups on the phenol rings are thought to be related to the relative toxicity to the microorganisms, with evidence that increased hydroxylation resulted in increased toxicity. In addition, there are reports about higher inhibitory activity for oxidized phenols (Scalbert 1991, Urs and Dunleavy 1985). The mechanisms thought to be responsible for phenolic toxicity to microorganisms include enzyme inhibition by the oxidized compounds possibly through reaction with sulfhydryl groups or through more non-specific interactions with proteins (Mason and Wasserman 1987). Flavones, flavonoids and flavonols are effective antimicrobial agents. Their activity is probably due to their ability to complex with bacterial cell walls. More lipophilic flavonoids may also disrupt microbial membranes (Tsuchiya et al 1996). Antimicrobial property of catechins and tannins are reported elsewhere. One of their molecular actions is to complex with proteins through so-called non specific forces such as hydrogen bonding and hydrophobic effects, as well as by covalent bond formation (Haslam 1996, Stern et al 1996). The probable mode of action of the MSCP against microbials may be possible due to the protein binding properties. Since the millet seed coat polyphenols extract is a rich source of the above mentioned classes of phenolic compounds, they may serve as potent antimicrobial agents. The SCM may also serve as a low cost, natural source of several antimicrobial compounds.

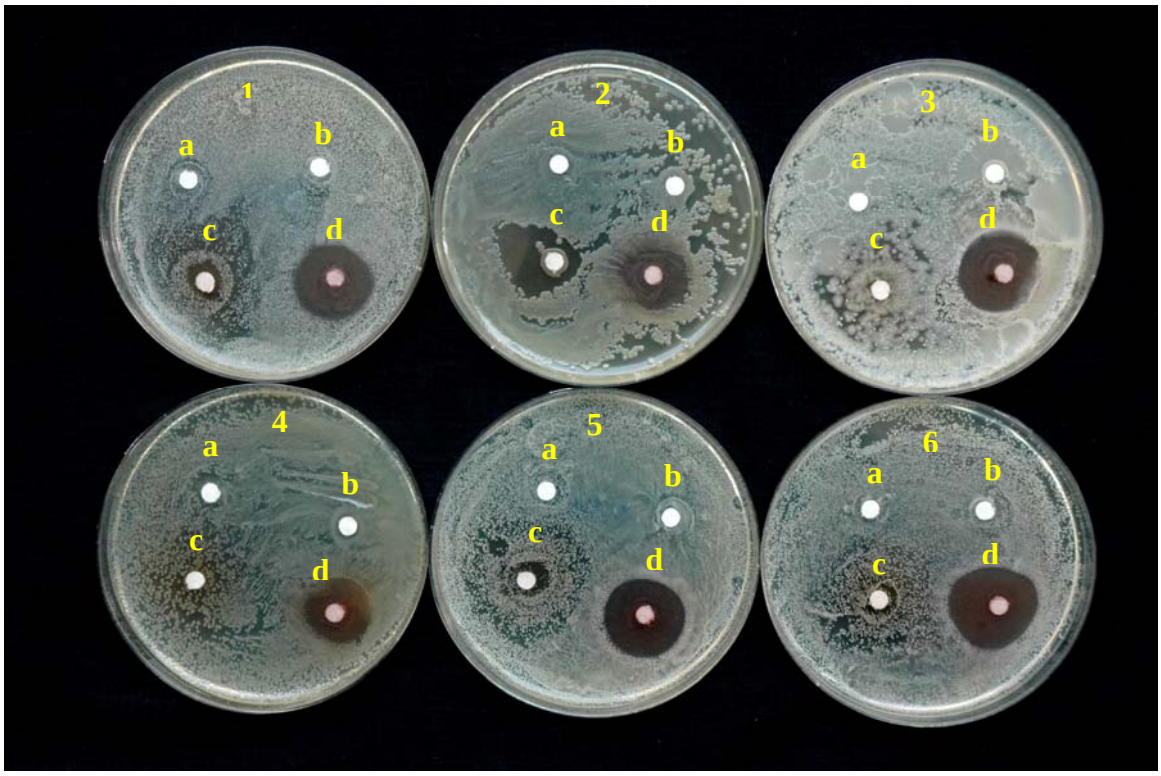


Figure 48. Antimicrobial properties of the millet seed coat polyphenols (MSCP)

a. 70% methanol (solvent control),
b. Acidic methanol (solvent control),
c. Millet seed coat polyphenols extracted in aqueous methanol,
d. Millet seed coat polyphenols extracted in acidic methanol (MSCP).

1. *Staphylococcus aureus* from pus
2. *Klebsiella pneumoniae* from urine
3. *Candida albicans* from sputum
4. *E Coli* from pus
5. *Staphylococcus aureus* from blood
6. *E Coli* from urine

SUMMARY AND CONCLUSIONS

- The millet seed coat polyphenols (MSCP) were tested for their inhibitory properties against rat intestinal α-glucosidase, porcine pancreatic amylase, fructose induced protein glycation, hydroxyl radical ($OH^{\cdot}$) mediated protein fragmentation as well as human pathogenic microflora in *in vitro* model systems. It was clearly observed that, the MSCP are effective non-competitive inhibitors of carbohydrate-hydrolyzing enzymes with an IC_{50} value of 16.9 µg for α-glucosidase and 23.5 µg for porcine pancreatic amylase respectively. The Michaelis - Menton constant (K_m) and V_{max} for α-glucosidase was 8×10^{-3} M for PNP-glycoside and 83.33×10^{3} µmoles of *p*-nitrophenol released/min. The K_m and V_{max} value for pancreatic amylase under the reaction conditions was found to be 1% starch and 66.0×10^{-2} µmoles of maltose eq released/min. The inhibitory constants (K_i) as determined from the Dixon plot for α-glucosidase and pancreatic amylase were 5.0 and 10 µg of the MSCP respectively. The dissociation constant (K_i') as determined from Bowden plot for α-glucosidase and pancreatic amylase were 2.5 µg and 7 µg of MSCP respectively.
- The MSCP were found to be potent inhibitors of fructose induced protein (BSA) glycation and inhibited both the changes in protein conformation as well as AGE formation. The MSCP at 20 and 45 µg level produced an 14 and 24 % restoration in the tryptophan fluorescence respectively compared to 10% restoration produced by 15 mM AG (aminoguanidine, standard glycation inhibitor). In addition, with the same levels, MSCP produced 20 and 41 % inhibition in AGE formation respectively compared to 54% inhibition produced by 15 mM AG. At 45 µg level, the MSCP prevent BSA crosslinking as evidenced by SDS-PAGE.
- The MSCP were also efficient DPPH radical scavengers (IC_{50} 2 µg) and also inhibitors of $OH^{\cdot}$ mediated protein fragmentation (2.5 µg of MSCP prevented protein fragmentation). The anti-glycation property of the MSCP may be due to the interference of MSCP in the first phase of glycation cascade and hence subsequent inhibition of down stream reactions involved in AGE formation. The anti-protein glycation property of the MSCP may also be partly attributed to its free radical scavenging abilities.

- The MSCP are effective inhibitors of human pathogenic microflora such as *E Coli, Klebsiella pneumonia, Staphylococcus aureus and Candida albicans.*

These observations reveal that, the millet seed coat polyphenols may serve as a natural source of postprandial blood glucose modulators, protein glycation inhibitors and antioxidants. The millet polyphenols also exhibited inhibitory property against some of the human pathogenic microflora. Hence, the MSCP may be useful as a nutraceutical for the dietary management of diabetes and its associated complications.

INTRODUCTION

Diabetes mellitus is a metabolic disorder characterized by hyperglycemia resulting from a defect in insulin action or deficiency of insulin or both with alterations in carbohydrate, lipid and protein metabolism. Uncontrolled diabetes leads to several microvascular (retinopathy, nephropathy and neuropathy) and macrovascular (atheroma) complications that affect many organs of the body and these complications play a detrimental role in the final outcome of the disease. The incidence of diabetes is alarmingly high (2.8%) world wide and India ranks first with large number of diabetics and the diabetic population in India in 2004 was around 66.6 million (Wild et al 2004).

The use of herbs and medicinal plants for the treatment of diabetes *mellitus* dates back to 1550 B.C as quoted in the Ebers papyrus. A multitude of herbs, spices and other plant materials have been advised for the treatment of diabetes throughout the world (Grover et al 2002). Besides, proper nutrition can help in prevention as well as management of diabetes *mellitus*. In this context, the health beneficial effects of dietary fibre and antioxidants derived from plant foods have been extensively studied and their role has been established to some extent.

Whole grain cereals form the most important sources of dietary fibre, minerals and phytochemicals with antioxidant activity such as polyphenols and phytate. Consumption of whole grains has been associated with lower incidence of diabetes (Jensen et al 2006) and cardiovascular diseases (Liu et al 1999). These possible health beneficial effects may be attributed to the presence of dietary fibre and phytochemicals such as polyphenols and phytates. Of late, there has been a renewed interest in search of several natural fibre supplements enriched with hypoglycemic phytochemicals, antioxidants and minerals. In cereals, the phytochemical constituents possessing health beneficial attributes are largely concentrated in the seed coat and several methods have been developed to prepare the phytochemical rich fraction or to isolate the seed coat components.

Finger millet foods are known for their higher sustaining power, lower glycemic response and higher satiety scores compared to other cereal foods and are usually recommended for diabetics from olden days. Recently, Hegde et al (2005a) studied

the effect of feeding finger millet and kodo millet on the antioxidant and glycemic status of alloxan-induced diabetic rats and reported a 36 and 42% reduction in the blood glucose levels, and 13 and 27% reduction in cholesterol levels, respectively, besides improving the antioxidant status. Finger millet seed coat is a reserve of several phenolic compounds such as phenolic acids, flavonoids, polymeric tannins and anthocyanins and are effective inhibitors of pancreatic amylase and intestinal α-glucosidase (Chethan and Malleshi 2007b, Shobana et al 2009). Finger millet seed coat is also a rich source of phytate and minerals (Shobana et al 2006). Dietary polyphenols and phytates are known for their ability to reduce the carbohydrate digestibility and thereby regulate postprandial glycemic response (Thompson et al 1983, Thompson et al 1987). Moreover, polyphenols are known to inhibit glucose absorption and also prevent AGE (advanced glycation end product) formation (Scalbert et al 2005).

However, reports on the ameliorative effect of the millet seed coat on the metabolic abnormalities and complications associated with diabetes are scanty. In view of this, the influence of finger millet seed coat matter on the metabolic abnormalities of diabetes and its associated complications in streptozotocin induced diabetic rats were studied.

MATERIALS AND METHODS

Chemicals: Streptozotocin, glucose oxidase, o-dianisidine, horse radish peroxidase, Triton X-100, human serum albumin, and Trizma base were purchased from the Sigma Chemical Co., St. Louis, USA. Thiosemicarbazide, diacetyl monoxime, cholesterol, Bernhart-Tommeralli salt mixture, and sodium azide were purchased from the SISCO Research Laboratories Pvt Ltd, Mumbai, India. Succinic acid was procured from SD Fine Chemicals Pvt Ltd, Mumbai, India. All other chemicals and solvents used were of analytical grade from Qualigens Fine chemicals, Mumbai, India. The solvents were distilled prior to their use whenever required.

The millet seed coat matter: Finger millet seed coat matter was prepared from the native millet (abbreviated as SCM) on a pilot scale following special protocol as described in Chapter II (Figure 11).

Animals: Male *Wistar* albino rats (weighing 150-250 g) raised at the Experimental Animal Production Unit of CFTRI, Mysore, were utilized for the studies. The animal study was conducted taking all the precautions to minimize pain or discomfort to the animals. The due approval from the Institutional animal ethics committee was obtained for the studies.

Induction of diabetes was carried out by a single i.p. injection of streptozotocin (40 mg/kg body weight as 1 ml freshly prepared solution in 0.1M citrate buffer, pH 4.5) and followed by that, the animals were given 5% glucose solution for 48 h to prevent the initial drug-induced hypoglycemic mortality. Three days after the injection, blood was drawn from the retro-orbital plexus of the animals after overnight fasting and was used to assess their diabetic status by the estimation of blood glucose levels. The animals with the fasting blood glucose levels higher than 250 mg/dl were considered as diabetic animals.

The animals were grouped into four groups (8 rats in each group) out of which two groups of animals were diabetic and the other two groups of animals were non-diabetic. The animals were housed in individual stainless steel metabolism cages with free access to diet and water. The feeding trial was of 6 week duration. One of the diabetic group received the experimental (the millet SCM containing diet) diet (Diabetic experimental abbreviated as DE) and the other received the control (corn starch based semi-synthetic diet) diet (Diabetic controls abbreviated as DC). Similarly, one of the non-diabetic group received the experimental diet (Non-diabetic experimental abbreviated as NE) and the other received control diet (Non-diabetic controls abbreviated as NC). The composition of control and experimental diet is given in Table 29.

Table 29. Composition of the animal diets (g per 100 g)

Ingredient	Control diet	Experimental diet
SCM	-	20
Casein	21	21
Cane sugar	10	10
Corn starch	54	34
Refined peanut oil	10	10
Salt mixture[a]	4	4
Vitamin mix[b]	1	1

[a] Bernhardt FW, Tommarelli CM (1966) Journal of Nutrition 89:495-500

[b] Anon (1978) Publication No. 10, National Academy of Sciences, Washington D.C, USA, p 7-37

The 24 h urine samples were collected under few ml of toluene at weekly intervals for the determination of the urinary metabolites. The urine samples were filtered, the volume was noted, and stored frozen until further analysis. At the end of the experimental period, to observe cataract formation, ophthalmological examination was conducted using slit lamp. The rats were sacrificed under mild ether anaesthesia by exsanguination from the hearts. The blood samples were collected by puncturing the heart and the serum was separated by centrifugation and stored at -20^{0}C until further analysis. Few ml of blood was collected with EDTA and was immediately utilized for the analysis of HbA1c levels. The eye lens and kidneys were excised and used for the determination of aldose reductase (AR) activity and histological studies respectively.

Analytical methods

The serum samples were analyzed for the biochemical parameters such as glucose, urea, creatinine, total protein, albumin, total cholesterol, HDL cholesterol, triglycerides, AGE (advanced glycation end-products) whereas, the EDTA blood samples were utilized for the determination of HbA1c levels. The urine samples were used for the determination of metabolites namely, glucose, urea, creatinine and protein.

Glucose: Plasma as well as urinary glucose was measured by glucose oxidase method reported by Huggelt and Nixon (1957). Deproteinized blood filterate (0.5 ml) or the undiluted urine (0.1 ml) were mixed with 4 units of glucose oxidase, 0.5 units of peroxidase, 3 µmol of o-dianisidine and 10 µmol triton X-100 and incubated for 1 h at 37^{0}C. The chromogenic product formed after the incubation was quantitated at 420 nm using glucose standard.

Urea: The serum and also the urinary urea was estimated by the method of Wybenga et al (1971). An aliquot of serum (0.1 ml) or the diluted urine sample (1 ml) was added to 5 ml of urea reagent (add 44 ml of concentrated H_2SO_4, 66 ml of 85% orthophosphoric acid to about 100 ml of distilled water in a 1 L flask, cool to room temperature and add 50 mg of thiosemicarbazide, 2.0 g of cadmium sulphate hexahydrate and 10 ml of urea solution (26 mg/L of water) mix and dilute to 1 L with distilled water) and mixed well. This was followed by the addition of 0.5 ml of 2%

diacetyl monoxime reagent with mixing. The tubes were placed in boiling water bath for exactly 12 min. The intensity of color developed was measured at 540 nm and compared with that of standard urea N (25 - 150 µg).

Creatinine: Creatinine was estimated from the serum as well as urine by reacting samples with alkaline picrate solution following Folin method as described by Oser (1965). Three ml of 1:5 tungstic acid blood filtrate or 10 to 20 fold diluted urine was treated successively with 1 ml of 0.04 M picric acid and 1 ml of 0.75N NaOH. The intensity of orange-red color developed was measured after 15 min and within 30 min at 520 nm. The optical density was compared with creatinine standards (5 - 20 µg).

Total protein: An aliquot of serum or the urine (0.1 ml) was added to 5 ml of protein reagent (100 mg of Coomassie brilliant blue G 250 was dissolved in 50 ml of 95% ethanol, and to that 100 ml of 85% (w/v) phosphoric acid was added and the resulting solution was diluted to 1 litre) and mixed thoroughly and the absorbance of the resulting blue color was measured at 595 nm after 2 min and before 1 h against reagent blank. Bovine serum albumin (20-100 µg) served as the reference standard (Bradford 1976).

Albumin: Serum albumin was quantitated by the dye binding method of Cooper (1972). An aliquot of serum (20 µl) was added to 3 ml of buffered dye solution (8.85 g succinic acid, 108 mg bromocresol green sodium salt, 100 mg sodium azide dissolved in 950 ml of water and 4 ml of 30% Brij-35 was added and mixed thoroughly and the pH of the resulting solution was adjusted to 4.2 and the resulting solution was made up to 1 L with water), and the color of the resultant solution was measured at 630 nm and compared with that of reference albumin standards (200 - 600 µg).

Serum cholesterol: Cholesterol was estimated by the $FeSO_4$ /H_2SO_4 method of Searcy and Bergquist (1960). An aliquot of the lipid extract was dried under a stream of nitrogen and the residue dissolved in 6 ml of saturated solution of $FeSO_4$ in glacial acetic acid. After mixing thoroughly, 2 ml of conc. H_2SO_4 was added, mixed immediately and cooled to room temperature. The color developed was measured at 490 nm and compared with the cholesterol standards (30 - 150 µg) treated similarly.

Serum HDL and LDL cholesterol: Serum HDL cholesterol was determined in the supernatant after precipitation of the apolipoprotein-B containing lipoproteins with heparin-Mn^{++} according to the procedure of Warnick and Albert (1978). The precipitate was extracted with chloroform-methanol (2:1 v/v) solvent mixture and an aliquot of the extract was used for the cholesterol determination of LDL+VLDL cholesterol.

Serum triglycerides: Serum triglycerides were determined according to the procedure of Fletcher (1968) using triglyceride purifier (neutral alumina) to remove phospholipids. An aliquot of the total lipid extract was added to a centrifuge tube containing approximately 2 g of the triglyceride purifier. The triglycerides were extracted with 6 ml of isopropanol, vortexed and allowed to stand for 15 min and then centrifuged. The supernatant (2 ml) was transferred into a separate test tube and 0.6 ml of 5% KOH was added, stoppered and incubated at 65^0C for 15 min. The tubes were cooled to room temperature and 1 ml of sodium m-periodate (0.025M) solution was added, mixed and again incubated at 50^0C for 30 min and cooled to room temperature. The intensity of resulting colour was read at 405 nm and compared with the color obtained with triolein standard.

Lens aldose reductase (AR) activity: Eye lens were collected from rat eyes and homogenized with sodium potassium phosphate buffer (0.135 M, pH 7.0) containing 0.5 mM of phenyl methyl sulphonyl fluoride (PMSF) and 10 mM of β-mercaptoethanol. The homogenate was centrifuged at 10,000 rpm for 30 min at 4^0C and the supernatant was used for the assay of the enzyme activity. The enzyme reaction was carried out at 30^0C in a quartz cuvette with a 1 cm light path. The assay mixture contained 0.32 mM nicotinamide adenine dinucleotide phosphate tetrasodium salt, 5.5 mM DL-glyceraldehyde in sodium potassium phosphate buffer (0.135 mM, pH 7.0). The reaction was initiated by the addition of lens homogenate and the decrease in the absorbance was monitored for 3 min at 340 nm. One unit of enzyme activity is defined as the amount of enzyme required for forming 1 µmol of the product per min at 30^0C under the assay conditions (Kim and Oh 1999).

Serum advanced glycation end products (AGE): The serum samples were appropriately diluted with 0.05 M sodium phosphate buffer (pH 7.4) to adjust the

protein concentration to ≈ 10 µg/ml and was used for the fluorescence measurement. The fluorescence of the advanced glycation end-product was measured by obtaining the emission fluorescence spectra of diluted serum samples from 400-500 nm with excitation at 370 nm in a spectrofluorometer (Monnier and Cerami 1981).

Glycosylated Hemoglobin (HbA1c): Glycosylated hemoglobin levels were estimated using the specified kit (Nycocard Axisheild, Norway). Nycocard HbA1c is a rapid *in vitro* test for the measurement of glycated hemoglobin in human blood. The method is based on boronate affinity assay. The kit contains test device with a porous membrane filter, test tubes pre-filled with reagent and washing solution. The reagent contains agents that lyse erythrocytes and that precipitate the hemoglobin specifically, as well as a blue boronic acid conjugate that binds *cis*-diols of glycated hemoglobin. When blood is added to the reagent, the erythrocytes immediately lyse and hemoglobin precipitates. The boronic acid conjugate binds to the *cis*-diol configuration of glycated hemoglobin. An aliquot of the reaction mixture is added to the test device, and all the precipitated hemoglobin, bound and unbound conjugate, remains on top of the filter. Any excess of the colored conjugate is removed with the washing solution. The precipitate is analyzed by measuring the blue (glycated hemoglobin) and the red (total hemoglobin) color intensity with the Nycocard reader II, the ratio between them being proportional to the percentage of HbA1c in the sample.

Glomerular filteration rate (GFR): Glomerular filteration rate was calculated by the formula:

$$\frac{\text{Urinary creatinine (mg/dl)} \times \text{Urine volume (ml)} \times 1000\text{g}}{\text{Serum creatinine (mg/dl)} \times \text{Body weight (g)} \times 1440\ (\text{min})} = \ldots\ldots\ldots\ldots \text{ml/min}$$

Atherogenic Index (AI): AI was calculated according to the formula followed by Muruganandan et al (2005).

$$\text{Atherogenic index} = \frac{\text{Total cholesterol} - \text{HDL cholesterol}}{\text{HDL cholesterol}}$$

Ophthalmological examination: Eyes of the animals were examined for cataract associated changes at the end of the experimental period in a slit lamp with a built-in camera (Carl Zeiss Meditech AG, 07740 Jena, Germany) and the specific changes observed were photographed.

Histological studies of the kidney: Kidneys, soon after excision were fixed in 10% formalin, dehydrated and embedded in paraffin wax for sectioning (5±1 µm thick). Sections taken using microtome were stained with haematoxylin and eosin, and were examined for the changes in the glomerulus and the tubules.

Statistical Analysis: The results are expressed as mean ± SEM. All the data were analyzed by one way analysis of variance followed by multiple comparison test (Tukey's test) at 5% level of significance. A value of $p<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

The SCM of finger millet prepared following the special milling protocol (as mentioned in chapter II) contained 11.2 % polyphenols, 13.6 % protein, 3.2 % fat, 19% starch and 48% dietary fibre. The experimental diet containing 20% of SCM provides 2.24% of polyphenols which is nearly the same as that of the polyphenol content of the millet whole meal (2.3 g%). The experimental diet also contained about 9.6% of dietary fibre. Hence, the animals on an average (for 15 g of diet consumed/day) consumed about 0.34 g of the millet seed coat polyphenols and 1.5 g of dietary fibre per day.

Alloxan and streptozotocin (STZ) have been widely used for the induction of experimental diabetes mellitus and are known to selectively damage pancreatic β-cells and exhibit most potent diabetogenicity. Okamato and coworkers proposed a model called "Okamoto model" (Okamoto 1985), which proposes the fragmentation of nuclear DNA of the pancreatic β-cells as a result of the accumulation of superoxide or hydroxyl radical through H_2O_2 generation. These agents besides damaging DNA in pancreatic β- cells, also stimulate nuclear poly (ADP-ribose) synthetase for DNA repair, thereby depleting intracellular NAD levels and inhibiting proinsulin synthesis (Yamamoto et al 1981).

Different levels of STZ ranging from 35 - 55 mg/kg body weight of the animals were injected initially to arrive at the optimal dosage required to induce hyperglycemia and it was noticed that, the animals injected with 40 mg dosage of STZ, developed hyperglycemia with more than 80% survival rate. Hence, 40 mg/kg body weight dosage of STZ was administered to the experimental animals.

All diabetic rats fed with the experimental diet (DE group) survived the experimental period of 6 week whereas, 25% of the diabetic rats fed with the control diet (DC group) died in the course of the experimental period. The cause of mortality is presumed to be severe hyperglycemia and associated complications.

The animals consumed the control as well as experimental diets normally and the dietary intake was comparatively higher in diabetic groups compared to the non-diabetic groups. However no significant differences were observed with respect to the dietary intake between DC and DE groups and also between NC and NE groups (Figure 49).

The body weights of the diabetic rats remained much lower than those of corresponding non-diabetic animals. Experimental diet significantly improved the weight gain in the diabetic animals (Figure 50). There was no difference in the body weight gain pattern among the non-diabetic group (NC and NE) of animals, whereas significant ($p<0.001$) weight loss was observed in the DC group of animals.

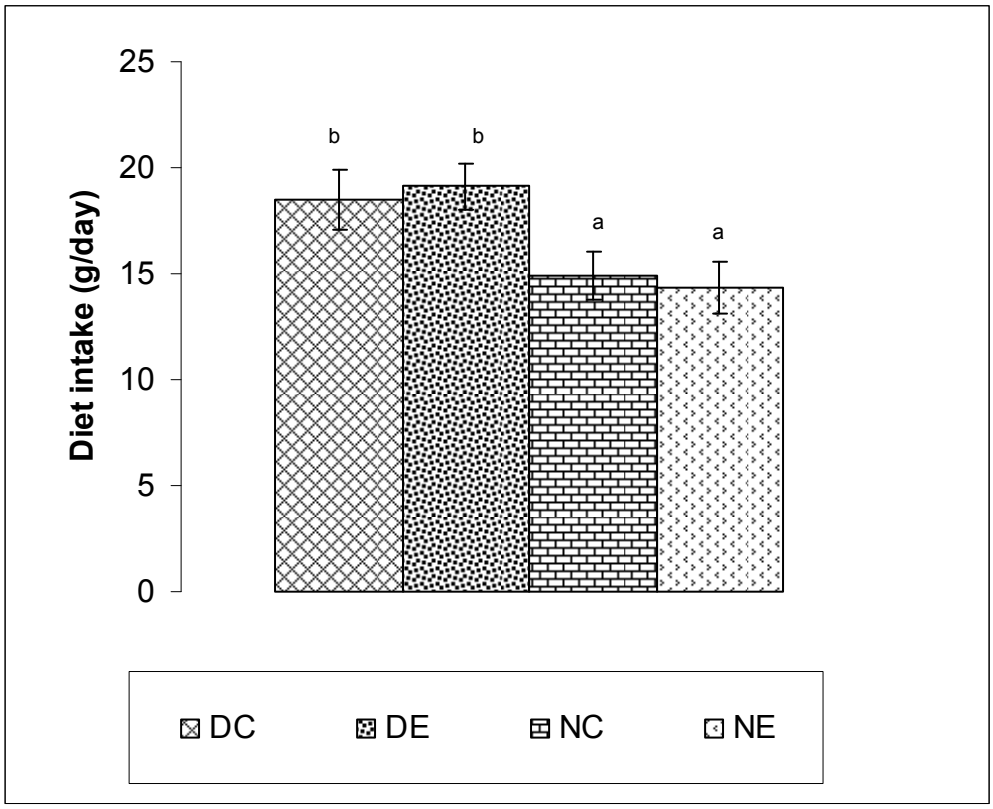


Figure 49. Average dietary intake of the diabetic and non-diabetic animals during the experimental period

Values with the same superscript are statistically not significantly different at $p<0.05$

DC - Diabetic control
DE - Diabetic experimental
NC - Non-diabetic control
NE - Non-diabetic experimental

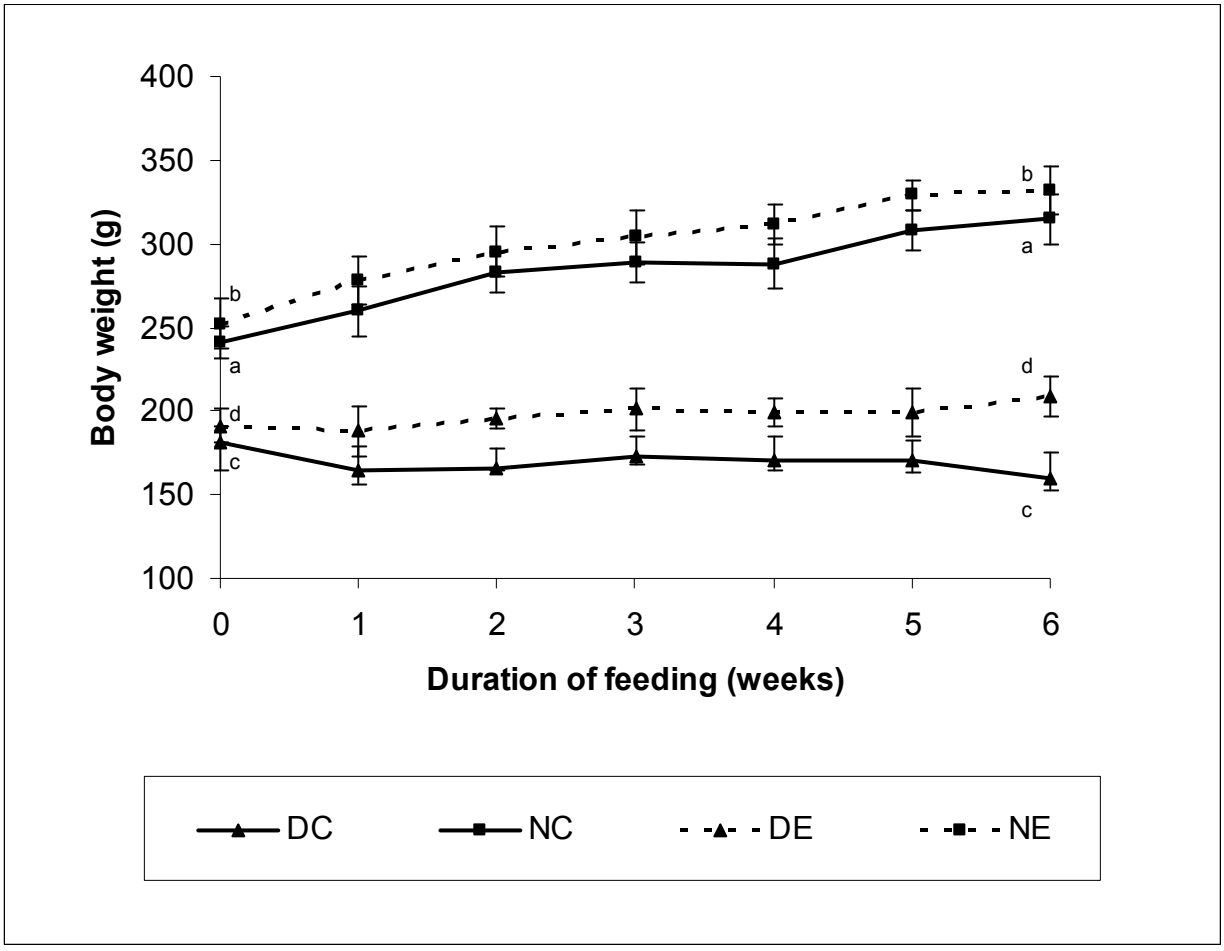


Figure 50. Influence of dietary finger millet seed coat matter on the body weights of the diabetic and non-diabetic animals

Values with the same superscript are statistically not significantly different at $p<0.05$

DC - Diabetic control
DE - Diabetic experimental
NC - Non-diabetic control
NE - Non-diabetic experimental

The diabetic animals maintained on experimental diet showed a significant (at $p<0.001$) and consistent improvement in the body weight throughout the period of experiment as compared to the animals fed on the control diet (DC group).

The liver weight was considerably higher in the DC group (3.89 g/100 g body weight) compared to that of NC group (2.56 g/100 g body weight) indicating that, diabetes resulted in hepatomegaly (about 30 % increase in liver weight) (Table 30). On the other hand, the animals fed on the experimental diet (DE group), exhibited relatively lower liver weights as compared to the animals maintained on the control diet (DC). In fact, the liver weight of the DE group of animals were 9% lower than that of the diabetic controls (DC). The relatively lower liver weights of the DE group as compared to that of DC group clearly indicates the ameliorative characteristics of the SCM.

Likewise, the kidney weights of the DC group of animals were higher than that of DE group. The kidney weights (% body weight) were higher in diabetic animals (2.4 fold higher than those of NC group) (Table 30) and DE group of animals exhibited a 21% lower kidney weights (1.25 g/100 g body weight) compared to that of DC animals (1.59 g/100g body weight).

Table 30. Effect of dietary finger millet seed coat matter on the liver and kidney weights of diabetic and non-diabetic animals (g/100 g body weight)

Animal group	Liver	Kidney
DC	$3.89^{b} \pm 0.23$	$1.59^{b} \pm 0.15$
DE	$3.53^{b} \pm 0.10$	$1.25^{c} \pm 0.17$
NC	$2.56^{a} \pm 0.17$	$0.67^{a} \pm 0.02$
NE	$2.50^{a} \pm 0.05$	$0.68^{a} \pm 0.04$

Values are mean ± SEM of 8 animals in each group

Values with the same superscript are statistically not significantly different at $p<0.05$

DC - Diabetic control
DE - Diabetic experimental
NC - Non-diabetic control
NE - Non-diabetic experimental

The mean fasting blood glucose concentration of the DC group of animals was 343 mg/dl at the beginning of the study and it did not change appreciably till the end of the study (Figure 51), whereas, the mean fasting blood glucose levels of the DE group of animals decreased by 31 % (from 313 mg/dl at the beginning of the experimental period to 216 mg/dl at the end of the experimental period) and the mean fasting blood glucose value of DE group of animals was nearly 39% lower than that of the DC group of animals ($p<0.001$). The result clearly shows that, feeding the diabetic animals with the diet containing the millet SCM helps in controlling the hyperglycemia.

The results of this study indicate that, some of the abnormalities associated with diabetes mellitus in the rats were ameliorated by feeding the millet SCM diet for about six weeks. The improved metabolic status on feeding the experimental diet was featured by a marked decrease in hyperglycemia and a significant improvement in body weight gain. These benefits were observed in the absence of any insulin treatment. The millet seed coat apart from being a rich source of dietary fibre, phytate and minerals (Shobana et al 2006) is a reserve of many health beneficial phenolic compounds (Chethan and Malleshi 2007b). It has been reported that caffeic acid, isoferulic acid, prodelphinidin and catechin reduce the fasting hyperglycemia and also attenuate the postprandial blood glucose response in rats (Scalbert et al 2005). The above mentioned phenolic compounds have been identified in the millet seed coat (Chapter II, Shobana et al 2009). Moreover, several *in vitro* studies on cultured cells have shown that, polyphenols may increase glucose uptake by peripheral tissues. Caffeic acid increases glucose uptake by rat adipocytes and mice myoblasts. Black and green tea extracts and EGCG also increased glucose uptake by rat epididymal adipocytes, both in the presence or absence of insulin. Isoferulic acid increased glucose uptake by soleus muscle isolated from streptozotocin diabetic rats (Scalbert et al 2005). Hence, the observed health benefits in the DE group of animals may be attributed to the synergistic effect of these phenolic compounds present in the millet SCM.

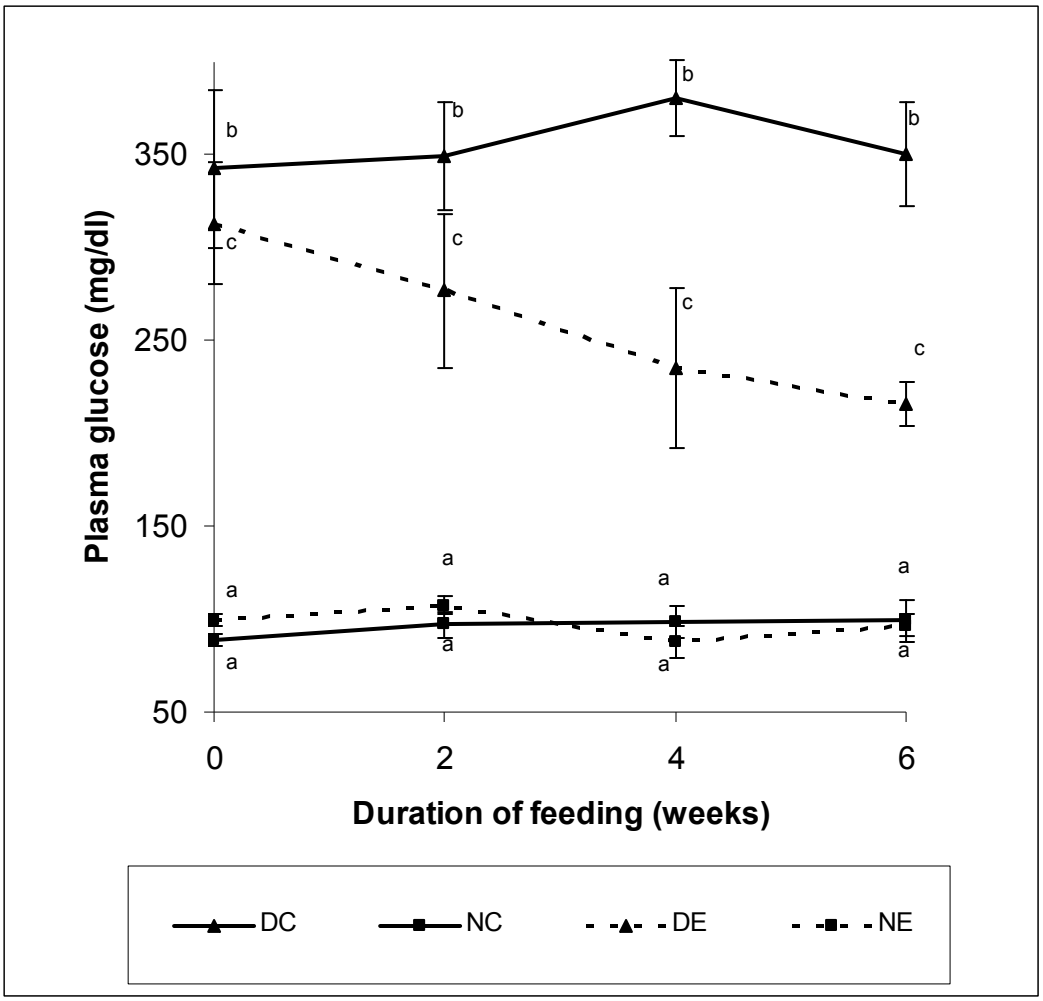


Figure 51. Changes in the blood glucose levels of the diabetic and non-diabetic animals maintained on finger millet seed coat matter diet during the experimental period

Values with the same superscript are statistically not significantly different at $p<0.05$

DC - Diabetic control
DE - Diabetic experimental
NC - Non-diabetic control
NE - Non-diabetic experimental

In addition, millet seed coat phenolics are non-competitive inhibitors of intestinal α-glucosidase and pancreatic amylase (Shobana et al 2009), as these inhibitors are proven modulators of postprandial glycemia and they play a significant role in the prevention of diabetic complications.

Phytate, the myo-inositol hexakis phosphate, is known for its amylase inhibitory property (Knuckles and Betschart 1987). Hence, the phytate present in the SCM along with the polyphenols of the SCM might have synergistically contributed towards the regulation of postprandial glycemic responses and also the glucose absorption in the small intestine. Rondini et al (2004), studied the pharmacokinetic profile of ferulic acid from wheat bran and reported that the bioavailability of the bran associated ferulic acid was comparatively higher than that of free compound. Reports indicate that, phytates (inositol hexakis phosphates) stimulate non Ca^{2+} mediated and primes Ca^{2+} mediated exocytosis of insulin by activation of protein kinase C in pancreatic beta cells (Efanov et al 1997). Thus, they regulate insulin secretion through modulation of calcium channels in pancreatic beta cells. Moreover, phenolic compounds are known to enhance insulin activity (Anderson and Polansky 2002), regulate intestinal glucose transporters (Shimizu et al 2000) and also inhibit sodium dependant D-glucose uptake by dissipation of the sodium electro-gradient (Welsch et al 1989), and hence glucose accumulation, it also increases muscle glucose uptake and reduces hepatic gluconeogenesis (Liu et al 2000). Hence, the phytate content of the SCM might have complimented, the positive role of polyphenols towards ameliorating complications associated with diabetes, as both phytate (Sandberg et al 1987; Sakamoto et al 1993) and polyphenols (Manach et al 2004; Manach et al 2005) are absorbed and metabolized.

Insoluble fibre has been reported to be more effective in the glycemic control in dogs with naturally occurring insulin dependent diabetes mellitus (Kimmel et al 2000). Moreover, Seki et al (2005) reported that, the insoluble fibre fraction from the pre-germinated brown rice lowered both the postprandial blood glucose and insulinemic responses in normal male *Wistar* rats and Qureshi et al (2002) observed that the water solubles and fibre concentrates of rice bran was more effective in lowering serum glucose and serum total and LDL cholesterols and also apo-

lipoprotein B levels in both type I and type II diabetic subjects. Most of the dietary fibre present in the millet seed coat is insoluble in nature (about 92% insoluble and 8% soluble) and hence, the anti-hyperglycemic action of the millet seed coat in the experimental animals could be due to the synergistic action of dietary fibre, phytate and phenolic compounds present in the millet SCM.

Total protein, albumin, urea and creatinine content of serum in the diabetic and non-diabetic animals are shown in Table 31. Diabetic condition resulted in a decrease in total protein and albumin and increase in urea and creatinine levels. DE group of animals exhibited improved protein and albumin levels in addition to the lowered levels of urea and creatinine compared to that of the DC group of animals. Serum creatinine (0.89 mg/dl) and urea (49 mg/dl) levels were lower in the DE group (by 36 and 13% respectively) compared to their levels in the DC group (1.4 and 57 mg/dl creatinine and urea respectively). The DE group of animals exhibited higher levels of serum total protein (5.7 mg/dl, $p<0.01$), as well as albumin levels (2.8 mg/dl, $p<0.05$) compared to their corresponding values (4.4, 1.9 mg/dl total protein and albumin respectively) in the DC group of animals. The total protein and albumin levels were higher by 30 and 47% respectively in the DE group of animals compared to the DC group of animals. The Albumin:Globulin (A/G) ratio of the DC group (0.76) was significantly lower than that of both the NC and NE group. However, the diabetic animals maintained on the experimental diet (DE group) exhibited an A/G ratio (0.97) almost comparable to that of both the NC and NE group. There were no significant differences among the two non-diabetic (NC and NE) groups of animals in the serum concentrations of total protein, albumin, urea and creatinine (Table 31).

The blood lipid profile of the non-diabetic and diabetic animals maintained on the control and the experimental diet regimens are presented in Table 32. The diabetic animals maintained on the experimental diet exhibited significantly lower serum cholesterol levels (as much as 43%) as compared to that of the DC group of animals ($p<0.01$). The lower levels of cholesterol observed in the DE group was mainly from the LDL+VLDL fraction of lipoproteins (68 % lower compared to that of DC group). On the other hand, HDL associated cholesterol fraction of the DE group was nearly 2.0 fold higher compared to that of the DC group of animals ($p<0.001$).

The LDL+VLDL cholesterol to HDL cholesterol ratio usually increase in the diabetic condition (the ratio was 5.6 in the DC group of animals) and this increase was countered by the experimental diet to a marked extent. Infact, the LDL+VLDL cholesterol to HDL cholesterol ratio observed for the DE group of animals (0.9) was slightly lower than that of both NC (2.6) and NE (1.2) group of animals. Significantly lower serum triglyceride levels ($p<0.001$) were observed (62 % lower) in the DE group as compared to that of the DC group (Table 32). One more interesting observation is that, the lower values for total cholesterol, LDL+VLDL fraction, HDL fraction and LDL+VLDL to HDL ratio was not only observed in the case of DE group but was also apparent with the NE group of animals.

The atherogenic index (AI) is an indicator of the tendency to develop atherosclerosis. Lower this index, lower the chances of incidence of atherosclerosis. The atherogenic indices (AI) exhibited by the DE group of animals (1.35) was significantly lower compared to that of the animals in DC group (10.3). This lower AI was not only observed in diabetic animals, but was also observed in the non-diabetic animals (Table 32).

Table 31. Effect of dietary finger millet seed coat matter on blood urea, creatinine and protein levels of the diabetic and non-diabetic animals

Animal groups	Urea (mg/dl)	Creatinine (mg/dl)	Protein (g/dl)			
			Total	Albumin	Globulin	A/G ratio
DC	$56.8^{b} \pm 6.9$	$1.40^{b} \pm 0.11$	$4.4^{b} \pm 0.32$	$1.9^{b} \pm 0.25$	$2.5^{b} \pm 0.35$	$0.76^{b} \pm 0.15$
DE	$49.3^{b} \pm 2.70$	$0.89^{a} \pm 0.17$	$5.7^{c} \pm 0.53$	$2.8^{c} \pm 0.45$	$2.9^{ab} \pm 0.35$	$0.97^{b} \pm 0.12$
NC	$23.2^{a} \pm 2.39$	$0.88^{a} \pm 0.18$	$7.5^{a} \pm 0.66$	$3.8^{ac} \pm 0.55$	$3.7^{a} \pm 0.55$	$1.0^{ab} \pm 0.12$
NE	$21.7^{a} \pm 2.50$	$0.76^{a} \pm 0.10$	$7.3^{a} \pm 0.23$	$3.5^{a} \pm 0.25$	$3.8^{a} \pm 0.25$	$0.9^{ab} \pm 0.11$

Values are mean ± SEM of 8 animals in each group

Values with the same superscript are statistically not significantly different at $p<0.05$

NC - Non-diabetic control
DC - Diabetic control
NE - Non-diabetic experimental
DE - Diabetic experimental

Table 32. Influence of dietary finger millet seed coat matter on the blood lipid profile and atherogenic index in the diabetic and non-diabetic animals.

Animal group	Cholesterol (mg/dl)				Triglycerides (mg/dl)	Atherogenic Index (units)
	Total	LDL+ VLDL	HDL	LDL+VLDL / HDL		
DC	$95.8^{b} \pm 9.40$	$80.6^{c} \pm 5.21$	$15.2^{c} \pm 2.82$	$5.3^{b} \pm 2.11$	$161.3^{b} \pm 14.5$	$10.3^{b} \pm 4.6$
DE	$55.3^{c} \pm 5.10$	$25.7^{d} \pm 4.04$	$29.6^{d} \pm 2.63$	$0.9^{c} \pm 2.21$	$61.2^{c} \pm 6.4$	$1.35^{c} \pm 0.3$
NC	$52.2^{ac} \pm 4.70$	$37.7^{a} \pm 3.92$	$14.5^{ac} \pm 2.01$	$2.6^{a} \pm 1.93$	$81.1^{ac} \pm 1.98$	$2.94^{ac} \pm 0.1$
NE	$53.0^{ac} \pm 1.57$	$29.1^{b} \pm 4.31$	$23.9^{bd} \pm 2.91$	$1.2^{ac} \pm 2.52$	$50.2^{ac} \pm 4.75$	$1.04^{ac} \pm 0.1$

Values are mean ± SEM of 8 animals in each group

Values with the same superscript are statistically not significantly different at $p<0.05$

DC - Diabetic control
DE - Diabetic experimental
NC - Non-diabetic control
NE - Non-diabetic experimental

Hyperlipidemia is a recognized complication in diabetes mellitus characterized by elevated levels of cholesterol, triglycerides and phospholipids with altered lipoprotein composition (Howard & Howard, 2005). Atherosclerosis is the most serious consequence of long-term diabetes and the major cause of death in these patients. It is characterized by deposition of atherosclerotic plaques inside the arterial walls causing occlusion of blood flow and eventual myocardial infarction. In our study, STZ induced diabetic animals exhibited clear cut abnormalities in lipid metabolism as evidenced from the significant elevation of serum total cholesterol, LDL-cholesterol, triglycerides, atherogenic index and reduction of HDL-cholesterol levels. The results of the present study reveal that, the lipid and lipoprotein abnormalities developed in diabetic condition were significantly countered by feeding the experimental diet. Pore and Magar, (1976) in their study, fed male albino rats with finger millet whole meal diet for 8 weeks and reported the cholesterol lowering effect of the millet. Moreover, the hypocholestrolemic effect of the finger millet as well as kodo millet was shown by Hegde et al (2005a). The authors reported that, the alloxan induced diabetic rats maintained on the millet whole meal containing diet (at the level of 55% in the basal diet) for 28 days, exhibited a 13% lower cholesterol levels compared to their corresponding controls. In addition, the lipid lowering effect of other millet brans were also shown by Rooney et al (1992). The lipid lowering effect of the millet SCM may be mainly due to its dietary fibre and polyphenol content. There are good number of reports indicating the hypocholestrolemic effect of dietary fibre. Nandini et al (2000) reported 18% reduction in blood glucose in diabetic animals fed with 10% wheat bran containing diet. There are a few studies wherein, the effect of utilizing cereal bran as a constituent of diet has been examined on the hyperglycemic subjects, in the case of feeding wheat bran at the level of 19 g/day, Jenkins et al (2002) did not observe any significant effect in type 2 diabetic patients with respect to the glycemic control and cardiovascular disease risk factors such as lipids, lipoproteins, C-reactive protein and homocysteine levels in humans. Several reports on the hypoglycemic and hypocholestrolemic properties of the polyphenols isolated from cereals are available. Anti-obesity and hypoglycemic properties of cyanidin 3-o-β-D-glucoside, an anthocyanin isolated from the purple corn are reported by Tsuda et

al (2003). Xia et al (2006) in their study established the anti-atherogenic properties of anthocyanin extracts from black rice. Red and black rice feeding is known to decrease atherosclerotic plaque formation and increase antioxidant status in rabbits (Ling et al 2001).

Hence, the hypoglycemic and hypolipidemic effects of the experimental diet clearly indicate the beneficial effect of the SCM in regulation of glucose homeostasis and prevention of dyslipidemia. Hence, the lower lipid levels and AI observed in the DE group of animals may be partly attributed to the anthocyanin contents of the millet SCM.

The urine volumes of the different group of animals presented in Figure 52. clearly indicates that, the diabetic rats excreted nearly 6 fold higher volumes of urine compared to the non-diabetic rats throughout the experimental period (Figure 52). In the case of diabetic animals, although the urine volumes were comparable in the beginning of the study, the volume excreted by the animals maintained on the experimental diet decreased progressively and at the end of the experiment, the decrease was significant ($p<0.05$). The urinary excretion of various metabolites such as glucose, protein, urea and creatinine were monitored at weekly intervals in the urine samples collected over 24 h duration. Urinary excretion of glucose by diabetic animals remained high throughout the experimental period. However, the DE group of animals exhibited lower urine volume (40 ml/24h) and also a significant reduction in the urinary glucose excretion (4.5 g/24 h at the end of the experimental period compared to 11 g/24 h at the beginning of the experimental period) (Figure 53). Similarly the DE group of animals excreted significantly lesser protein (15 g/24 h) compared to the DC group (28 g/24 h) of animals ($p<0.001$) (Figure 54). Lower urinary glucose excretion observed in DE group is consistent with blood glucose lowering effect of the millet SCM. The hypoglycemic nature of the millet SCM observed in the present study is in conformity with Hegde et al (2005a), who observed 36% reduction in blood glucose levels in alloxan induced diabetic rats maintained on the millet whole meal incorporated (55% of the basal diet) diet.

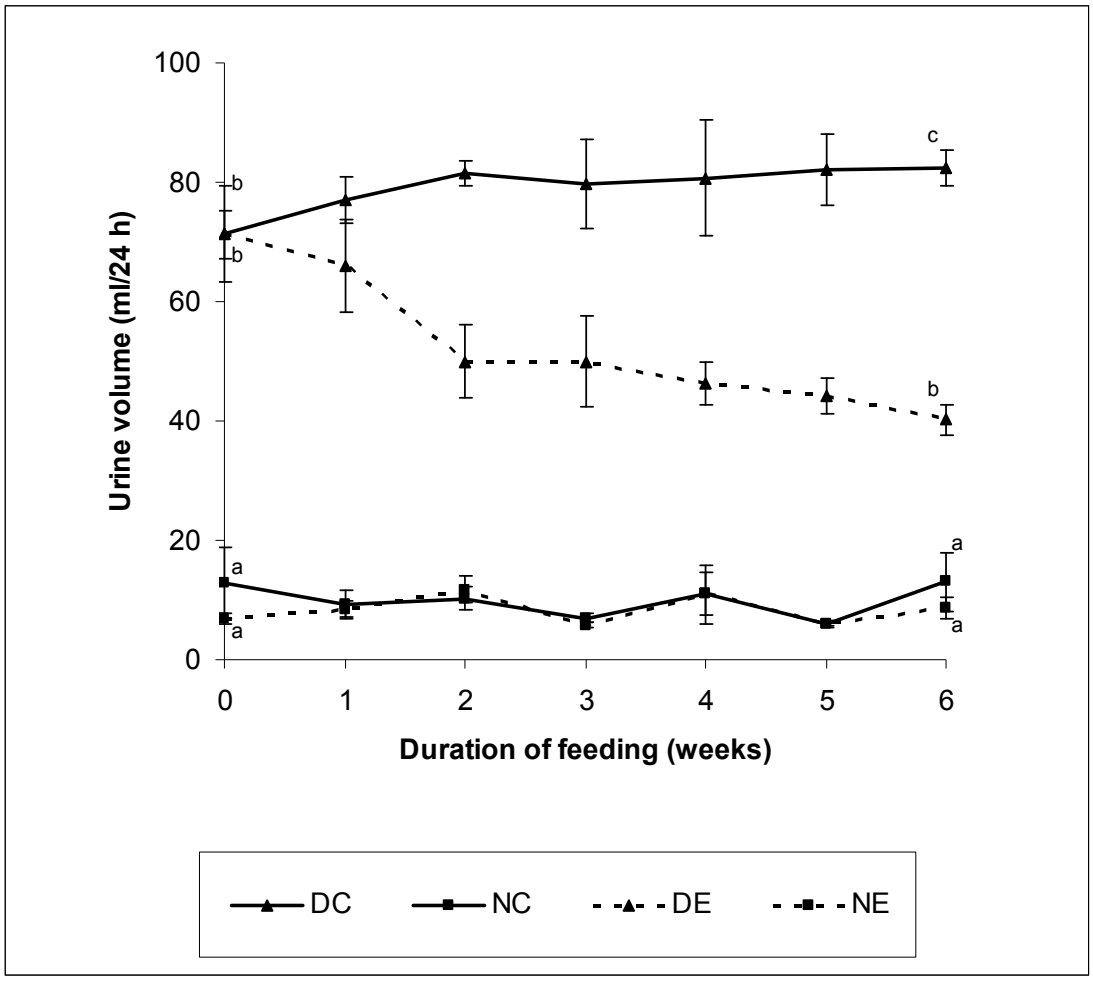
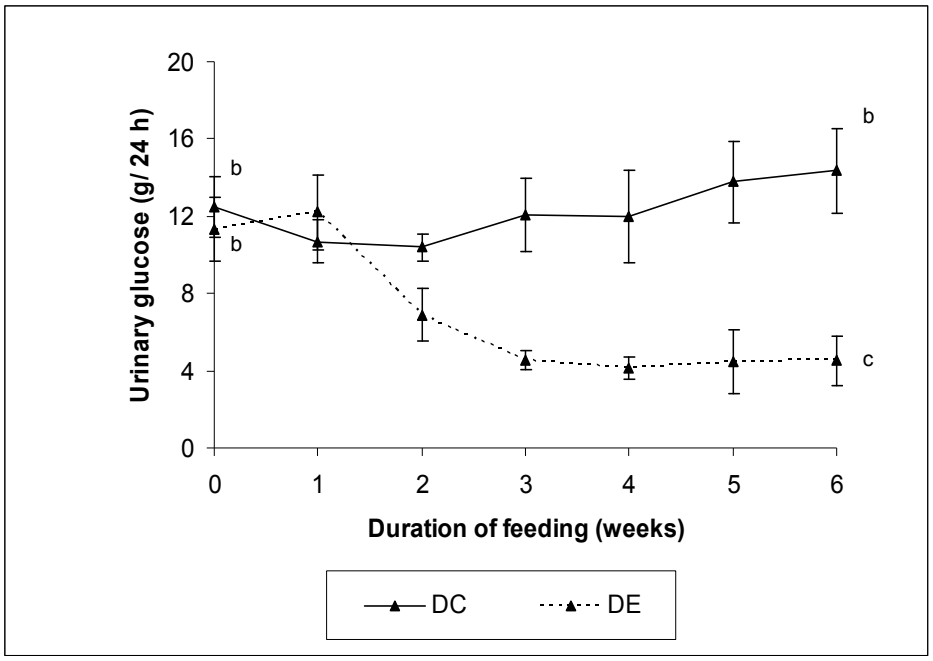


Figure 52. Effect of dietary finger millet seed coat matter on the urine volumes in the diabetic and non-diabetic animals

Values with the same superscript are statistically not significantly different at $p<0.05$

DC - Diabetic control
DE - Diabetic experimental
NC - Non-diabetic control
NE - Non-diabetic experimental

A



B

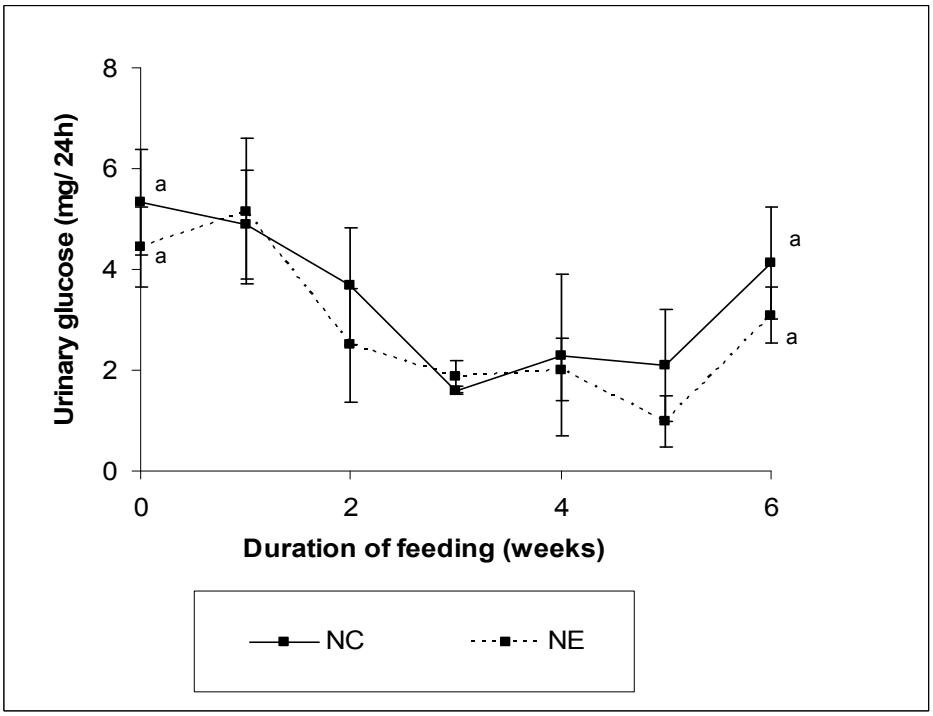


Figure 53. Effect of dietary finger millet seed coat matter on urinary glucose level in diabetic (A) and non-diabetic animals (B)

Values with the same superscript are statistically not significantly different at $p<0.05$

DC- Diabetic control, DE - Diabetic experimental, NC- Non-diabetic control, NE- Non-diabetic experimental

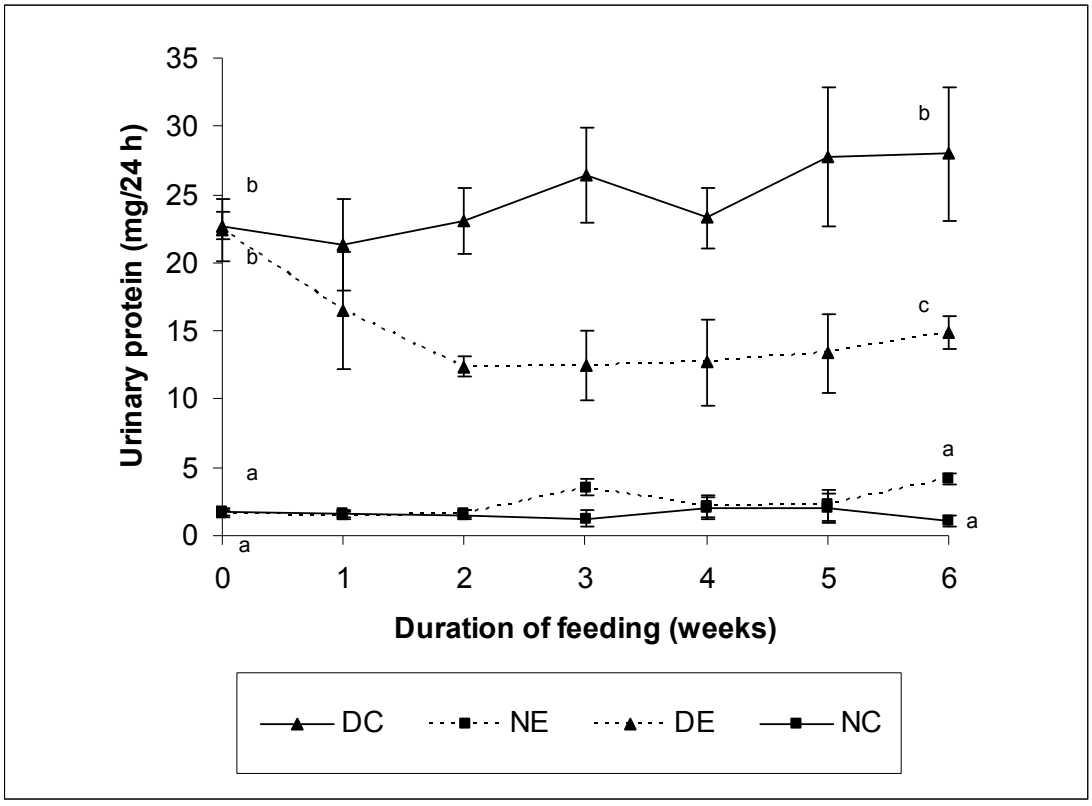


Figure 54. Influence of dietary finger millet seed coat matter on urinary protein excretion in the diabetic and non-diabetic animals

Values with the same superscript are statistically not significantly different at $p<0.05$

DC - Diabetic control
DE - Diabetic experimental
NC - Non-diabetic control
NE - Non-diabetic experimental

Figure 55 shows that, the diabetic rats excreted about 15 fold higher urea (249 mg/24 h) compared to the NC group of animals (22 mg/24 h). The urinary urea levels decreased significantly from 230 mg/24 h (at the beginning of the experimental period) to 132 mg/24 h (at the end of the experimental period) in the DE group of animals ($p<0.001$). Similarly, the DE group of animals exhibited significantly lowered levels of urinary creatinine (the levels decreased from 27 mg/24 h at the beginning of the experimental period to 18 mg/24 h at the end of the experimental period). The values were significantly lower compared to the DC group of animals ($p<0.01$) (Figure 56).

DE group of animals showed only marginal increase in the weight of the liver. Moreover, they exhibited higher serum protein, albumin and lower serum urea levels accompanied by the lower excretion of protein and urea as compared to the DC group of animals. This indicates a lesser degree of protein catabolism in the DE group of animals. The serum creatinine is widely interpreted as a measure of the glomerular filteration rate (GFR) and used as an index of renal function in clinical practice (Perrone et al 1992). Lower levels of blood creatinine, urinary creatinine, nephromegaly in the DE group of animals is suggestive of improved renal function in the particular group of animals.

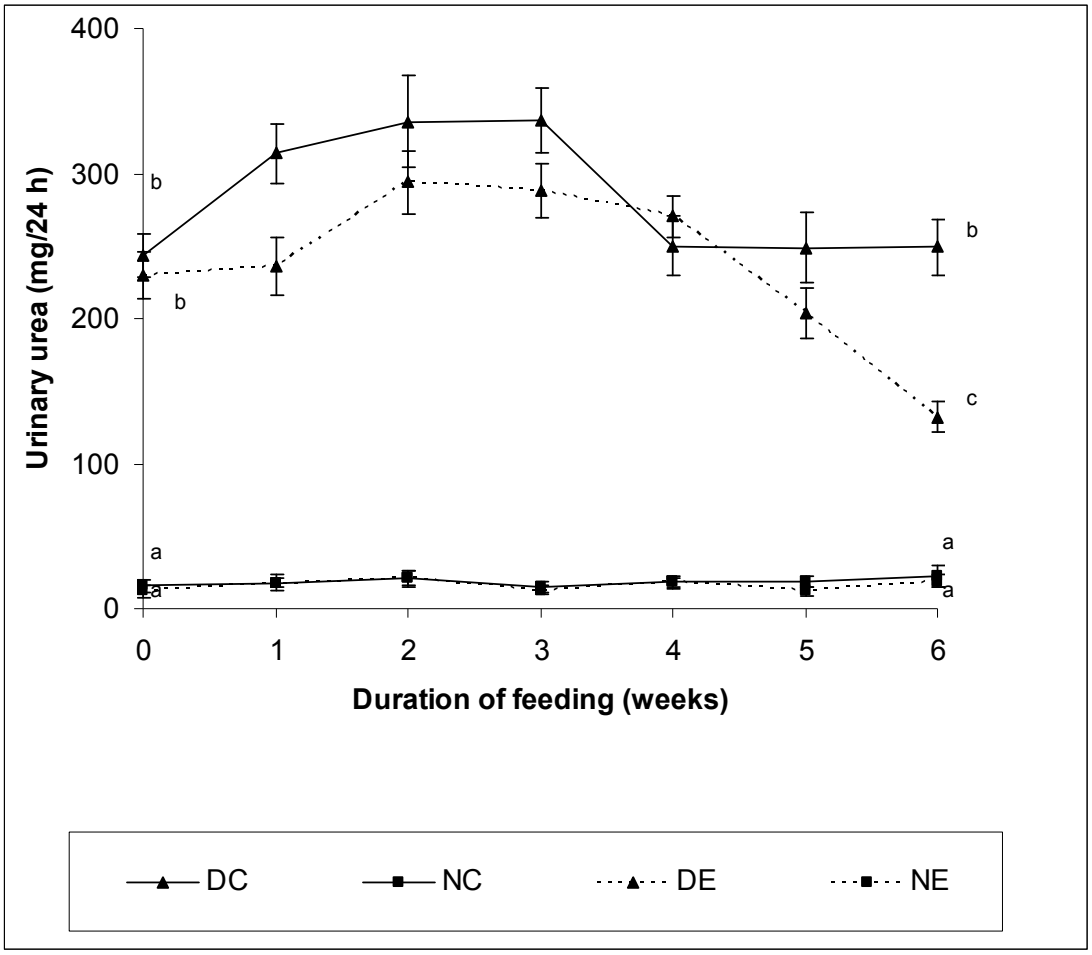


Figure 55. Effect of dietary finger millet seed coat matter on the urinary urea levels in the diabetic and non-diabetic animals

Values with the same superscript are statistically not significantly different at $p<0.05$

NC - Non-diabetic control
DC - Diabetic control
NE - Non-diabetic experimental
DE - Diabetic experimental

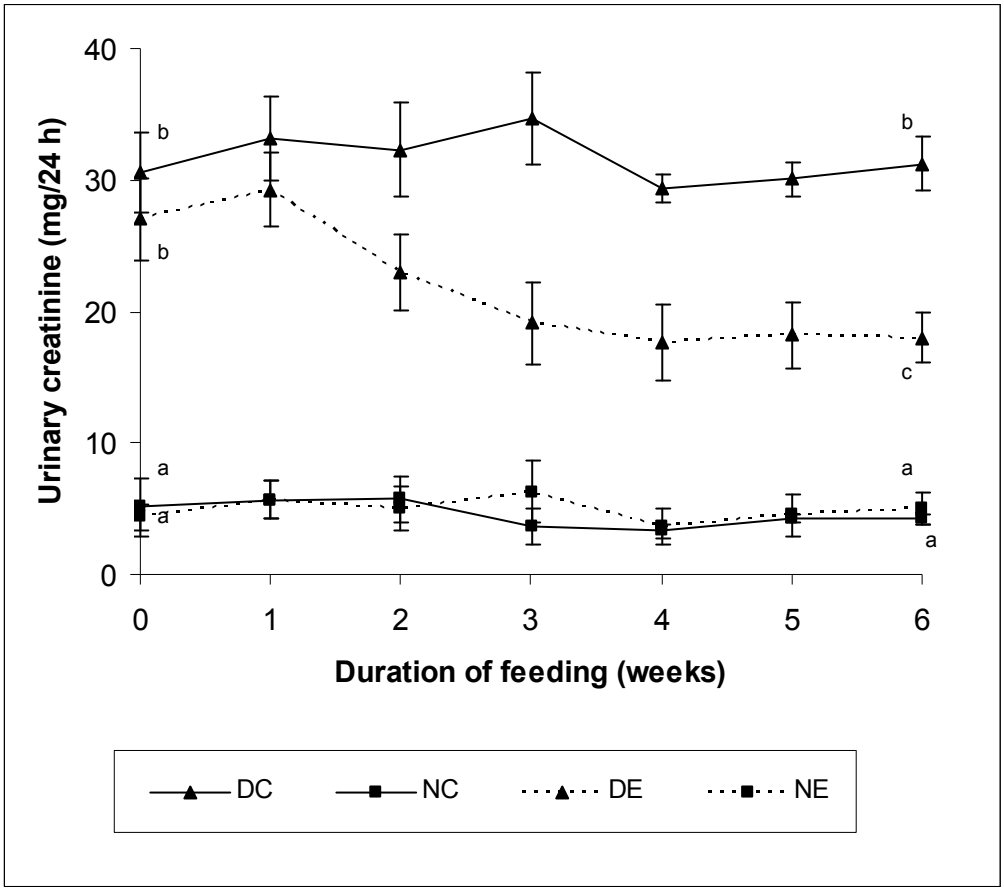


Figure 56. Effect of dietary millet seed coat matter on the urinary creatinine levels in the diabetic and non-diabetic animals

Values with the same superscript are statistically not significantly different at $p<0.05$

NC - Non-diabetic control
DC - Diabetic control
NE - Non-diabetic experimental
DE - Diabetic Experimental

The incidence of mature cataract was considerably lower in the diabetic animals maintained on the experimental diet as only 10% of the DE group of animals developed mature cataract compared to the DC group of animals (90% of the DC group exhibited mature cataract) at the end of the experimental period. Slit lamp examination is widely used for the diagnosis of ophthalmological disorders. Hence the slit lamp examination of the rat eyes was conducted to observe the cataract associated changes in the eye lens and the observations clearly revealed the presence of very mild lenticular opacity and posterior subcapsular cataract (immature cataract) in the DE group of animals in contrast to the significant lenticular opacity (mature cataract) and corneal vascularisation observed in the DC group of animals. Whereas, no lenticular opacity was observed with the non-diabetic animals (Figures 57, 58 and 59). These observations clearly indicate that, the SCM constituents delay the onset of cataractogenesis in the diabetic animals.

In order to get further insight into the mechanism of anti-cataract action of the millet SCM, activity of aldose reductase (AR), a key enzyme involved in cataractogenesis (through polyol pathway) was determined in the eye lens. The AR activity was lower by 25% in DE group of animals in comparison to that observed in the DC group (Figure 60).

Chronic hyperglycemia plays an important role in the pathogenesis of vascular complications leading to atherosclerosis, a variety of neuropathies, cataract and also the end stage renal failure, all of which account for disabilities and higher mortality rates in diabetics (Yamagishi and Imaizumi, 2005). Diabetic cataract is characterized by opacification of the lens, eventual loss of vision that occurs at a much earlier age than senile cataract. The three possible mechanisms involved in cataract formation as a result of hyperglycemia are the polyol pathway (Figure 61), oxidation and non enzymatic glycation (Spector, 1995). Through the polyol pathway, glucose is converted into sorbitol by aldose reductase (AR), the first rate limiting enzyme in the pathway, concomitant with the conversion of NADPH into $NADP^+$. Another enzyme sorbitol dehydrogenase oxidizes sorbitol to fructose. In diabetes *mellitus*, the increased glucose level results in sorbitol being produced at a faster rate than its oxidation to fructose, and as a result the accumulation of sorbitol in blood vessels, nerves, retina and kidney occurs.

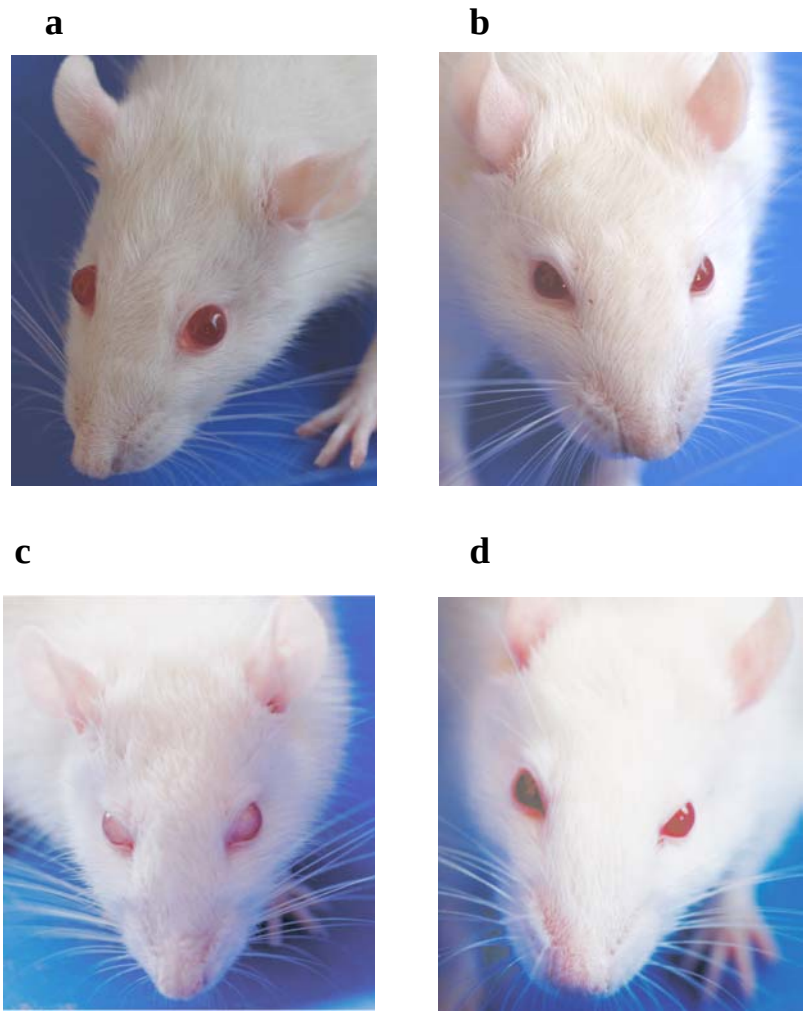


Figure 57. Effect of dietary finger millet seed coat matter on cataractogenesis in the diabetic animals

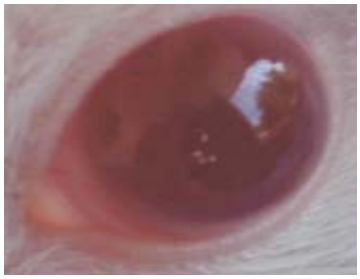
a. Non-diabetic control (NC) - showing normal eyes

b. Non-diabetic experimental (NE) - showing normal eyes

c. Diabetic control (DC) – showing mature cataract development in both the eyes

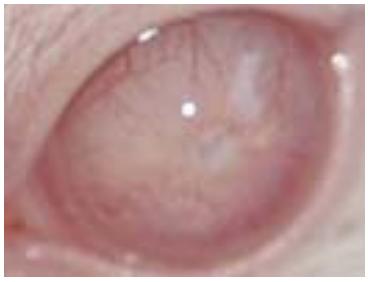
d. Diabetic experimental (DE) - showing normal eyes

A



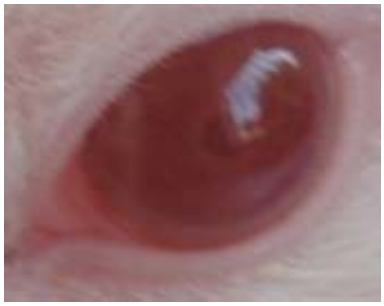
Non-diabetic control (NC) - normal eyes

B



Diabetic control (DC) - increased lenticular opacity with corneal vascularisation

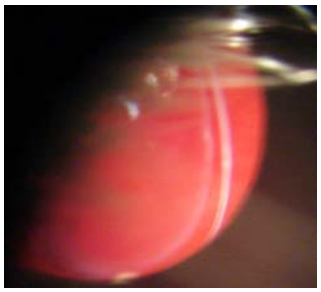
C



Diabetic experimental (DE) - no significant lenticular opacity

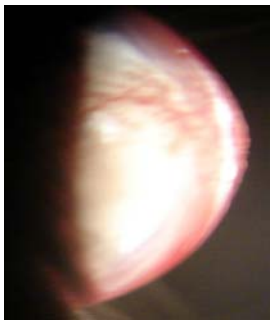
Figure 58. Influence of dietary finger millet seed coat matter on the cataractogenesis and lenticular opacity of the diabetic rat eyes

A



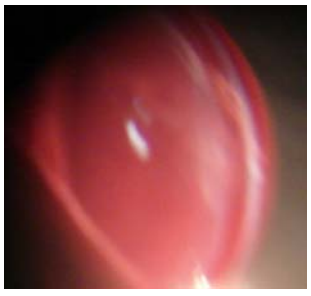
Non-diabetic control (NC) - no lenticular opacity - normal eyes

B



Diabetic control (DC) - corneal vascularisation, significant lenticular opacity, mature cataract

C



Diabetic experimental (DE) - early cataract changes (immature posterior subcapsular cataract)

Figure 59. Slit lamp examination of the eyes of diabetic and non-diabetic animals

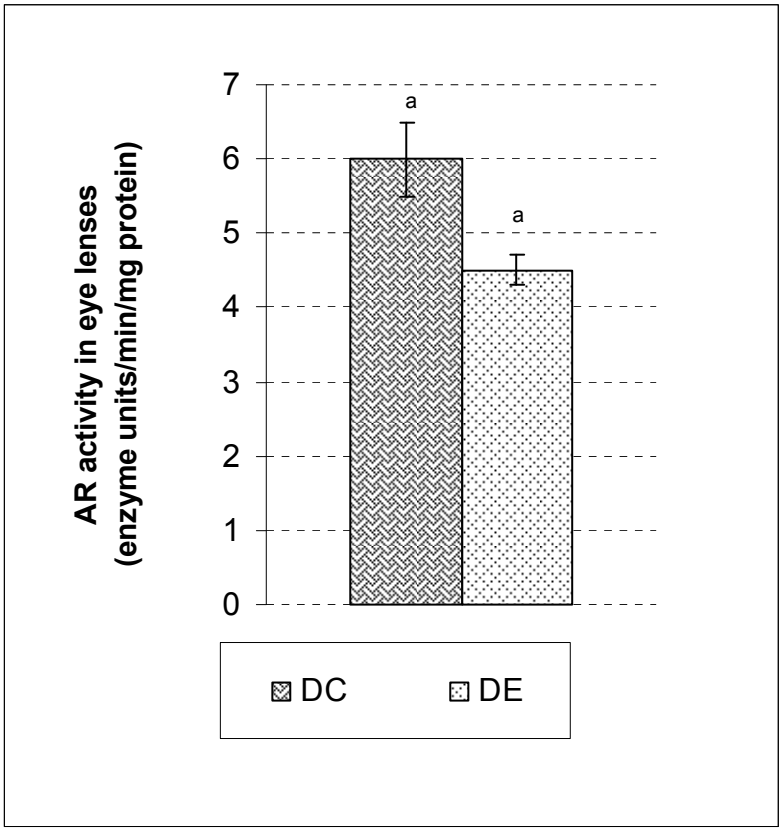


Figure 60. Effect of dietary finger millet seed coat matter on the eye lens aldose reductase (AR) activity in the diabetic animals

Values with the same superscript are statistically not significantly different at $p<0.05$

DC - Diabetic control
DE - Diabetic experimental

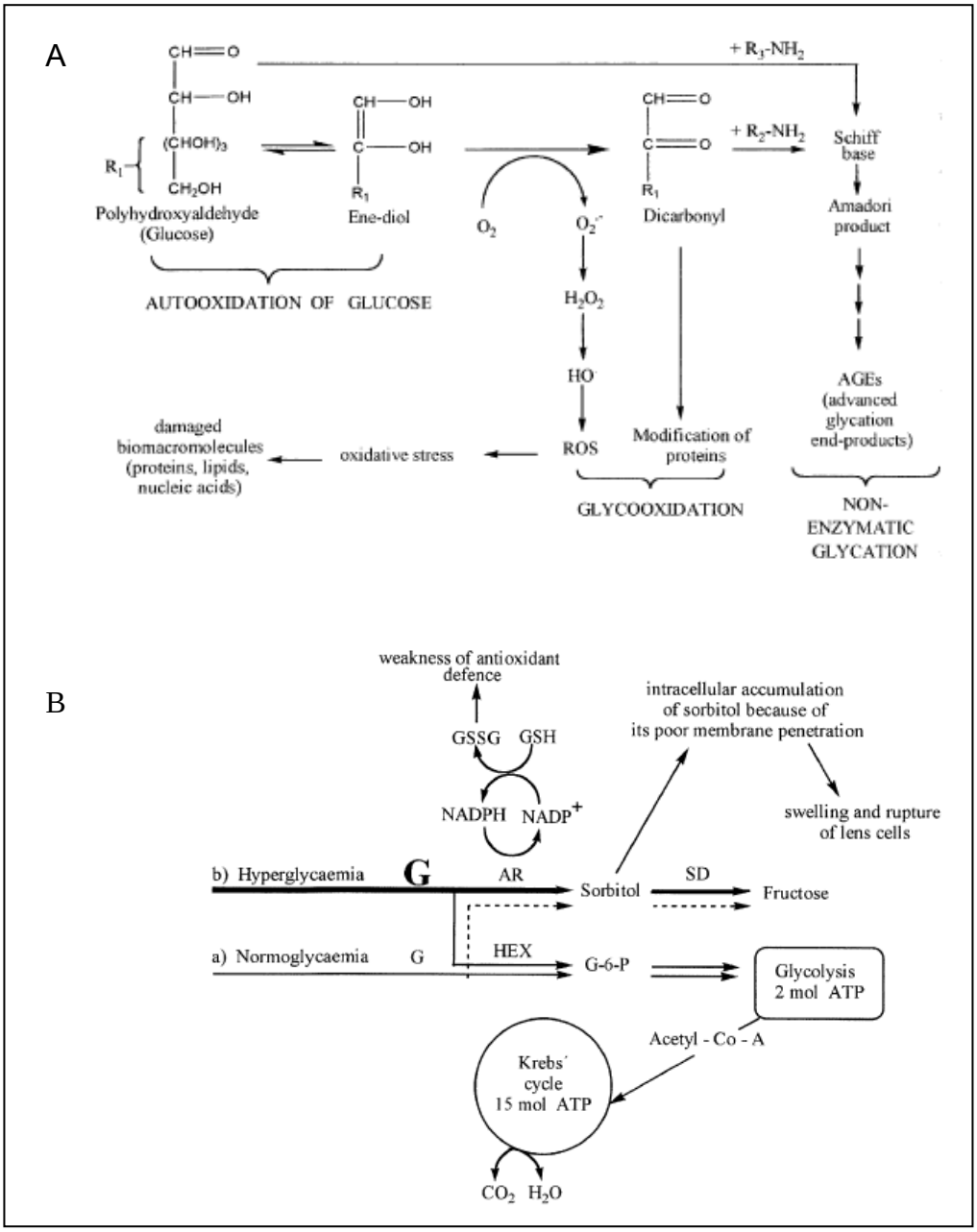


Figure 61. Non enzymatic reactions of excessive glucose in diabetic state (A), Polyol pathway in hyperglycemic and normoglycemic state (B)

O_2 – oxygen, O_2 - superoxide anion radical, H_2O_2 - hydrogen peroxide, $HO^{\cdot}$ - hydroxyl radical, ROS – reactive oxygen species, R_2-NH_2, R_3-NH_2 - polypeptidic chains, G - glucose, G-6-P - glucose 6 phosphate, AR - aldose reductase, SD - sorbitol dehydrogenase, HEX -hexokinase, GSSG - oxidised glutathione, GSH - reduced glutathione, bold line arrow - accelerated process, dotted line arrow - physiological rate of processes.

Source: Kyselova et al 2004

Accumulated sorbitol can produce a hyper osmotic effect, leading to membrane permeability changes (Figure 62) and the onset of cellular pathology (Kador et al 1985; De la Fuente and Manzanaro 2003). Glycation of lens protein-crystallins may cause conformational changes resulting in exposure of thiol groups to oxidation and cross-link formation (Ansari et al 1980). Furthermore, the lens crystallins have virtually no turnover and hence readily accumulate AGEs which in turn cause aggregation of the lens crystallins producing the high molecular weight material responsible for opacification as demonstrated by animal studies (Perry et al 1987). It has been shown by *in vitro* studies that, increased glycation of Na^+,K^+ - ATPase reduces its activity, altering intracellular ion concentration and subsequent water movement via osmosis (Stevens, 1998). Such an effect *in vivo* may contribute towards cataract formation in diabetes (Ahmed, 2005). Since the millet SCM is a rich source of phenolic compounds with antioxidant properties, the millet seed coat feeding may delay cataractogenisis in the diabetic rats. The aldose reductase inhibitory property of the millet seed coat polyphenols (in *in vitro*) has been already reported by Chethan et al (2008). Thus, the enzyme inhibitory property of the millet seed coat polyphenols may also be attributed to the anti-cataractogenisis property of the millet SCM.

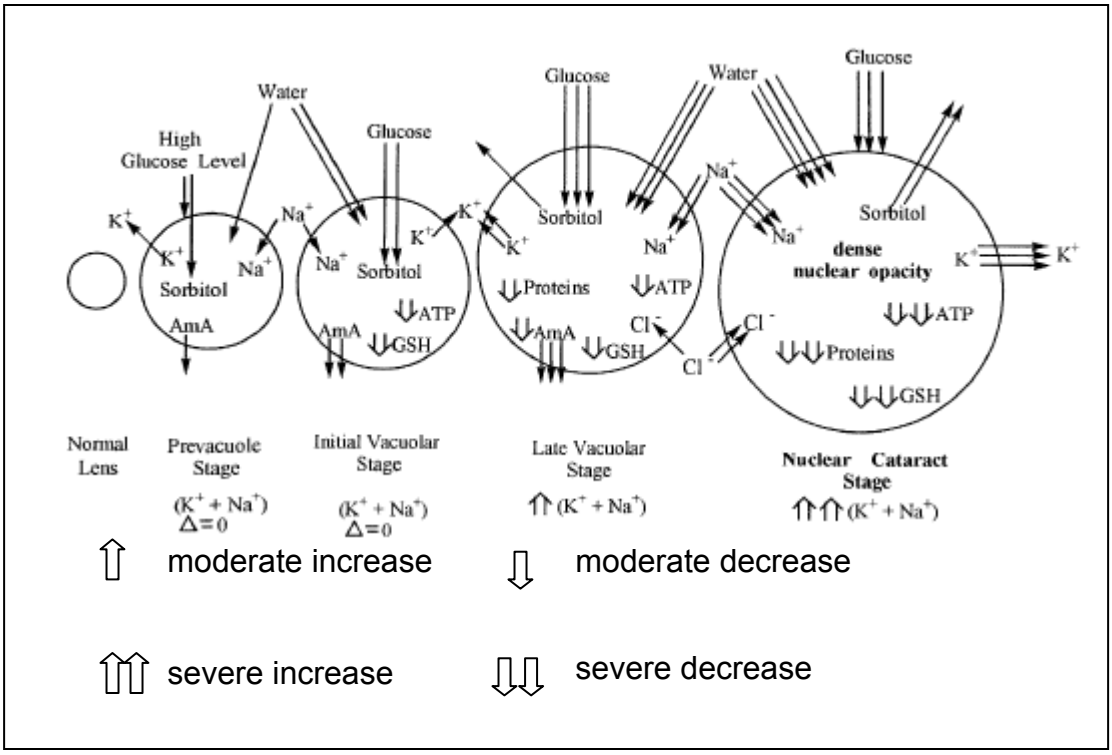


Figure 62. Biomorphological changes of eye lens during cataract formation

K^+- potassium, Na^+ - sodium, Cl^- - chlorine, $\Delta = 0$ - no change in total K^+, Na^+, Cl^-, AmA - amino acids, GSH - reduced glutathione

Source: Kyselova et al (2004).

The serum AGE fluorescence spectra (Figure 63) reveals that, the AGE levels in the diabetic animals were higher compared to the non-diabetic animals. It was also observed that the serum AGE and HbA1c levels of DE group of animals were lower by 20% and 33% respectively compared to the levels observed in the DC group of animals. The HbA1c levels of DE group were significantly different ($p<0.001$) from the DC group of animals (Figure 64). The increased levels of serum AGE and HbA1c, higher lens aldose reductase activity and also the incidence of mature cataract in the DC group indicate increased conversion of glucose to sorbitol by polyol pathway and higher rate of protein glycation inducing cataractogenesis. In contrast, lower AGE, HbA1c levels, AR activity and lesser lenticular opacity as well as the absence of mature cataract in the DE group of animals reveal that, the millet SCM prevent protein glycation and in addition, delays cataractogenesis in the diabetic animals. Chethan et al (2008) reported that, the millet seed coat matter polyphenols inhibit aldose reductase. In chapter II, it has been observed that, the millet seed coat phenolics are effective inhibitors of fructose induced protein glycation. Reduced rat tail tendon collagen glycation was also observed in the diabetic rats maintained on the millet diet (Hegde et al 2005a). Hence, the anti-cataract properties of the millet seed coat may partly be attributed to the enzyme inhibitory as well as to the anti-protein glycation properties of the millet seed coat phenolic compounds, which may influence either directly or by indirect regulation of glucose homeostasis.

Protein glycation and AGE formation are closely associated with artherosclerosis. Increased glycation of low-density lipoprotein (LDL) occurs in diabetes. Glycated LDL is not recognized by the LDL receptor but its uptake by macrophages is enhanced. Due to this, the hyperlipidaemia and accelerated foam cell formation occurs in diabetes. LDL is also modified by AGE and LDL–AGE complex increase in diabetic condition and has reduced serum clearance. Glycation and AGE formation are also accompanied by increased oxidation of LDL and as a result, increase in the atherogenic oxidized LDL occurs in the diabetic condition (Ahmed, 2005). Hence, the lower AI observed in the diabetic animals maintained on the SCM diet may also be partly attributed to the anti-glycation properties of millet seed coat phenolics.

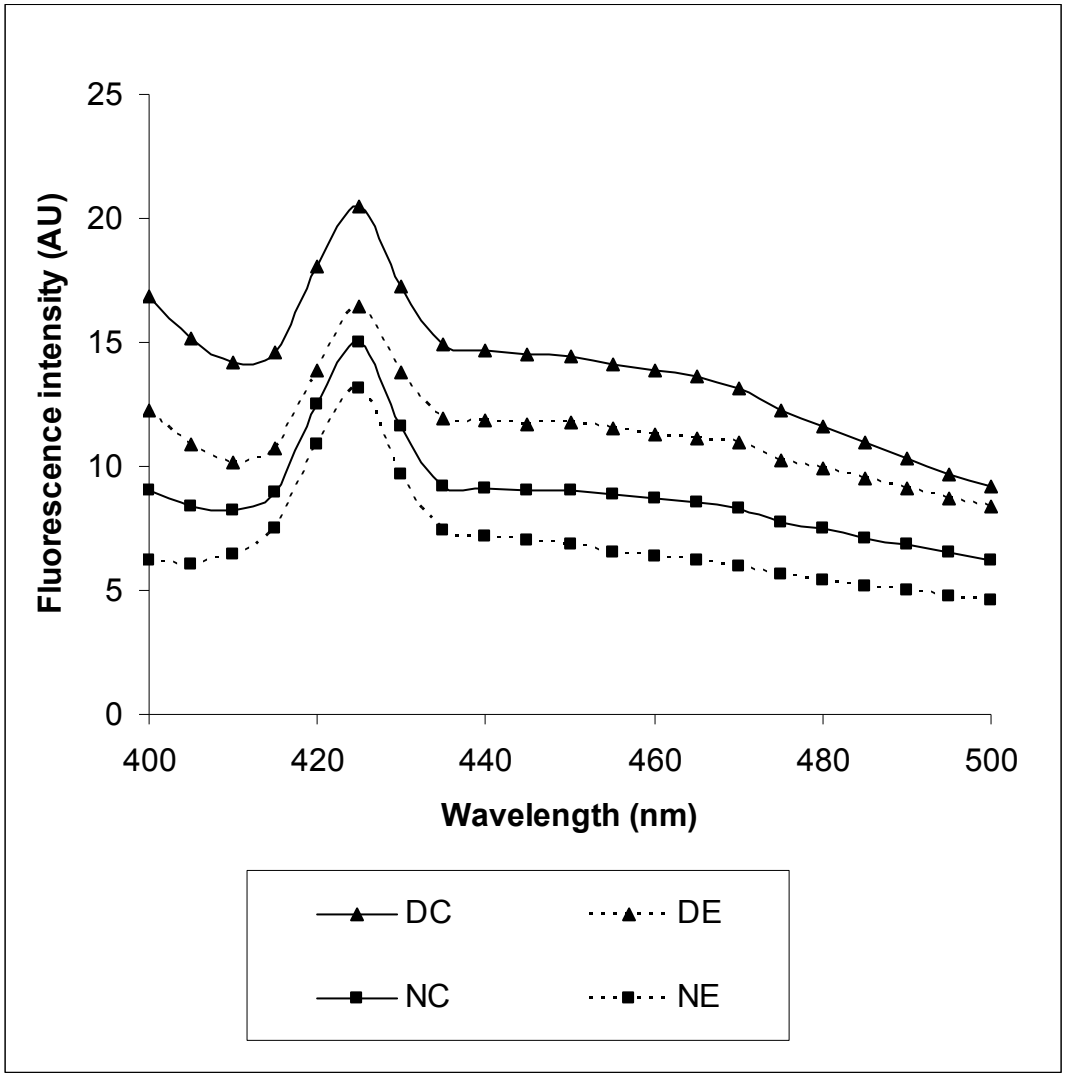


Figure 63. Serum AGE fluorescence spectra of diabetic and non-diabetic animals maintained on the control and experimental diet

Protein 10 µg/ml in 0.05 M sodium phosphate buffer pH 7.4 was excited at 370 nm and emission was monitored at 400 - 500 nm.

Data represent average of three such observations

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NC - Non-diabetic control
DC - Diabetic control
NE - Non-diabetic experimental
DE - Diabetic experimental

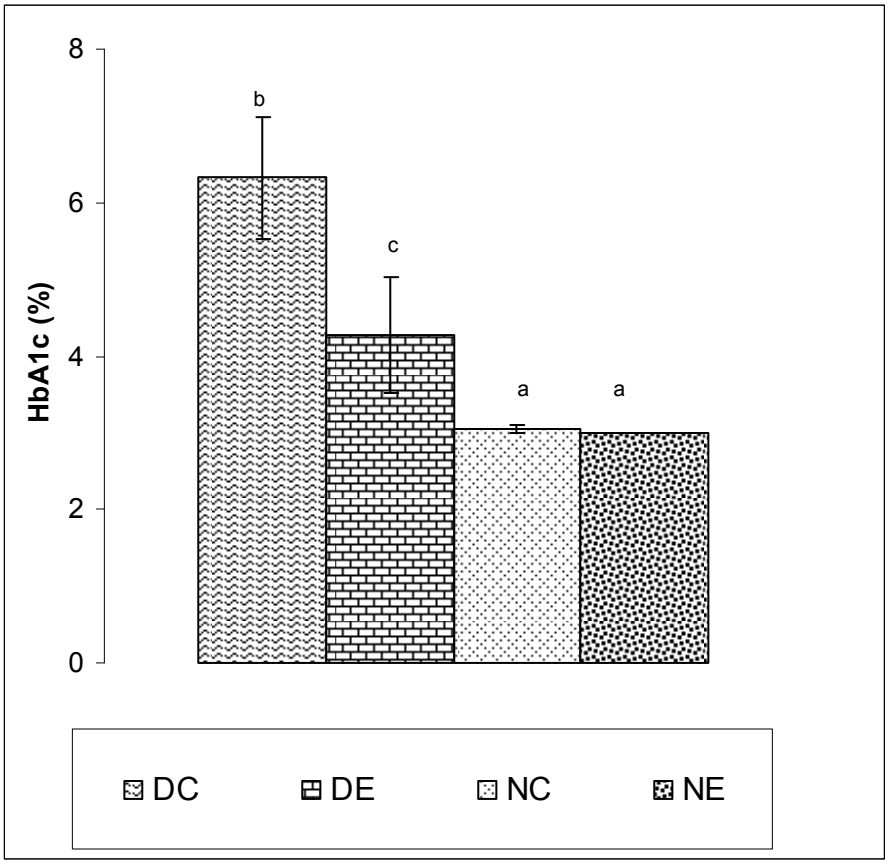


Figure 64. Glycosylated hemoglobin (HbA1c) levels of non-diabetic and diabetic animals maintained on the control and experimental diets

Values with the same superscript are statistically not significantly different at $p<0.05$

NC - Non-diabetic control
DC - Diabetic control
NE - Non-diabetic experimental
DE - Diabetic experimental diet

Diabetic rats were characterized by markedly large kidneys i.e, nephromegaly (Figure 65), the weights of the kidney being about 2.4 - fold higher than those of non-diabetic controls (Table 30). DE group of animals exhibited lower (by 21%) kidney weights compared to the DC group (Table 30). The glomerular filteration rates (GFR) of the DC group were markedly high (7.3 ml/min), compared to the NC group of animals (0.1-0.2 ml/min) and among the DE and DC groups (Figure 66), the GFR of the DE group (2.7 ml/min) was significantly lower ($p<0.001$).

Histological examination of the kidney sections revealed the presence of normal glomerulus in the DE group of animals (Figures 67A and B) compared to the shrunken glomerulus observed in the DC group of animals (Figure 67C). Moreover, normal glomerular, tubular structures and absence of mucopolysaccharide depositions were observed in the kidney sections of the DE group of animals (Figures 68A and B). However, mucopolysaccharide depositions (Figure 68C) and higher degree of tubular clarifications or vacuolations (Figure 68D) were observed in the kidney sections of DC group of animals.

Diabetic nephropathy is characterized by thickening of the basement membrane, expansion of the mesangium, reduced filtration, albuminuria and ultimately renal failure. AGEs have been detected in renal tissues in amounts that correlate with the severity of diabetic nephropathy (Sugiyama et al 1996). Hyperglycaemia and AGEs enhance the release of transforming growth factor-b (TGF-b) which in turn stimulates synthesis of collagen matrix components and this may account for, at least in part, the thickening of the basement membrane in diabetic nephropathy. The accumulation of AGEs on collagen in the basement membrane together with their ability to trap plasma proteins may also contribute towards thickening of the basement membrane as well as altered filtration and ultimately loss of glomerular function (Monnier et al 1992). The reduced ability to filter in diabetic nephropathy is, in part, due to an expansion of the mesangial layer that causes compression of capillaries and a reduction in the surface area over which filtration takes place. Cultured mesangial cells possess AGE receptors and respond to AGEs by increased synthesis of matrix proteins and type IV collagen. Animal studies have shown thickening of the basement membrane and expansion of the

mesangial layer following injection of AGE-altered protein, thus supporting the involvement of AGEs in diabetic nephropathy (Vlassara et al 1994).

Normal glomerulus and absence of mucopolysaccharide depositions in the kidney tissues of the DE group of animals may possibly be due to the improved glycemic status and lowered hyperglycemia induced protein glycation.

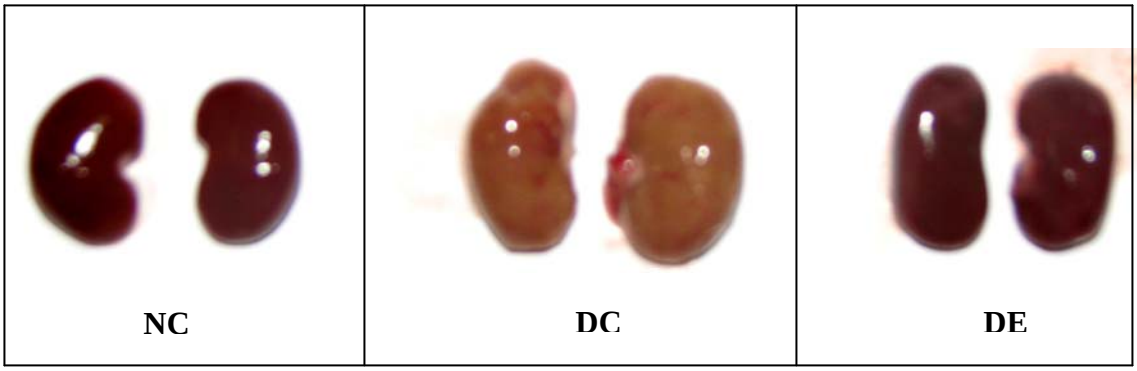


Figure 65. Morphological changes in the kidneys of the non-diabetic and diabetic animals maintained on the control and experimental diets

NC - Non-diabetic control
DC - Diabetic control
DE - Diabetic experimental

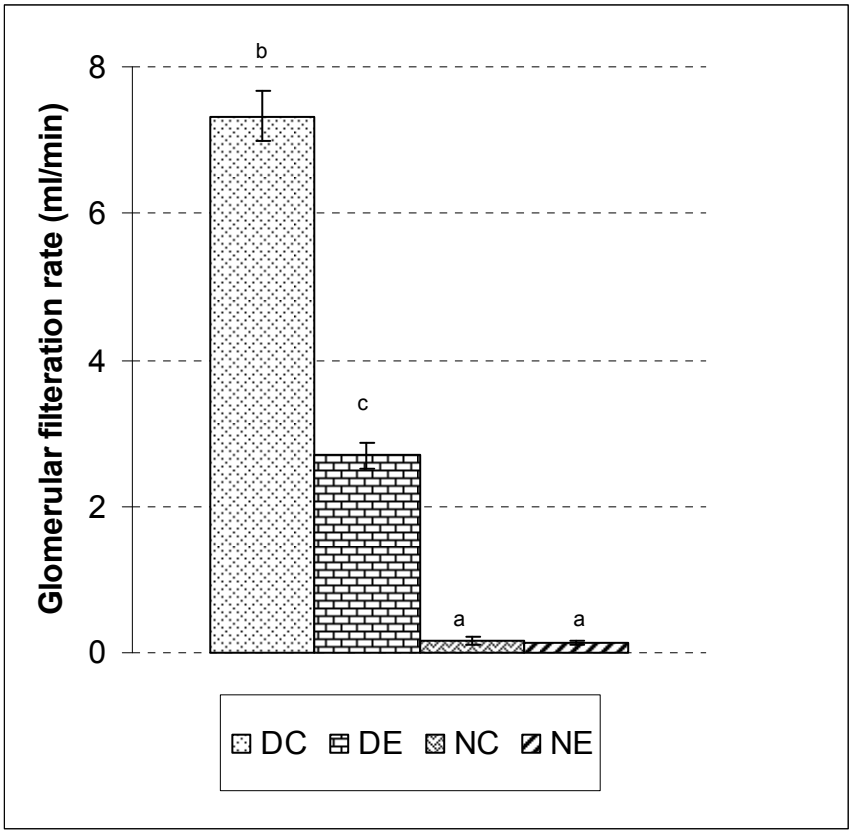
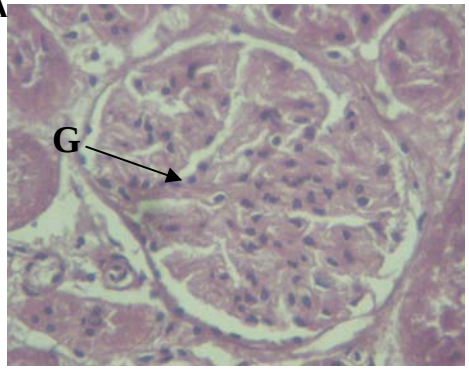


Figure 66. Glomerular filtration rate (GFR) of the diabetic and non-diabetic animals maintained on the control and experimental diet

Values with the same superscript are statistically not significantly different at $p<0.05$

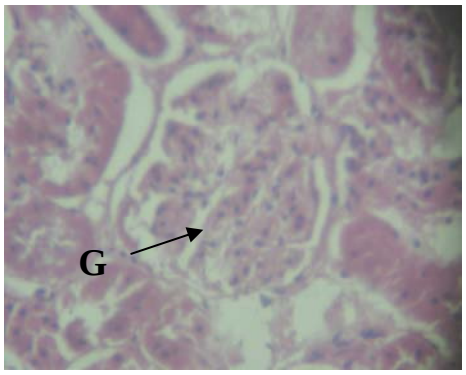
NC - Non-diabetic control
DC - Diabetic control
NE - Non-diabetic experimental
DE - Diabetic experimental

A



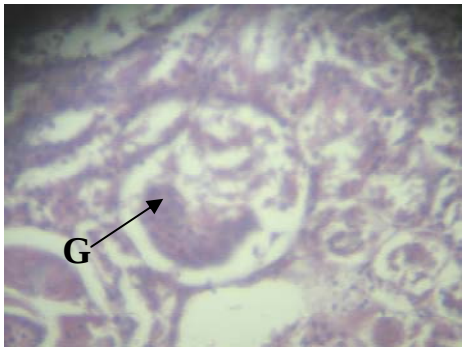
Normal glomerulus (G) in the non-diabetic control (NC) kidney

B



Normal glomerulus (G) in the diabetic experimental (DE) kidney

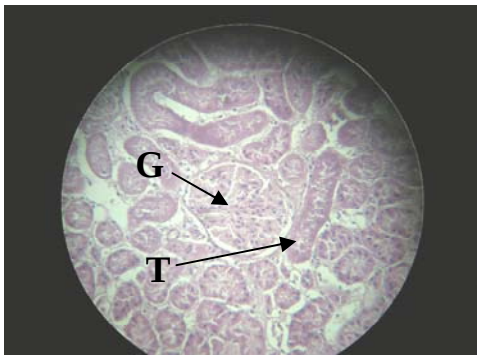
C



Shrunken glomerulus (G) in the diabetic control (DC) kidney

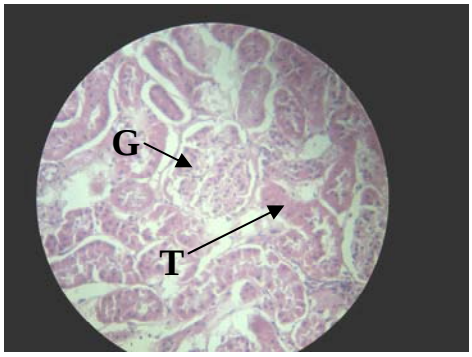
Figure 67. Effect of dietary finger millet seed coat matter on the kidney glomerular structures of the diabetic and non-diabetic animals

A



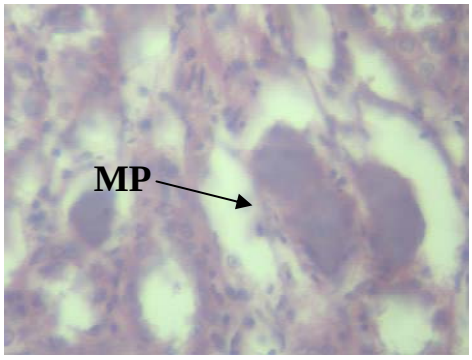
Non-diabetic control (NC) kidney showing normal glomerulus, proximal tubules

B



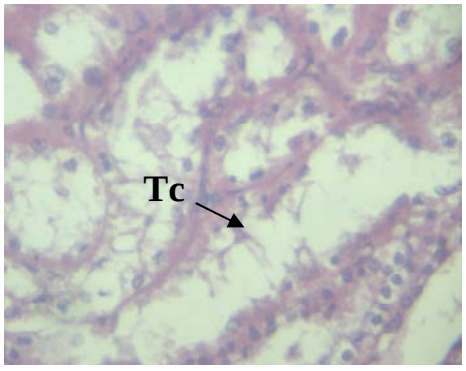
Diabetic experimental (DE) kidney showing normal glomerulus, proximal tubules with no mucopolysaccharide depositions

C



Mucopolysaccharide depositions in the diabetic control (DC) kidney

D



Clearing and vacuolation in tubules of the diabetic control (DC) kidney

Figure 68. Effect of the dietary finger millet seed coat matter on the kidney structures of the diabetic animals

G - glomerulus, T – tubules, MP - mucopolysaccharide depositions, Tc – tubular clarifications

SUMMARY AND CONCLUSIONS

- To evaluate the hypoglycemic properties of the finger millet seed coat matter, the semi-synthetic diet containing 20% seed coat matter (SCM) was fed to the streptozotocin (STZ) induced diabetic rats for six weeks period. The adult albino rats (*Wistar*) were rendered diabetic by single i.p. STZ injection and two groups of diabetic animals (8 animals in each group) and parallely 2 groups of healthy or non-diabetic animals (8 animals in each group) were used for the feeding trials.
- One of the diabetic groups and one of the non-diabetic groups received the experimental diet (SCM diet) and the other two groups received the control diet (semi-synthetic diet). The changes in the body weight and urinary metabolites (glucose, protein, urea, creatinine) were monitored at weekly intervals, the blood glucose levels were determined at fortnightly intervals. Whereas, at the end of experimental period, the eye lens of the animals were examined for cataractogenesis by slit lamp examination after which the animals were sacrificed and the important biochemical parameters of the blood and status of liver and kidney were assessed.
- It was observed that, the intake of the experimental diet was normal in both the diabetic and non-diabetic animals. The urinary excretion of metabolites (glucose, protein, urea, creatinine) were considerably lower in the diabetic animals receiving the experimental diet (diabetic experimental, DE) as compared to the diabetic animals receiving the control diet (diabetic controls, DC).
- The levels of blood glucose, urea, creatinine, total cholesterol, triglycerides, AGE (advanced glycation endproducts), HbA1c (glycosylated hemoglobin) and atherogenic index of the DE group of animals were significantly lower compared to that of DC group of animals. The levels of serum total protein, albumin, globulin and HDL cholesterol in the DE group of animals were higher when compared to their corresponding levels in the DC group of animals.

- The slit lamp examination of eyes of the DE group of animals indicated immature subcapsular cataract changes compared to the mature cataract, significant lenticular opacity and corneal vascularisation observed in the DC groups of animals. In concurrence with this the aldose reductase (AR) activity in the former group of animals were lower than the later group.
- Histochemical examination of the kidneys of the diabetic animals revealed the presence of normal glomerular and tubular structures in the kidneys of DE group of animals compared to the shrunken glomerulus, tubular clarifications and mucopolysaccharide depositions observed in the DC group of animals.

The study clearly brought out the ameliorative characteristics of the millet SCM towards hyperglycemia, dyslipidemia, kidney dysfunction, hyperglycemia induced protein glycation and AGE formation in STZ induced diabetic animal models. In addition, the millet SCM also delays the onset of cataractogenesis in the diabetic experimental animals.

Since the millet SCM is an edible component and rich source of dietary fiber, polyphenols, calcium and other micronutrients with health beneficial properties, its has very high potential for utilization as a functional ingredient in health foods for diabetic and obese population.

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