FINGER MILLET (*Eleusine coracana*) POLYPHENOLS AND THEIR NUTRACEUTICAL POTENTIAL

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

The work embodied in the thesis entitled "***Finger millet (Eleusine coracana) polyphenols and their nutraceutical potential***'' submitted to the University of Mysore, Mysore, for the award of Degree of "**Doctor of Philosophy**" was carried out by me at the Department of Grain Science and Technology, Central Food Technological Research Institute, Mysore, under the guidance of **Dr. N. G. Malleshi**, Head, Department of Grain Science and Technology, Central Food Technological Research Institute, Mysore, during the period 2003 – 2008.

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

S. Chethan

Place : Mysore
Date :

Dedicated to……

My parents, whose love, affection, blessings and encouragement, made it possible to fulfill my ambition, and my guide ***Dr. N. G. Malleshi,*** *without whom nothing would have been possible.*

Dr. N. G. Malleshi
Head,
Department of Grain Science and Technology

CERTIFICATE

This is to certify that the thesis entitled "***FINGER MILLET (Eleusine coracana) POLYPHENOLS AND THEIR NUTRACEUTICAL POTENTIAL***" is the result of the work carried out by Mr. S. Chethan, CSIR-SRF, during the period from June 2003 to December 2008, under my guidance at the Department of Grain Science & Technology, Central Food Technological Research Institute, Mysore, India.

N. G. Malleshi

Place :
Date :

Acknowledgements

It gives me immense pleasure to express my sincere thanks and deep sense of gratitude to my guide **Dr. N. G. Malleshi**, Head, Department of Grain Science and Technology, Central Food Technological Research Institute, Mysore, for his valuable guidance, keen interest, constant encouragement, help and cooperation throughout the course of this work and under whose guidance the thesis has been prepared.

My sincere thanks goes to **Dr. V. Prakash**, Director, CFTRI, Mysore, for kindly approving the R&D work on the nutraceutical aspects of finger millet and allowing me to carryout this work in the Institute, besides providing all the necessary facilities and encouragement to complete the work.

I gratefully acknowledge the help and support received from **Dr. Shylaja M. Dharmesh, Dr. P. Srinivas, Dr. Y. N. Sreerama and Dr. M. S. Meera,** Scientists, CFTRI, Mysore.

I am thankful to **Dr. Vasudeva Singh, Dr. Jaydeep, Dr. Sila Bhattacharya, Dr. Manisha, Mr. B. V. Satyendra Rao, Mr. A. Srinivas, Mr. V. M. Pratape, Mrs. Usha Dharmaraj, Mrs. V. B. Shashikala, Mrs. Mallika P. Kumar, Mr. Ashwathnarayan and Mr. G. Santhosh,** the staff of Grain Science and Technology Department of the Institute for their support during my entire stay in CFTRI, Mysore.

I am grateful for the help rendered by **Dr. E. Rati Rao, Dr. Negi, Krishnamurthy, Mukund Laxman, Jayprakashan, Girish** and **Shivaprasad**.

The piece of work would not have been a success without the help of **Dr. B. S. Vishwanath**, Department of Biochemistry, University of Mysore, Mysore and **Dr. Padma**, Mysore Race Course Eye Hospital, Mysore.

I remember with pleasure the unfailing help received from my friends **Anitha, Chandrashekar, Chidanand, Devraj, Gangadhara, Gajendra, Harsha, Jagadeesh, Kanakaraju, Komala, Latha, Manjunath, Mohan, Pradeep, Police Gowda, Rateesh, Sandya, Sanjay, Sangeeta, Satish**

Kumar, Shivaprasad, Smita, Soumya Banerjee and Venkatesh Kumar of CFTRI, Mysore.

I gratefully acknowledge CSIR, New Delhi, for the award of Senior Research Fellowship, which enabled me to carry out this research work.

Last but not the least, I am grateful to my **parents**, **brother** and my **wife** for their never-ending encouragement and moral support.

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

Chethan

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ABBREVIATIONS

α	alpha
ABTS	2, 2'- azinobis (3 – ethylbenzothiazoline – 6 – sulfonate)
ARIs	aldose reductase inhibitors
Å	angstrom
AP-1	activator protein 1
APS	ammonium persulphate
ADP	adenosine diphosphate
ATCC	American type cell culture
Asp	aspartate
β	beta
BSA	bovine serum albumin
BSS	British standard sieves
BHA	butylated hydroxyanisole
BHT	butylated hydroxytoluene
Ca	calcium
cm	centimeter
cm-sephadex	carboxymethyl sephadex
cpm	counts per minute
Cu	copper
ºC	degree centigrade
Cox	cyclooxygenase
DNA	deoxyribose nucleic acid
DMSO	dimethyl sulfoxide
DIPF	diisopropyl phosphorofluoridate
DNS	3, 5-dinitrosalicylic acid
DPPH	1, 1-diphenyl-2-picrylhydrazyl
EDTA	ethylenediaminetetraacetic acid
EC	Enzyme Commission
ERK	extracellular signal-regulated protein kinase
E-S complex	enzyme-substrate complex
E-S-I complex	enzyme-substrate-inhibitor complex

Fig	figure
Fe	iron
F/C reagent	Folin-Ciocalteau reagent
γ	gamma
g	gram
GPC	gel permeation chromatography
h	hour
HPLC	high pressure liquid chromatography
HCl	hydrochloric acid
H_2O_2	hydrogen peroxide
His	histidine
I	inhibitor
K	potassium
kg	kilogram
K_m	Michaelis constant
JNK	c-jun N-terminal kinase
L	liter
LC-MS	liquid chromatography mass spectrometry
LB plot	Lineweaver-Burk plot
Li_2SO_4	lithium sulfate
Lys	lysine
Lox	lipoxygenase
mg	milligram
Mn	manganese
µg	microgram
µmol	micromole
min	minute
µl	microliter
Mmol	millimole
M	molar
mol	mole
MeOD	deuteramethanol

MU	maltose units
Max	maximum
MAPK	mitogen-activated protein kinase
MTCC	microbial type cell culture
N	normality
nm	nanometer
NMR	nuclear magnetic resonance
ηM	nano molar
NaOH	sodium hydroxide
NF-κB	nuclear factor-kappa B
NGO	Non-Governmental Organization
Nrf2	nuclear factor erythroid 2 related factor 2
$NaHCO_3$	sodium bicarbonate
NADPH	nicotinamide adenine di-nucleotide phosphate (reduced)
Na_2HPO_4	di-sodium hydrogen phosphate
Na H_2PO_4	sodium di-hydrogen phosphate
OD	optical density
ODS	octadecylsilane
PMSF	phenylmethanesulfonylfluoride
Pb	lead
%	percent
ppm	parts per million
PI3	phosphoinositide 3
PRSCF	polyphenols rich seed coat fraction
PLA_2	phospholipase A_2
PBS	phosphate buffer saline
PAF	platelet aggregation factor
RFF	refined flour fraction
ROS	reactive oxygen species
RP-HPLC	reverse phase high pressure liquid chromatography
sec	seconds
SCF	seed coat fraction

SDS-PAGE	sodium dodecyl sulfate polyacyrlamide gel electrophoresis
s/v	substrate vs velocity
TEMED	N,N,N′,N′-tetramethylethylenediamine
TMS	tetramethylsilane
TRIS	tris(hydroxymethyl)aminomethane
TFA	trifluoroacetic acid
TNBS	trinitrobenzenesulfonic acid
TBA	thiobarbituric acid
TCA	trichloroacetic acid
UV	ultraviolet
Vis	visible
V/V	volume/volume
V_{max}	maximum rate of reaction
W/V	weight/volume
Zn	zinc

ABSTRACT OF THE THESIS

Finger millet is one of the minor cereals and is known for its health benefits and extremely good storage quality. These special features of the millet are generally attributed to its polyphenols content. However, the scientific information to substantiate theses attributes and also the information on the nature of the millet polyphenols is scanty. Hence, R & D work towards isolation and characterization of the millet polyphenols and its nutraceutical potential were undertaken, and the salient features of the work presented in the thesis are as follows;

The scientific and technological information on cereals in general and finger millet in particular with special reference to polyphenols, published in peer reviewed scientific journals, proceedings of the scientific conferences, book chapters and popular articles are presented in chapter I.

The investigations on the extraction, purification, fractionation and characterization of the millet polyphenols are dealt in chapter II. Since, nearly 70 % of the millet polyphenols are concentrated in the seed coat tissue, a special milling protocol was developed to prepare the seed coat rich fraction of the millet and the same was used for the studies. Various polar and non-polar solvents were tried for extraction of the polyphenols and HCl–methanol (1:99 v/v) was identified as very effective extractant. The polyphenols of the acidic methanol extract were sensitive to the changes in pH and precipitated as well as denatured at alkaline conditions. Fractionation of the extracted polyphenols by high performance liquid chromatography (HPLC) showed a large number of constituent phenolics but only a few of them namely, gallic, proto-catechuic, *p*-hydroxy benzoic, *p*-coumaric, syringic, ferulic and *trans*-cinnamic acids and quercetin a flavonoid were identified. The

identity of the component phenolics was confirmed by LC-MS and also by NMR spectra. The information generated may facilitate isolation of specific phenolics of pharmaconutritional importance.

The nature of inhibition of the millet polyphenols on some of the plant and animal enzymes is reported in chapter III. Finger millet is extensively processed for preparation of malt, and the malt amylases play a major role with respect to the functional properties of malt based foods. Hence, the effect of millet polyphenols on the nature of inhibition on its amylases was investigated by the kinetic studies using Michaelis-Menten and Lineweaver-Burk equations. It was observed that the K_m remained constant (0.625 %), but the maximum velocity (V_{max}) decreased in the presence of a crude polyphenols extract, indicating mixed non-competitive type of inhibition. In contrast to that, some of the constituent phenolics, namely, gallic acid, vanillic acid, quercetin and *trans*-cinnamic acid isolated from the polyphenols extract showed uncompetitive inhibition. Gallic acid and quercetin exhibited highest affinity whereas *trans*-cinnamic acid showed lower affinity for the enzyme-substrate complex with K_i' of 4.6×10^{-7} M and 7.3×10^{-7} M, respectively. The studies indicated the presence of secondary binding sites in malted finger millet amylases similar to other cereal amylases. These observations add to the basic science of the finger millet enzymes and its phytochemicals besides may help for the usage of millet as a source of amylase or as amylase rich food (ARF), in various food formulations.

Normally diabetes induced cataract is characterized by accumulation of polyol which is mediated by the up regulation of a key enzyme aldose reductase (AR). Since, the whole meal millet is consumed, it was of interest to know the positive role of the millet polyphenols in minimizing the incidence as well as the intensity of cataractogenesis. Hence, the crude polyphenols of the millet and also some of the

individual phenolics isolated from it were evaluated for their AR inhibitory activity. Out of the phenolics tested, quercetin was more potent inhibitor with IC_{50} of 14.8 ηM. The inhibition of AR was non-competitive. Results thus provide a stronger evidence for the potential of the millet polyphenols in minimizing the cataractogenesis in human eye lenses.

Exploratory investigations were conducted on the inhibitory activity of millet polyphenols on snake venom PLA_2, and it was observed that, the crude polyphenols extract and also the gallic acid as well as quercetin isolated from that, exhibited considerable inhibition with IC_{50} values of 83, 62 and 17 µg/ml, respectively.

The antioxidant and the antimicrobial activity of the millet polyphenols were assayed and the same is described in chapter IV. The IC_{50} value for the antioxidant potential of the millet phenolics on DPPH, hydroxyl and ABTS radical scavenging activity were 62.7, 109 and 114 µg/ml, respectively. Gallic, caffeic, ferulic, proto-catechuic and *p*-hydroxy benzoic acids isolated from the crude extract of the millet polyphenols exhibited inhibitory activity against *K. pneumoniae, Y. enterocolitica, S. pyogenes, P. mirabilis* and *S. marcescens* however, the degree of inhibition varied among the phenolics. Quercetin, inhibited the growth of all the pathogenic microbes tested even at a very low concentration. The antioxidant and also the antimicrobial activity of the polyphenols were dose dependent. These observations revealed that, the millet polyphenols may serve as natural antioxidants as well as antimicrobial agents, useful as a nutraceutical for reducing the intensity of some of the diseases arising out of oxidative deterioration in humans if not preventing them, and also for enhancing the safety of foods from microbial spoilage.

From the studies it may be concluded that, finger millet seed coat is a rich source of polyphenols, and acidic methanol is very effective solvent for extraction of polyphenols. The millet polyphenols are complex mixture of several phenolics acids, out of which only a few could be identified. The millet polyphenols inhibit the millet malt amylases and aldose reductase from human eye lenses, the inhibition being uncompetitive and noncompetitive, respectively. The polyphenols exhibit antioxidant and antimicrobial activity also. Thus, the seed coat matter of the millet as such could be utilized for biofortification of various foods as a source of antioxidant rich ingredient or the polyphenols isolated from the millet may find application as a source of nutraceuticals.

Finger millet (*Eleusine coracana*) or ragi is one of the important minor cereals in Indian subcontinent and also in several African countries. Some of the health beneficial qualities, excellent shelf-life and the anti-fungal characteristics of the millet could be attributed to its polyphenols content. The seed coat amounting to 12–15 % of the millet kernel is a rich source of polyphenols and micronutrients. Since, the whole grain millet is edible, it is imperative that, the millet polyphenols also form part of the millet foods. Moreover there are no reports of any kinds of adversaries on regular consumption of millet meal. Even though, the millet has been a traditional staple food for a large segment of population, the millet polyphenols have received very little attention from the scientific community.

Polyphenols are important phytochemicals in food materials including cereals. Now a days cereal polyphenols are gaining prominence worldwide because of their nutraceutical characteristics. Although, a large number of phytochemicals have been identified as constituents of food materials, many more are being discovered untiringly. They are involved in many biochemical processes including the ones that are reported to help hinder cell damage as well as cancer cell replication, decrease cholesterol levels and act as free radical scavengers in humans. They are associated with reducing the progress of at least four of the leading causes of human death, namely, cancer, diabetes, cardiovascular disease and hypertension. Finger millet being low cost minor cereal, scientific information on its nutraceutical abilities would augment its consumption by the non-traditional consumers also and that may improve its economic value. Moreover, the generation of scientific information on the biochemical aspects of the millet polyphenols could be useful in identifying parmaconutritional uses for the millet.

Now-a-days the millet seed coat is readily available as a by-product due to the advent of the millet processing technologies, and exploiting its utilities as a source of food additive or as a pharmaconutrient would be highly remunerative. Hence, investigations on isolation, characterization as well as the biochemical and nutraceutical qualities of the millet polyphenols were under taken with the following objectives;

1. Concentration of the polyphenols rich fraction of the kernels and isolation as well as characterization of the constituent phenolics,
2. Evaluation of the biochemical characteristics of the millet polyphenols with special reference to the inhibitory activity on some of the enzymes of plant and animal origin, and
3. Nutraceutical properties of polyphenols with respect to antioxidant and antimicrobial activities.

The research work conducted with these objectives will be presented in the form of a thesis. The thesis will comprise of four chapters, beginning with the review of literature on the nutritional, biochemical and technological qualities of the millet (Chapter 1). Experimental aspects and the results pertaining to distribution of the polyphenols in the millet kernel and their isolation and characterization will be presented in Chapter II. The role of the millet polyphenols on the inhibition of the amylases developed during germination of the millet, and also on aldose reductase from the cataracted human eye lenses as well as on snake venom neutralization will be reported in Chapter III. The nutraceutical potential of the millet polyphenols with special reference to their antioxidant as well as antimicrobial potential will be discussed in Chapter IV. Finally, the literature cited in the thesis will be compiled under reference section.

A brief accounts of the experimental work and the results obtained are as follows;

CHAPTER I

INTRODUCTION

This chapter consists of review of the scientific and technological information on cereals in general and finger millet in particular, published in peer reviewed scientific journals, proceedings of the scientific conferences, book chapters and popular articles. The production of finger millet, its grain morphology, processing, products, nutritional composition, food uses and the nutraceutical properties with emphasis on polyphenols are covered in detail. The information available on cereal polyphenols, their isolation, characterization and their role on the health beneficial aspects of the millet are also briefly described in this chapter.

CHAPTER II

ISOLATION AND CHARACTERIZATION OF THE MILLET POLYPHENOLS

The investigations on the extractability of the millet polyphenols and purification of the extracted polyphenols and fractionation in to the constituent phenolics and their characterization were carried out. To concentrate the polyphenols rich fraction of the millet, histochemical examination and analysis of the millet milling fractions were carried out. The histochemical examination of the millet kernels and also analysis of the seed coat as well as the endosperm fractions of the millet for polyphenols content revealed that, nearly 70 % of the millet polyphenols are concentrated in the seed coat tissue, which amounts to 12–15 % of the seed mass. In view of that, the seed coat rich fraction of the millet was prepared following a special milling protocol developed. The polyphenols of the seed coat fraction were extracted

with different polar and non-polar solvents to identify a suitable extractant and it was observed that, 1 % HCl–methanol (v/v) was very effective for extraction of the millet polyphenols and the extract contained about 12 % assayable polyphenols of the millet. Hence, the polyphenols extracted with acidic methanol were studied for various properties such as, constituent phenolic acid contents, their stability at different temperatures and different pH. The results indicated that, the polyphenols content of the extract were stable to the changes in the temperature up to 95 ºC tested but were sensitive to the changes in pH. The extract being highly acidic, its pH was less than 1. At highly acidic conditions, the polyphenols extracted completely remained in soluble form, as the extract was clear solution. But neutralization of the extract caused precipitation of some of the extracted matter. It was noticed that, as the pH increased the quantum of precipitate formed increased proportionately. The quantity of the precipitate increased from 4 ± 0.5 % to 40 ± 3 % of the extracted matter, as the pH of the extract increased from 1 to 10. However, the polyphenols content of the extract decreased, being marginal up to pH 7 and significant at alkaline pH. Fractionation of the extracted polyphenols by high performance liquid chromatography (HPLC) showed a large number of constituent phenolics but only a few of the constituent phenolics, such as the derivatives of benzoic acid (gallic, proto-catechuic, and *p*-hydroxy benzoic acids) and cinnamic acid (*p*-coumaric, syringic, ferulic and *trans*-cinnamic acids) were identified. It was note worthy that a flavonoid (quercetin) was also detected. However, in highly alkaline condition (pH 10) of the extract, only gallic acid and proto-catechuic acid were detected. These observations indicated that, the millet polyphenols are of highly complex nature and are stable only at strong acidic conditions and loose their stability at alkaline conditions. The relative proportion of these phenolic compounds was ferulic (33 %), *p*-hydroxy benzoic

(18 %), protocatechuic (15 %), gallic (13 %), *p*-coumaric (4 %), syringic (4 %), vanillic (4 %), *trans*-cinnamic (3 %) acids and quercetin (6 %). The identity of the component phenolic compounds was confirmed by LC-MS and also by NMR spectra of a few phenolics. Extensive investigations may be needed to identify the large number of the phenolics detected but not yet identified in acidic methanol extract of the millet.

CHAPTER III

ENZYME INHIBITORY ACTIVITY OF FINGER MILLET POLYPHENOLS

Polyphenols are known to inhibit the enzyme activity. This characteristic features of the polyphenols bears direct relevance with the digestive enzymes of the human gastrointestinal tract and also the food values of the millet products. Finger millet is extensively processed for preparation of malt, mainly for food uses. The millet malt is a rich source of amylases and the enzyme play a major role with respect to the functional properties of malt based foods. The millet malt contains considerable proportion of polyphenols also. Hence, the effect of millet polyphenols on the nature of inhibition on its amylases in terms of starch hydrolysis catalysed by amylases developed during germination was investigated. The native millet contained about 2.5 % polyphenols and polyphenols content decreased as germination progressed. On the other hand the amylase activity of the millet increased rapidly on germination up to 96 h and subsequently decreased. Normally, to prepare specialty foods from the millet malt, the whole meal malt is suspended in water and the slurry is heated to boiling. During heating the malt enzymes hydrolyse the starch even though the polyphenols derived from the seed coat as well as the endosperm also are present in the reaction mixture. In view of this, the amylases extracted from 96 h germinated

millet and the polyphenols extracted from the seed coat matter of the millet were used for the kinetic studies using Michaelis-Menten and Lineweaver-Burk equations. It was observed that the K_m remained constant (0.625 %) but the maximum velocity (V_{max}) decreased in the presence of a crude polyphenols extract, indicating mixed non-competitive type of inhibition. In contrast to that, some of the constituent phenolics, namely, gallic acid, vanillic acid, quercetin and *trans*-cinnamic acid isolated from the polyphenols extract of the millet seed coat matter showed uncompetitive inhibition. Gallic acid and quercetin exhibited highest affinity for enzyme-substrate complex with K_i' of 4.6×10^{-7} M and *trans*-cinnamic acid showed lower affinity for the enzyme-substrate complex with K_i' of 7.3×10^{-7} M. Kinetic studies on the inhibition of the amylases by phenolic compounds indicated the presence of secondary binding sites in malted finger millet amylases similar to other cereal amylases. These observations help to explain the ability of the millet malt to hydrolyse the starch and its usage as a source of amylase or a amylase rich food (ARF), in the formulation of amylase rich weaning foods.

Diabetes is the major risk factor for cataract, which is one of the main causes of blindness worldwide. Diabetes induced cataract is characterized by accumulation of polyol which is mediated by the up regulation of a key enzyme aldose reductase (AR). The crude polyphenols of the millet and also some of the individual phenolics, namely gallic, protocatechuic, *p*-hydroxy benzoic, *p*-coumaric, vanillic, syringic, ferulic, *trans*-cinnamic acids and quercetin were evaluated for their AR inhibitory activity of the cataracted human eye lenses. Out of the phenolics tested, quercetin was more potent inhibitor with an IC_{50} of 14.8 ηM. Structure function analysis of some of the phenolics isolated from the millet, revealed that, phenolics with hydroxyl group at 4^{th} position was important for aldose reductase inhibitory property. Also the presence

of neighboring *O*-methyl group in phenolics denatures the AR and inhibits its activity. The millet polyphenols inhibited the activity of AR reversibly by non-competitive inhibition. Results thus provide a stronger evidence for the potential of the millet polyphenols in preventing cataractogenesis in human eye lenses.

The enzyme inhibitory activity of the millet was also tested for phospholipases A_2 (PLA_2) from the snake venom. The snake venom is a complex mixture of enzymes and toxins having distinct biological activities. The venom of *Naja naja* (forest cobra) possesses the highest phospholipases activity. Exploratory investigations on the effect of the millet polyphenols extract on snake venom PLA_2 indicated inhibitory activity. Further it was observed that the inhibitory activity of crude polyphenol extract, gallic acid and quercetin on snake venom PLA_2 was significant with IC_{50} of 83, 62 and 17 µg/ml respectively.

CHAPTER IV

NUTRACEUTICAL PROPERTIES

The polyphenols are also known to possess antioxidant and antimicrobial activity and accordingly, these properties of the millet polyphenols were assayed. The IC_{50} value for the antioxidant activities of millet phenolics extracted in HCl-methanol on 1, 1-diphenyl-2-picrylhydrazyl (DPPH), hydroxyl and ABTS (2, 2′- azinobis (3 – ethylbenzothiazoline – 6 – sulfonate)] radical scavenging assay were 62.7, 109 and 114 µg/ml respectively. Among the various solvents used for extraction of polyphenols, the 1% HCl-methanolic extract showed the highest *in vitro* antioxidant activity, which increased with increasing the concentration of the phenolics in it. Hence, these extracts could be considered as natural antioxidants and may be useful for mitigating some of the diseases arising from oxidative deterioration in humans.

The antimicrobial activity of some of the millet phenolics isolated from the extract were screened and among the phenolics tested, only gallic, caffeic, ferulic, proto-catechuic and *p*-hydroxy benzoic acids exhibited inhibitory activity against *K. pneumoniae, Y. enterocolitica, S. pyogenes, P. mirabilis and S. marcescens.* On the other hand quercetin inhibited the growth of all the pathogenic microbes even at a very low concentration.

At the end, the relevant references cited in the thesis are compiled in the alphabetical order as BIBLIOGRAPHY.

SUMMARY AND CONCLUSIONS

The work reported in the thesis provides considerable information on the biochemical and nutraceutical aspects of finger millet polyphenols. The information will be useful to food scientists, nutrionists and also medical personnel besides adding to the biochemistry of finger millet. The salient features of the out come of the thesis work are;

- The millet polyphenols are concentrated in the seed coat tissue and present sparingly in the endosperm cell walls of the kernel. A suitable protocol for preparation of the millet seed coat matter that contains about 12 % polyphenols was developed.
- HCl-methanol (1:99 v/v) solvent system was identified as a suitable and effective solvent for the extraction of the millet polyphenols.
- The characterization of the isolated polyphenols indicated the presence of a large number of constituent phenolics but a few derivatives of benzoic acid (namely, gallic, proto-catechuic and *p*-hydroxy benzoic acids) and cinnamic acid (namely, *p*-coumaric, syringic, ferulic and

trans-cinnamic acids) and quercetin, a derivative of flavonoid were identified.

- The millet polyphenols are pH sensitive, but stable to changes in temperature.
- Investigations on the inhibitory activity of the millet polyphenols on the millet malt amylases indicated that the inhibition was of mixed non-competitive type, whereas some of the pure phenolic compounds isolated from the crude extract exhibited uncompetitive type of inhibition. The crude polyphenols extract and also some of the purified phenolics of the millet showed non-competitive inhibition on aldose reductase.
- The millet polyphenols were found to neutralize the phospholipases and exhibit anti-inflammatory activity, antioxidant and antimicrobial properties.

The studies indicated that, finger millet seed coat is a rich source of polyphenols and the seed coat as such could be utilized for biofortification of various foods or the isolated polyphenols may find application as nutraceuticals.

S. Chethan
Candidate

N. G. Malleshi
Guide

CHAPTER I

Review of Literature

CHAPTER II

Isolation and characterization of the millet polyphenols

CHAPTER III

Mode of enzyme inhibition by the millet polyphenols

CHAPTER IV

Nutraceutical properties of Finger millet polyphenols

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Synopsis

Cereals constitute to over 60 % of the total world food commodities. The important cereals are rice (*Oryza sativa* L.), wheat (*Triticum aestivum* L.), maize (*Zea mays* L.), sorghum (*Sorghum bicolour* L.), barley (*Hordeum vulgare* L.), oats (*Avena sativa* L.), rye (*Secale cereal* L.), pearl millet (*Pennisetum glaucum* L.), finger millet (*Eleusine coracana* L. Gaertn.) and the small millets namely, foxtail millet (*Setaria italica* L.*)*, proso millet (*Panicum milliaceum* L.*)*, little millet (*Panicum milliare* L.), kodo millet (*Paspalum scrobiculatum* L.) and barnyard millet (*Echinochola frumantacea*). Cereals contain 60 – 80 % carbohydrates and form the main source of energy and also protein to some extent to the human population worldwide and some of them also form the feed component in most of the developed countries. Free sugars, starch and non-starchy polysaccharides constitute the cereal carbohydrates. Free sugars and starch are available carbohydrates where as the non-starchy polysaccharides are unavailable carbohydrates (Kamath and Belavady 1980). Cereals contain 6 - 12 % protein, but the proteins are deficient in lysine, the important essential amino acid, but they compliment well with other lysine rich proteins of grain legumes, animal proteins and oil seed cake proteins to form the food with balanced amino acid profile (Prakash and Narasinga Rao 1988; Katiyar and Bhatia 1991). Cereals are poor sources of lipids (1.0 – 5.0 %) but are good source of minerals including trace elements and B-group vitamins, besides several phytochemicals with nutraceutical qualities.

Millets

The millets are minor cereals and comprise the species in several genera, mostly in the subfamily Panicoideae, 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 short duration, ability to survive under conditions of moisture stress and low soil fertility. In view of these, millets are called as the crops of food security. The advances in agricultural practices during the past few years on these crops have made them competitive with other cereals under the rain fed agriculture.

Millets are important food crops of the arid and semi-arid tropics, namely, India, China, Japan, Ethiopia, Kenya, Tanzania, Zambia and a few other African countries surrounding the Sahara and also some Central and South American countries (**Fig. 1**). The annual global production of all the millets including pearl millet 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. The annual production of millets in India amounts to about 12 million metric tones (**Table 1**) and out of this pearl millet accounts to 60 %, finger millet 25 % and other small millets form 15 % (**Table 2**).

Nutrient composition

The nutrient composition of millets compares very well with other cereals. Some of them are even nutritionally superior to rice and wheat (**Table 3**). They are rich source of carbohydrates (70 – 80 %) and contain 6 – 12 % protein and 1 – 6 % fat, besides vitamins and minerals, specially B group vitamins like niacin, B_6 and also several minerals.

Finger millet

Among the minor millets, finger millet stands unique because of its superior nutritional qualities. It is a near spherical small seeded caryopsis that is widely used in India as a food crop. It is an annual robust grass that grows to a height of 40 – 100 cm

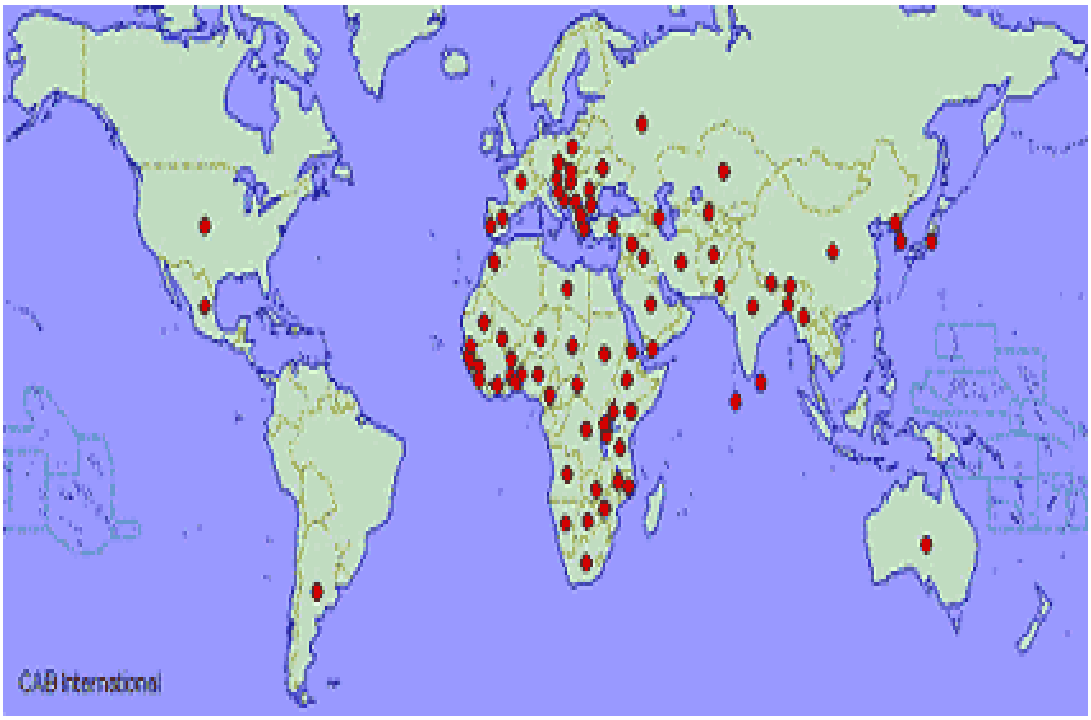


Fig. 1. Millets producing countries (producing at least 1 Metric ton) in the world.

Source: Food and Agriculture Organisation of the United Nations; http://faostat.fao.org

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

Country	Area (HA)	Yield (Hg/Ha)	Production (MT)
India	11,000,000	8,182	9,000,000
Nigeria	NA	NA	7,964,000
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 the United Nations, http://faostat.fao.org

Table 2. Nomenclature of millets largely grown in India

Botanical term	Common name (English)	Popular colloquial (Indian) names
Eleusine coracana	Finger millet	Ragi, nachni, nagli
Pennisetum glaucum	Pearl millet	Bajra, cambu, sajje
Setaria italic	Foxtail millet	Navana, tennai, kangani
Panicum milliaceum	Proso millet	Panivaragu, baragu, meneri
Panicum milliare	Little millet	Samai, mutaki, gindi
Paspalum scrobicultum	Kodo millet	Varagu, harak, kodra
Echinochola frumentacea	Barnyard millet	Banti, sanwa, shama

Source: Hulse et al. (1980)

Table 3. Nutrient composition of major cereals and millets (per 100 g edible portion; 12 % moisture basis)

Source	Protein (g)	Fat (g)	Ash (g)	Crude fibre (g)	Carbohydrates (g)	Ca (mg)	Fe (mg)	Thiamine (mg)	Riboflavin (mg)	Niacin (mg)
Rice (brown)	7.9	2.7	1.3	1.0	76.0	33	1.8	0.41	0.04	4.3
Wheat	11.6	2.0	1.6	2.0	71.0	30	3.5	0.41	0.1	5.1
Maize	9.2	4.6	1.2	2.8	73.0	26	2.7	0.38	0.2	3.6
Sorghum	10.4	3.1	1.6	2.0	70.7	25	5.4	0.38	0.15	4.3
Pearl millet	11.8	4.8	2.2	2.3	67.0	42	11.0	0.38	0.21	2.8
Finger millet	7.7	1.5	2.6	3.6	72.6	350	3.9	0.42	0.19	1.1
Foxtail millet	11.2	4.0	3.3	6.7	63.2	31	2.8	0.59	0.11	3.2
Little millet	9.7	5.2	5.4	7.6	60.9	17	9.3	0.3	0.09	3.2
Barnyard millet	11.0	3.9	4.5	13.6	55.0	22	18.6	0.33	0.10	4.2
Kodo millet	9.8	3.6	3.3	5.2	66.6	35	1.7	0.15	0.09	2.0

Source: Gopalan et al. (2000)

(**Fig. 2**). It's mainly grown in semi-arid tropics and sub-tropics of the world under rain fed conditions. It belongs to the genus *Eleusine* in the tribe Eragrostideae. *Eleusine coracana* species are the most cultivated species whereas *Eleusine africana* and *Eleusine indica* species have remained as wild grass. It is believed that this millet originated in the Ugandian region of Africa and was transported to India in the pre-Aryan period (1500 BC). In Sanskrit literature, it is referred to as *Nrutya-Kondaka* which means 'dancing grain'. Even today, in Uganda, numerous tribal rituals and religious ceremonies are associated with finger millet (Rachie and Peters 1977).

Finger millet is produced in several parts of the country. Karnataka state tops in its production. Tamilnadu, Andra Pradesh, Maharastra, Orissa, Uttar Pradesh and Uttarkhand also produce substantial quantity of this millet. The millet is known by different names in different regions of India and also in other countries (**Table 4**). It is adaptable to sea level as well as in high altitude (in the Himalayas) up to 3200 meters. The origin of finger millet in the country dates back to 1800 B.C. (Vishnu Mittre 1974). With the advent of agricultural technology, high yielding finger millet varieties, including crosses between Indian and African species (designated as Indaf), which have a production potential as high as 3 tonnes per hectare have been developed.

The name, "finger" millet is derived from the look of its earhead which consists of spikes, radiating mostly in a curving manner from a central point, resembling fingers of the human hand. The Indian varieties of the millet normally have long spikes whereas the African cultivars have shorter curving spikes.

a

b

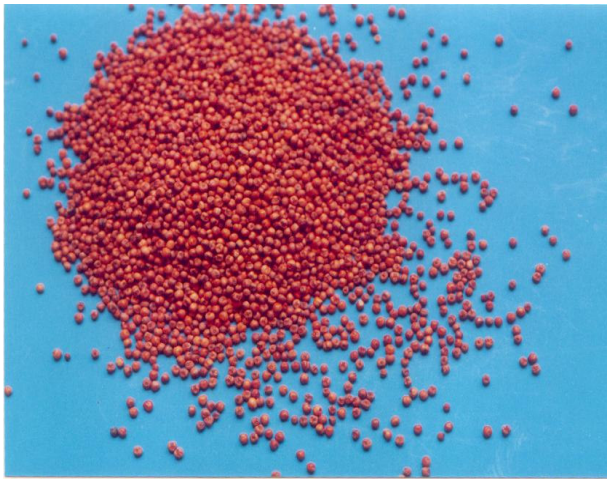


Fig. 2. a. Earhead of finger millet, b. finger millet grains

Classification of finger millet

Kingdom	Plantae – Plants
Subkingdom	Tracheobionta - Vascular plants
Superdivision	Spermatophyta - Seed plants
Division	Magnoliophyta - Flowering plants
Class	Liliopsida – Monocotyledons
Subclass	Commelinidae
Order	Cyperales
Family	Poaceae - Grass family
Genus	*Eleusine* Gaertn.- Goosegrass
Species	*Eleusine coracana* (L.) Gaertn.- Finger millet

Source : USD/ARS NPGS'S taxonomic database; http://plants.usda.gov

Table 4. Common names for finger millet

Country/Language	Common name
Arabic	Tailabon
English	Finger millet, African millet, Ragi, Koracan
Ethiopia	Dagussa (Amharic/Sodo), Tokuso (amharic), Barankiya (Oromo)
French	Eleusine cultivee, Coracan, Koracan
German	Fingerhirse
India	Ragi (Kannada, Telugu), Kezhvaragu (Tamil), Maduva (in some parts of north INDIA), Nachani (Marathi)
Kenya	Wimbi (kiswahili), Ugimbi (Kikuyu)
Nepal	Koddo
Sri Lanka	Kurakkan
Swahili	Wimbi, Ulezi
Tanzania	Mwimbi, Mbege
Uganda	Bulo
Zambia	Kambale, Lupoko, Mawele, Majolothi, Amale, Bule
Zimbabwe	Rapoko, Zviyo, Njera, Rukweza, Mazhovole, Uphoko, Poho

Source: Germplasm Resources Information Network (GRIN); http://www.ars-grin.gov

Morphological features of finger millet kernel

Finger millet grains are smaller in size with 1.2 – 1.8 mm diameter and 1000 kernel weight ranges from 2.0 to 3.5 g. The color of the seed coat of the millet varies from dark red to purple but brick red is the most common color. A few varieties of finger millet with a white seed coat have also been released but they have not become productive and popular mostly because of their poor keeping qualities and bland taste (Mahudeshwaran et al. 1966; Mallanna and Rajasekhara 1969). The seed coat, embryo and endosperm form main structural or botanical tissues of the millet kernel **(Fig. 3)**. The endosperm and the seed coat accounting for about 85 % and 13 % of the seed mass, respectively whereas the embryo forms only 1 - 2 % of it. The kernel consists of a single aleurone layer that completely encircles the endosperm. The aleurone cells are rectangular with thick cell walls and contain considerable proportion of the seed protein, oil, minerals and enzymes. The peripheral as well as the corneous and floury endosperm areas are embedded in the protein matrix and the protein bodies are distributed throughout the matrix. The starch granules are spherical in the floury area and become progressively polygonal in the corneous and peripheral endosperm. The entire seed is edible.

Nutrient composition of finger millet

Finger millet is a good source of dietary carbohydrates. Free sugars (1 – 2 %), starch (75 – 80 %) and non-starch polysaccharides (NSP; 15 – 20 %) form the main constituents of finger millet carbohydrates (Malleshi 2005). Amylose to amylopectin ratio in the millet is normally 20:80 and there are no reports of very low amylose (<15 %) or very high amylose (>30 %) millet cultivars. The slow digestibility of the millet-based diets normally could be attributed to the intrinsic hypoglycemic characteristics of its starch, as well as to the high proportion of NSP contents.

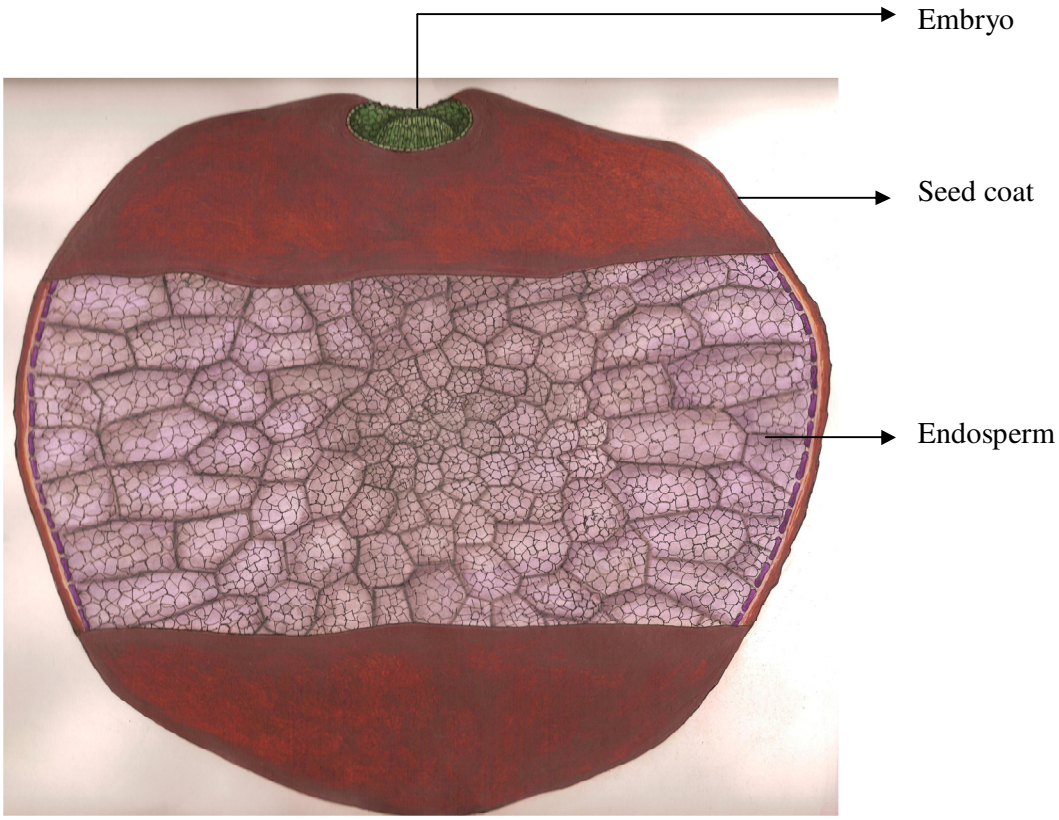


Fig. 3. Morphology of finger millet kernel

The millet contains about 6 - 8 % protein. Varietal as well as the agronomical conditions of its cultivation influence the protein and other nutrient contents (Hulse et al. 1980). The sulfur-based amino acids (0.35 mg/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. Similar to other cereal proteins lysine, one of the essential amino acids, forms the limiting amino acid 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 – 28 % 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 predominant fatty acids. Major components of the free lipids are triglycerides (Mahadevappa and Raina 1978). Finger millet is exceptionally rich in calcium (300 – 400 mg %) besides, it is also a good source of many other micronutrients such as iron, magnesium, zinc, chromium, iodine and thiamine.

The millet is also a very good source of phytochemicals such as dietary fibre, polyphenols, pigments and phytate (Hulse et al. 1980). The whole grain millet is edible and the traditional foods are generally prepared from the whole meal. This indicates that, the millet phytochemicals including polyphenols are edible and do not cause any adversities on the human health. On the other hand, some of the known health benefits associated with the millet, such as its hypoglycemic (Lakshmi Kumari and Sumathi 2002), anti-glycating (Hegde et al. 2002), hypocholestrolemic (Kurup and Krishanmurthy (1993) and also anti-ulcerative properties (Tovey 1994) besides the excellent storage quality of the millet (Iyengar et al. 1945) could be attributed to a large extent to its polyphenols content. In recent years, the millet polyphenols have

received a considerable interest in view of their antioxidant and other nutraceutical properties.

Processing of finger millet

Milling, malting, popping and fermentation are the primary processing technologies applied to the millet extensively, although the application of contemporary food processing technologies are being explored for the preparation of specialty health foods and value added products from finger millet.

Flour milling

The most commonly practiced primary processing for the millet is pulverization or milling for preparation of whole meal or flour. Although, the whole meal of the millet is used for food purpose, the glumes or the thin pericarp which loosely envelopes the kernels of the high yielding varieties is separated by abrasion in huller or ragi polishers, since it is a non-edible tissue. The debranning method followed for most of the cereals is not at all effective in the case of finger millet owing to its intactness of bran with soft and highly friable endosperm. Hence, to prepare refined flour, the grains are moistened with about 5 % additional water, pulverized in any of the cereal pulverizers and sifted through about 50 mesh sieves to separate the coarse seed coat as overtails (Kurien and Desikachar 1962; 1966; Malleshi and Desikachar 1981b). The flour with very little admixture of pulverized bran (refined flour) happens to be fairly white in color and may find usage in bakery and composite flour mix useful for various food and allied products (Crabtree and Dendy 1976).

Decortication

Recently, a process for decortication of finger millet has been developed. The process involves parboiling the millet (to harden the endosperm that enables it to withstand the impact during milling) and decortications in emery disc mill (Malleshi 2006). The decorticated millet is unique among the finger millet products, as it could be cooked in to discreet grains similar to rice (Shobana and Malleshi 2007). The seed coat of the millet forms a major by-product of the decortication and is a rich source of calcium and other phytochemicals and may serve as a source of calcium for biofortification of foods.

Malting

Malting of finger millet is one of the traditional processes largely practiced for preparation of local beer in Africa and also at the Himalayan belt. But in India, the millet malt is mainly used to prepare specialty foods. Malting is nothing but *in vivo* biotransformation process, which converts the seed into a storehouse of hydrolytic enzymes. During this process, the bioavailabilty of proteins, carbohydrates, vitamins and minerals are enhanced and the concentrations of anti-nutritional factors are considerably reduced (Malleshi and Klopfenstein 1998). The malted millet is nutritionally superior to that of native millet and is a good source of amylases. When the malt flour mixed with water or milk is heated to boiling, the amylases hydrolyze the starch to simple sugars there by reduce its water holding capacity and as a result form low bulk and nutrient-dense foods. This natural process of enhancing the nutritional density has been used for developing various specialty and health foods such as infant food, weaning food, enteral food and milk based beverages and also in confectionary (Malleshi 2002). In view of this, the millet malt could be a new ingredient for the food industry.

Popping

Popping of finger millet is one of the important popular traditional technologies followed to prepare ready-to-eat products (Malleshi and Desikachar 1981a). Popping involves high temperature and short time (HTST) treatment to the millet using sand or salt or air as heat transfer media. Popped millet can be prepared at household, community or industrial levels. Popped millet is a crisp product with highly desirable aroma and would be suitable for preparation of nutritious food supplements for the Integrated Child Development Schemes (ICDS) or such other nutrition intervention programs of Government or supported by Non-Governmental Organization (Prasannappa and Jagannath 1985). "*Sattu*" is a popular popped product of north India, whereas, *hurihittu* is a very popular popped food of finger millet in south India. Diversification of millet in the form of popped food offers an advantage because of its ease of preparation, desirable sensory qualities and better shelf-life. Recently, a process has been developed to prepare expanded finger millet which resembles expanded rice (Ushakumari et al. 2007). This product finds utilization as snacks and also as an ingredient of confectionary products such as chocolate and also in health bars.

Fermentation

Indigenous fermented foods prepared from major cereals are common in many parts of Africa and mainly used as beverages. Fermented millet is also used for preparation of breakfast cereals or snack foods and also weaning foods. Traditionally fermentation of finger millet is an auto-natural process causing a significant reduction of phytate, polyphenols, tannins and increases the starch and protein digestibility (Usha and Chandra 1998). Normally, in Africa, lactic fermented 'bantu-beer' is

produced but in India, thin porridge or '*Ambali*' a mild fermented gruel is commonly prepared from finger millet.

Contemporary food processing technologies

A variety of ready-to-eat traditional snacks, breakfast cereals, supplementary foods and also pet foods are prepared by extrusion cooking technology (Malleshi et al. 1996). The millet grits equilibrated to about 18 % moisture content on extrusion cooking form well expanded ready-to-eat food products with porous and crunchy texture. Extruded products are crisp and crunchy similar to deep-fried foods, without actually deep-frying in oils or fat. Roller drying of millet finds applications as a base for soup (Manisha and Malleshi 2006). Extrusion cooking and roller drying of finger millet blended with other food ingredients holds promise for preparation of snacks and supplementary foods, besides feed formulations.

Storage of millet

Finger millet is known for its excellent storage property. The small and flinty grains are highly resistant to both pests and fungi. There are reports of finger millet being stored for over 25 years in traditional underground pits (*hageu*) in India (Iyengar et al. 1945). The good storage properties of the millet could mainly be attributed to the antifungal activities of its seed coat phytochemicals as well as to its distinctive morphology. Probably the compactness of the stored millet does not provide sufficient spaces between each grain, which is necessary for the proliferation of insect pests and fungi. In view of this, no pesticide or such other treatments need to be applied to finger millet during storage.

Phytochemicals

The millet is known for its richness in many of the edible phytochemicals, such as dietary fibre, polyphenols, pigments and phytate. The contents of these in the

millet is about 15, 2 and 1 % of the seed matter, respectively. The dietary fibre (DF) of the millet comprises of both soluble and insoluble fibre. Cellulose forms the major portion of insoluble dietary fibre whereas hemicelluloses, pectin etc are important soluble fibre (Malleshi et al. 1986). The millet seed coat being colored, contains considerable levels of anthocyanins and such other pigments. The phytate content in the millet varies from 0.5 – 2.0 %, but most of it is concentrated in the seed coat matter (Ravindran 1991). Among the millet phytochemicals, polyphenols are most important in view of their nutraceutical characteristics. Hence, the matter of polyphenols is dealt in detail in the subsequent pages.

Polyphenols

Polyphenols are secondary metabolic of plant and are termed as phytochemicals that normally provide protection to plants against pathogens and predators by acting as phytoalexins or by increasing food astringency, thus making the food rather unpalatable. They also protect the millet crop from plague and pre-harvest seed germination. The food application of polyphenols has not been completely explored but as on date, its usage is limited as natural colorants and to some extent as preservatives. Polyphenols were termed as anti-nutritional factors earlier, but now a days they are considered as nutraceuticals. Some of the phenolic compound as well as flavonoids (Kuhnau 1976) are utilized as antibiotics, anti-diarrheal, anti-ulcer (Saito et al. 1998) and anti-inflammatory agents, and also in the treatment of hypertension, vascular fragility, allergies, hypercholesterolemia and similar disorders (Chung et al. 1998).

Chemistry of polyphenols

Polyphenols arise biogenetically from two main synthetic pathways namely, the shikimate pathway and the acetate pathway (Harborne 1989).

Natural polyphenols range from simple molecules, such as phenolic acids to highly polymerized compounds such as tannins. They occur primarily in conjugated form, with one or more sugar residues such as monosaccharides, disaccharides or even as oligosaccharides linked to hydroxyl groups, although direct linkages of the sugar unit to an aromatic carbon atom exist. Glucose is the most common sugar residue, but galactose, rhamnose, xylose, and arabinose as well as glucuronic and galacturonic acids are the other residues. Associations with other compounds such as carboxylic and organic acids, amines, and lipids and linkages with other phenols are also common.

Simple phenols and flavonoids

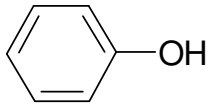
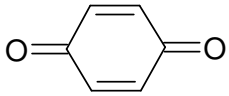
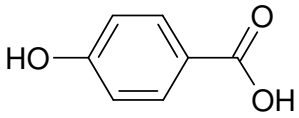
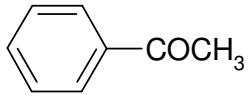
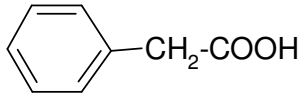
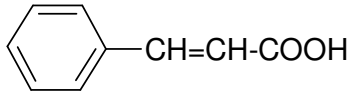
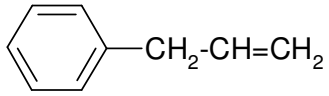
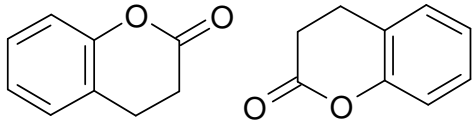
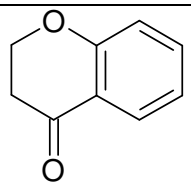
Simple phenolic derivatives and flavonoids form the most common and important low-molecular weight polyphenols. Simple phenols (C_6), such as phenol itself, cresol, thymol, resorcinol, orcinol, etc., including hydroquinone and their derivatives (e.g., arbutine, sesamol) and phloroglucinol are wide spread among different plant species. Phenolics with a C_6-C_1 structure, such as phenolic acids (e.g., gallic, vanillic, syringic, *p*-hydroxybenzoic) and aldehydes (e.g., vanillin, syringaldehyde, *p*-hydroxybenzaldehyde), are fairly common in higher plants and ferns. All of these compounds are generally phenylpropanoid derivatives (C_6-C_3) and form an important group of phenolics of lower molecular weight compounds. Chromones are less known than coumarins, with the latter occurring naturally as glycosides (e.g., umbilliferone, aesculetin, scopoletin). The most important phenylpropanoids are the hydroxycinnamic acid derivatives (*p*-coumaric, caffeic, ferulic, sinapic acids). Cinnamyl alcohols (coniefryl alcohol or guaiacyl, sinapyl alcohol or syringyl and *p*-coumaryl alcohol or *p*-hydroxyphenyl) form the basic constituent of lignins and thus represent one of the major groups of plant phenolics.

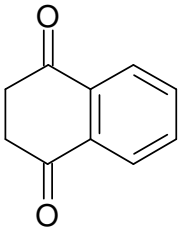
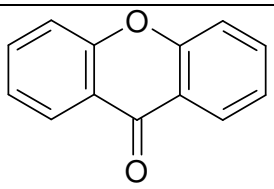
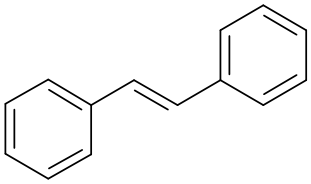
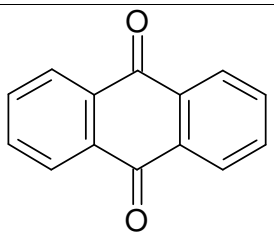
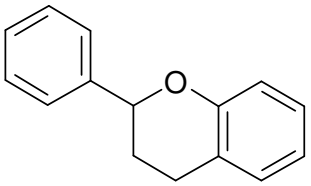
Phenylpropanoids and more simple phenols (benzoic acid and benzaldehyde derivatives) are usually covalently linked to cell wall polysaccharides (predominantly ester-linked to arabinose units of hemicellulose) or to the so-called core lignin (Wallace et al. 1991).

Flavonoids represent the most common and widely distributed group of plant phenolics. Their common structure is that of diphenylpropanes (C_6-C_3-C_6) which consists of two aromatic rings linked through three carbons that usually form an oxygenated heterocycle. The basic structure and the system used for carbon numbering of the flavonoid nucleus are presented in **Table 5**. Biogenetically, the 'A' ring usually comes from a molecule of resorcinol or phloroglucinol synthesized in the acetate pathway, whereas ring 'B' is derived from the shikimate pathway (Harborne and Mabry 1982).

Flavonoids occasionally occur in plants as aglycones, although they are most commonly found as glycoside derivatives. Among the flavonoids, flavones (e.g., apigenin, luteolin, diosmetin), flavonols (e.g., quercetin, myrcetin, kaempferol) and their glycosides are the most common compounds. They are widespread in plant kingdom, with exception of algae and fungi. Flavonols occur as O-glycosides, but flavone O-glycosides and C-glycosides are very common, with the latter characterized for possessing a carbon-carbon linkage between the anomeric carbon of a sugar molecule and the C_6 or C_8 carbon of the flavone nucleus. Unlike O-glycosides, sugars in C-glycosides are not cleaved by acid hydrolysis. Flavanones (e.g., naringenin, hesperidin) also can occur as O- or C-glycosides and are especially abundant in citrus foods. The variability of this group of flavonoids is noteworthy, with about

Table 5. Main classes of polyphenolic compounds

Class	Basic skeleton	Basic structure
Simple phenols	C_6	
Benzoquinones	C_6	
Phenolic acids	C_6-C_1	
Acetophenones	C_6-C_2	
Phenylacetic acids	C_6-C_2	
Hydroxycinnamic acids	C_6-C_3	
Phenylpropenes	C_6-C_3	
Coumarins, isocoumarins	C_6-C_3	
Chromones	C_6-C_3	

Naftoquinones	C_6-C_4	
Xanthones	C_6-C_1-C_6	
Stilbenes	C_6-C_2-C_6	
Anthraquinones	C_6-C_2-C_6	
Flavonoids	C_6-C_3-C_6	
Lignans, Neolignans	$(C_6$-$C_3)_2$	
Lignins	$(C_6$-$C_3)_n$	

Source: Bravo (1998)

380 flavonol glycosides and 200 different quercetin and kaempferol glycosides described to date (Harborne 1993). Isoflavones especially, occur in legumes (e.g., genistein, diadzein) and their structural features are that, the ring 'B' of the flavone molecule is attached to the carbon 3 of the heterocycle.

Flavonoids (e.g., catechin, epicatechin, gallocatechin) are the monomeric constituents of the condensed tannins, although they are very common as free monomers. Anthocyanins are the most important group of water soluble plant pigments and are responsible for the color of flowers and fruits of higher plants. The term anthocyanin refers to the glycosides of anthocyanidin (e.g., pelargonidin, malvidin, cyaniding). In addition to glycosylation, common linkages with aromatic and aliphatic acids, as well as methyl ester derivatives, also occur. Anthocyanins and polymeric pigments formed from anthocyanins by condensation with other flavonoids are responsible for the color of red wine (Mazza 1995).

Simple phenols and flavonoids represent the vast majority of plant phenolics. Most of these compounds are of relatively low molecular weight and are soluble according to their polarity and chemical structure (degree of hydroxylation, glycosyaltion, acylation, etc). Some of them, however, can be linked to cell wall components (polysaccharides, lignin). Because of the nature of the ester linkages, these compounds can be solubilised in alkaline conditions or otherwise retained in the fiber matrix.

Tannins

Tannins are compounds of intermediate to higher molecular weight ranging from 5000 to 30000 Da (Wursch et al. 1984). The term "tannin" comes from the tanning capacity of these compounds in transforming animal hides into leather by forming stable tannin-protein complexes with skin collagen. Tannins with a molecular mass of up to 30,000 Da have been reported in leguminosae (Wursch et al. 1984). Tannins are highly hydroxylated molecules and can form insoluble complexes with carbohydrates and proteins. This function of plant tannins is responsible for the astringency of tannin-rich foods, mainly because of the precipitation of salivary proteins.

Plant tannins are normally subdivided into two major groups:

1. Hydrolysable, and
2. Condensed tannins

A third group of tannins, the phlorotannins, found only in marine brown algae and are not commonly found in plant materials.

Hydrolysable tannins

Hydrolysable tannins consist of gallic acid and its dimeric condensation product, hexahydroxydiphenic acid, esterified to a polyol, mainly glucose. These metabolites can oxidatively condense to other galloyl or hexahydroxydiphenic molecules and form high-molecular weight polymers (Porter 1989). As their names indicate, these tannins are easily hydrolysed by acid, alkali and even hot water and also by enzymatic action, and yield polyhydric alcohol and phenyl carboxylic acid. The hydrolysable tannins are further subdivided into gallotannins, which are derived from gallic acid, or ellagitannins, which are derived from hexahydroxydiphenic acid. These take their name

from the lactone ellagic acid. The best-known hydrolysable tannin is tannic acid which is a galotannin consisting of a pentagalloyl glucose molecule that can further esterify with another five gallic acid units.

Condensed tannins

Condensed tannins or proanthocyanidins are high molecular weight polymers. The monomeric unit is a flavan-3-ol (catechin, epicatechin, etc.,) with a flavan-3, 4-diol or leucoanthocyanidin molecule as its precursor. Oxidative condensation occurs between carbon C_4 of the heterocycle and carbons C_6 or C_8 of adjacent units (Porter 1989).

Much of the literature on the condensed tannin content of different plants refers only to oligomeric proanthocyanidins (dimers, trimers, tetramers), because of the difficulty in analyzing highly polymerized molecules. Proanthocyanidins however, can occur as polymers with degrees of polymerization of 50 or higher of flavan-3-ol. The most commonly described condensed tannins have molecular weights of approximately 5000 Da, although as previously mentioned, polymers with molecular weights greater than 30,000 Da have been reported (Wursch et al. 1984). Autooxidative or enzymatic polymerization of flavan-3-ol and flavan-3, 4-diol units has been suggested as the process leading to the formation of condensed tannins (Haslam 1966). Interflavanoid linkages are acid labile and yield anthocyanidins during acid hydrolysis in alcoholic solutions. This reaction is used for determination of proanthocyanidin molecules.

Oligomeric proanthocyanidins and low molecular weight hydrolysable tannins are soluble in different aqueous and organic solvents such as acetone, methanol and water. However, high molecular weight condensed and hydrolysable tannins are

insoluble in aqueous and organic solvents. In addition, they remain insoluble when they form complexes with proteins or cell wall polysaccharides.

Extraction and methods for assay

Finger millet polyphenols have not been investigated in detail and hence, in the absence of clear information about the nature of its phenolics, the methods normally followed for the assay of sorghum polyphenols is followed for the extraction and for assay of the millet polyphenols also.

Careful scrutiny of the literature on the extraction and method of assay followed for the millet polyphenols reveals that, the majority of the workers used 1% HCl in methanol as solvent for extraction of the millet polyphenols, but the duration as well as the mode of extraction differed considerably (**Table 6**). Ramachandra et al. (1977) treated the millet meal for 24 h with 1% HCl–methanol solvent system with occasional swirling at ambient conditions, but Rao and Deosthale (1988) used 1% HCl-methanol at ambient condition whereas on the other hand Sripriya et al. (1996) refluxed the millet with only methanol at 60 ºC for 2 h whereas Shankara (1991) used 1% HCl-methanol with refluxing for 30 min. Subba Rao and Muralikrishna (2001; 2002) used 70 % ethanol and 1 M sodium hydroxide containing sodium borohydrate under nitrogen atmosphere to extract the free and bound phenolics, respectively.

For assay of the polyphenols including tannins most of the reports indicate the use of Vanllin-HCl method of Burns (1971), Folin-Dennis method of Swain and Hillis (1959) and Folin-Ciocalteau method of Singleton et al. (1995). The Vanillin–HCl method involves the condensation of the aromatic aldehyde vanillin with monomeric

Table 6. Polyphenols and tannin contents of finger millet

	Reported as				Reference
	Total phenols	**Reference standard**	**Tannin**	**Reference standard**	
Indian brown					
(N = 13)	0.08 – 0.96%	Chlorogenic acid	0.12 – 1.05%	Catechin	Ramachandra et al. (1977)
(N = 01)	-	-	0.36%	Catechin	Rao and Prabhavati (1982)
(N = 85)	0.03 – 0.57% 0.06 – 0.67%	Tannic acid Chlorogenic	0.03 – 2.37% -	Catechin -	Shankara (1991)
(N = 1)	0.1%	Chlorogenic	-	-	Sripriya et al. (1996)
(N = 12)	-	-	0.35 – 2.39%	Catechin	Rao and Deosthale (1988)
(N = 1)	Free 0.77% Bound 0.57%	Gallic acid	-	-	Subba Rao and Muralikrishna (2002)
(N = 3)	0.55 – 0.59%	Chlorogenic	0.17 – 0.32%	Catechin	McDonough et al. (1986)
African brown					
(N = 10)	0.54 – 2.44%	Chlorogenic	0.46 – 3.47%	Vanillin – HCl	Ramachandra et al. (1977)
Indian white					
(N = 6)	0.06 – 0.09%	Chlorogenic	0.04 – 0.06%	Vanillin – HCl	Ramachandra et al. (1977)
(N = 1)	0.003%	Chlorogenic	-	-	Sripriya et al. (1996)

N indicates the number of varieties used for the studies

flavanols and their oligomers to form a red adduct that absorbs at 500 nm, but Folin-Ciocalteau is based on the oxidation of phenols with a mixture of phosphotungstic acid and phosphomolybdic acids in alkaline conditions leading the formation of blue colored compounds, the concentration of which is read at 760 nm. Ramachandra et al. (1977) used Folin-Dennis method to estimate the total phenols using chlorogenic acid as a reference standard but for assay of tannins they used Vanillin-HCl method using catechin as reference standard. But in contrast McDonough et al (1986) used Vanillin-HCl method for the assay of tannin and Folin-Ciocalteau method for total polyphenols.

Similar to many other cereals and grain legumes, varietal variations with respect to the polyphenol content of finger millet also have been reported (**Table 6**). Ramachandra et al. (1977), analyzed 32 varieties of the millet comprising of both brown and white seed coat material from Indian and African source and reported that the white grain varieties contained lower levels of polyphenols (0.04 – 0.09 %) than the brown grain varieties (0.08 – 3.47 %). The total polyphenols content of the Indian varieties ranged from 0.08 – 0.96 %, reported as chlorogenic acid equivalents and tannins in the range of 0.04 – 1.05 %, reported as catechin equivalents respectively. On the other hand, 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) where as McDonough et al. (1986) reported 0.55 - 0.59 % total polyphenols and 0.17 - 0.32 % tannins (catechin equivalent) in a small number of the millet varieties (n = 3). Subsequently, Rao and Deosthale (1988) showed that the tannin (catechin equivalents) contents in brown colored millet varieties (n = 12) but they did not detect tannins in white (n = 3) varieties. 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 polyphenols content 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 %).

This information on the polyphenols content 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.

The large variability in the polyphenols reported for the millet could be due to the different methods followed for the assay.

Distribution of polyphenols in the millet

A limited information on the distribution of polyphenols in the millet kernel indicates that, in the millet kernel, about 90 % of phenolics is concentrated in the seed coat and remaining is distributed in the endosperm cell walls. Fulcher et al. (1972), observed intense auto-fluorescence in the cell walls of the millet suggesting that they contain phenolic acids, probably ferulic acid. McDonough et al. (1986), also substantiated the observation of Fulcher (1982) with the aid of fluorescence microscopy by observing intense fluorescence in the testa and mild fluorescence in the cell walls of the endosperm. Shobana et al. (2006), have noticed by staining the phenolics with $FeCl_3$ identified the phenolics in the endosperm cell walls of the millet and also by chemical estimation of polyphenols in the milling fractions, namely the seed coat rich and endosperm rich fractions. According to Ramachandra et al. (1977),

dehulling of finger millet also reduced polyphenols by 80% from the high tannin finger millet varieties which indicates the concentration of the phenolics in the seed coat. Similarly, Malleshi (2006) reported that, decorticated finger millet contained 0.067% of polyphenols as against 0.24 % in the native millet.

Characterization of polyphenols

For the separation of the polyphenols, the HPLC method is normally used which include the use of a reversed-phase C18 column; UV-vis diode array detector (DAD), and a binary solvent system containing acidified water (solvent A) and a polar organic solvent (solvent B). The separation normally requires 1 h at a flow rate of 1.0 – 1.5 ml/min. The latest techniques for analysis of polyphenols includes

- LC-MS,
- LC-NMR and
- LC-coulometric detection

Cereal polyphenols

In cereals, the polyphenols are generally present in cell walls and are linked to hemicelluloses in different forms such as 2-O-(5'-O-(E)-feruoyl-β-D-xylopyranosyl)-(1-4) D-xylopyranose (Fincher and Stone 1986; Rybka et al. 1993). Phenolic acids and flavonoids are present in cereals in the free and conjugated forms. Phenolic acids are known to contribute to the antioxidative potential of cereal grains (Goupy et al. 1999; Adom et al. 2005), and may also be used for the production of the end-use of cereal products. Cereal grains with elevated levels of phenolic acid in caryopsis exhibit greater resistance to disease and insect (Arnason et al. 1992), but exhibit reduced extractability of endosperm (Pussayanawin and Wetzel 1987; Hatcher and Kruger 1997). The highest concentration of phenolic acids and flavonoids is normally present in the aluerone layer besides, in embryo and testa of the grains (Shirley 1998).

Moreover, cross-linking of arabinoxylans with phenolic acids lower the arabinoxylan solubility and swelling in water as well as reduces their microbial degradation in the human colon (Hatfield et al. 1993) and also exerts antioxidant as well as membrane modulating effects (Eldin et al. 2001). Significant amounts of alk(en)ylresorcinols containing non-isoprenoid side chain (15-25 carbons in length) attached to the hydroxybenzene ring have also been detected in barley, rye and wheat (Mattila et al. 2005). The nature of prominent polyphenols present in important cereals and millets are summarized in **Table 7**.

Very little information is available on the characteristics of finger millet polyphenols (**Table 8**). Hilu et al. (1978), characterized the flavonoids by HPLC and identified orientin, isoorientin, vitexin, isovitexin, saponarin, violanthin, lucenin-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 acid (14 µg/g), *p*-hydroxy benzoic (09 µg/g), syringic (07 µg/g) and sinapic acid (04 µg/g) as component phenolics in finger millet. Sripriya et al. (1996), hypothetically assumed that catechin is the major phenolics in the millet whereas, Subba Rao and Muralikrishna (2002), identified gallic, vanillic, coumaric and ferulic acids as free phenolics, and ferulic, caffeic and coumaric acids as bound phenolic acids in the millet.

Table 7. Nature of phenolics present in different cereals

Cereals	Phenolics reported	Reference
Wheat	Ferulic, vanillic, gentisic, caffeic, salicylic, syringic, *p*-coumaric acid & sinapic acid as well as vanillin and syringaldehyde. Campestanyl, sitostanyl ferulates, Ferulic acid dehydrodimers (DiFA), n-alkylphenols coupled to a resorcinol ring at the 5 position, tricin, 6-C-pentosyl-8-C-hexosylapigenin & 6-C-hexosyl-8-C-pentosylapigenin	Anderson and Perkin (1931); Sosulski et al. (1982); Feng et al. (1988); Herrmann (1989); Seitz (1989); Rybka et al. (1993); Faurot et al. (1995); Gracia- Consea et al. (1997); McKheen et al. (1999); Hakala et al. (2002); Adom et al. (2005); Pathirana et al. (2005; 2006)
Corn	Feruloylputerescine, *p*-coumarylputrescine, di-*p*-coumarylputrescine,diferuloylputrescine, *p*-coumarylspermidine, diferuloylspermidine diferuloylspermine, and steryl cinnamic acid derivatives	Sosulski et al. (1982); Sen et al. (1994); Norton (1995); Seitz (1990); Grabber et al. (2000)
Sorghum	Hydroxybenzoic acids, hydroxycinnamic acids, flavonoids (flavanols, flavones, flavanones, isoflavones and anthocyanins), chalcones, aurones (hispidol), hydroxycoumarins, lignans, hydroxystilbenes and polyflavans (proanthocyanidins and prodeoxyanthocyanidins).	Chung et al. (1998); Waniska et al. (2000); Krueger et al. (2003); Awika et al. (2004); Dykes and Rooney (2006)
Barley	Ferulic acid, *p*- hydroxy benzoic acid tyrosine, tyramine and its derivatives, anthocyanins, proanthocyanidins, lignans and substances related to lignin.	Briggs (1978); Nordkvist et al. (1984); Hernanz et al. (2001); Yu et al. (2001)
Oats	Quinones, flavones, flavonols, chalcones, flavanones, anthocyanins & amino phenolics. phenolics acids linked to sugars, polysaccharides, lignins, amines, long chain alcohols, glycerols, as well as long chain ω-hydroxy fatty acids. Avenanthramides (conjugates of cinnamic acid with anthranilicacids) and N-acylanthranilate alkaloids.	Durkee et al. (1977); Collins and Mullins (1988); Collins (1989); Emmons et al. (1999); Peterson (2001); Mattila et al. (2005)

Rice	flavone-C-glycosides, neocarlinoside (6-C-β-D-glucopyranosyl-8-C-β-L-arabinopyranosylluteolin), carlinoside (6-C- β -D-glucopyranosyl-8-C-α-L-arabinopyranosylluteolin) and isoorientin-2"-O- β -glucopyranoside	Hirotaka et al. (2006)
Pearl millet	Protocatechuic, *p*-hydroxybenzoic, gentisic, vanillic, syringic, ferulic, caffeic, *p*-coumaric, cinnamic and sinapic acids. Glucosylorientin, glucosylvitexin, tricin, vitexin	Riechert (1979), Dykes and Rooney (2007)

Table 8. Phenolic acids and flavonoids reported in finger millet

Phenolic acids	References
Hydroxy benzoic acid derivatives Gallic, protocatechuic, *p*-hydroxy benzoic, gentisic, vanillic, syringic acids and ***Hydroxycinnamic acid derivatives*** Ferulic, caffeic, *p*-coumaric, cinnamic, sinapic acids	McDonough et al. (1986), Subba Rao and Muralikrishna (2002)
Flavonoids Orientin, isoorientin, vitexin, saponarin, violanthin, leucin, tricin	Hilu et al. (1978)

Processing of millets

The information on the status of polyphenols of malted millet has received the attention since, the millet is used for malting to a significant extent (Malleshi and Amla 1988). Rao and Deosthale (1988), reported 0.91 % tannins in ungerminated grain which decreased by about 72 % on 72 h germination, whereas Sripriya et al. (1996), reported decrease in the total polyphenols on germination is by 35% but increases by 34% on fermentation. A two fold decrease in all the major phenolic acids after 96 h of germination was recorded by Subba Rao and Muralikrishna (2002). The decrease in the bound phenolics may be due to the action of esterases developed during germination which is known to act on various phenolic acid esters linked either to arabinoxylans or other non-starch polysaccharides. A threefold decrease in protocatechuic acid content but marginal loss in caffeic acid upon 96 h of malting was reported by these workers. Similar observations was made by Lakshmi kumari and Sumathi (2002) also. Malleshi (2006) reported that, hydrothermal treatment and decortication of finger millet decreases polyphenols by 74.7%.

Utilization of protein in animal and human diet is adversely affected by phenolic constituents since they have the ability to bind with and precipitate proteins. Growth retardation has been observed in chicks and rats fed on diets containing high tannin sorghum (Ramachandra et al. 1977). Availability of amino acids in high tannin sorghums results in increased weight gain and feed efficiency of chicks and rats (Ramachandra et al. 1977). The precipitation of polyphenols - protein complexes is due to the formation of sufficient hydrophobic surface on the complex (McManus et al. 1981). The interaction of salivary proteins with polyphenols has been implicated in the perception of astringent flavour (Baxter et al. 1997). Polyphenols may form soluble or insoluble complexes with proteins. This in turn may have detrimental effect

on the *in vivo* bioavailability of both phenolics and proteins (Wollgast and Anklam 2000). The precipitation of a polyphenols-protein complex is due to the formation of sufficient hydrophobic surface on the complex.

Health benefits of finger millet

The health benefits of the millet with respect to diabetes (Gopalan 1981) and duodenal ulcer (Tovey 1994) have been reported and it is generally believed that the millet offers several health benefits. But there is no clinically proven information on the health benefits of millets. Moreover the role of millet polyphenols towards these have not been investigated.

A very low concentration of finger millet polyphenols extract was found to inhibit glycation similar to that of the known antiglycating agent namely, aminoguanidine and well known synthetic antioxidant butylated hydroxyanisole (Hegde et al. 2002). The study on cross linking of collagen, analyzed by pepsin digestion and CNBr digestion strongly suggested the protective role of the methanolic extract of the finger millet.

Polyphenols are known to inhibit the enzymes and this has relevance with the human digestive enzymes such as amylases, glucosidase, pepsin, trypsin and lipases and the subject has been studied extensively (Rohn et al. 2002). The role of millet phenolics in mediating enzyme inhibition has potential benefits for management of type 2 *Diabetes mellitus*, which is characterized by high blood glucose levels (Toeller 1994; Saito et al. 1998). One therapeutic approach to decrease postprandial hyperglycemia is to retard absorption of glucose through the inhibition of carbohydrate digesting enzymes, e.g., α-amylase and α-glucosidase, in the digestive tract to decrease postprandial hyperglycemia by retarding the absorption of glucose.

Various biological and health-beneficial effects of polyphenols have recently been demonstrated. Oxidative stress induced inflammation is mediated by the activation of nuclear factor kappa B (NF-κB) and activator protein (AP-1). Polyphenols inhibited pro-inflammatory gene expression via inhibition of inhibitory κB (IκB), thus inhibiting NF-κB transactivation (Tsai et al. 1999). Polyphenols may act as inhibitors of amylase and glucosidase similar to acarbose, miglitol and voglibose leading to decrease in post-prandial hyperglycemia (Bailey 2001). Resveratrol could inhibit pancreatic bile salt-dependent lipase (BSDL) activity, expression and secretion in the rat pancreatic AR4-2J cells (Sbarra 2005). Cyanidin-3α-O-rhamnoside and quercetin-3α-O-rhamnoside could inhibit α-glucosidase and advanced glycation end product (AGE) formation *in vitro* (Hanamura et al. 2005). The inhibition of digestive enzymes by dietary polyphenols may represent an under-reported mechanism for delivering some of the health benefits attributed to a diet rich in fruit and vegetables.

Dietary polyphenols appear to have a protective effect on immune cell functions. They also inhibit hyaluronidase activity and increase in intracellular free calcium concentration in RBL-2H3 cells stimulated with the antigen (Kanda 1998). Anthocyanins inhibit α-glucosidase activity and reduce formation of blood glucose levels after starch-rich meals. This is a proven clinical therapy for controlling type II diabetes (McDougall et al. 2005). Alvarez et al. (2006), showed that leukocyte functions were improved in prematurely aging mice after five weeks of diet supplementation with polyphenol-rich cereals.

Antioxidant properties

The antioxidant properties of the millet polyphenols has received the attention of many researchers. Sripriya et al. (1996), investigated the antioxidant properties of

polyphenols extracted with methanol which was able to quench about 77 % of hydroxyl radicals. According to them the DPPH radical quenching ability of finger millet was 94 %, whereas its germinated and fermented counterparts showed only 22 and 25 %, on the other hand germination followed by fermentation showed only 10 % quenching. This showed that these kinds of processing of the millet reduce its free radical quenching capacity. The major antioxidant principles identified by them was catechin. The effect of malting on phenolic antioxidants were studied by Subba Rao and Muralikrishna (2002). They reported that, the antioxidant activity of free phenolic acids was higher compared to that of bound phenolic acids. They reported an increase in the antioxidant activity coefficient from 770 to 1686 in the case of free phenolic acids and a decrease from 570 to 448 upon 96 h of germination in bound phenolic acids. They compared various naturally occurring phenolic acids such as caffeic, coumaric, ferulic, gallic, gentisic, protocatechuic, syringic and vanillic acids with the synthetic antioxidants such as BHA and BHT, and it was observed that, the antioxidant activity of the millet polyphenols was slightly lower than the synthetic antioxidant compounds. Cinnamic acid derivatives such as ferulic, caffeic and coumaric acids exhibit antioxidant activities (AACs) to higher extent than their corresponding benzoic acid derivatives namely, vanillic, protocatechuic and *p*-hydroxy benzoic acids respectively. Among the cinnamic acid derivatives, caffeic and ferulic acids were found to be stronger than coumaric acid. Gallic acid, which contains three 'OH' groups is stronger than protocatechuic acid, gentisic and syringic acids with respect to the antioxidant properties. Finger millet polyphenols exhibit the antioxidative properties effectively on super oxide, hydroxyl and nitric oxide radicals also (Bindu and Malleshi, 2003). Asharani et al. (2005), have shown that the millet contains 199 $\pm$ 77 µg/100g, 4 $\pm$ 1 mg % and 15.3 $\pm$ 3.5 TE/g for carotenoids,

Vitamin E and the total antioxidant activity respectively. They have identified the isomers of these and reported that the antioxidant activity of whole meal of finger millet is considerably higher than other millets. Varsha et al. (2008), determined the antioxidant activity of the polyphenol extracts from finger millet seed coat and the whole meal, and reported that the seed coat extract exhibits about 5 times higher activity compared to whole meal assayed in terms of reducing power assay and the β-carotene bleaching method.

Antimicrobial activity

Polyphenols are well documented to have microbicide activities against a large number of pathogenic bacteria (Scalbert 1991). The mechanism of polyphenols toxicity against bacterial growth may be related to inhibition of hydrolytic enzymes (proteases and carbohydrolases) or interactions to inactivate microbial adhesions, cell envelope transport proteins, non-specific interactions with carbohydrates, etc (Damintoti Karou et al. 2005). Phenolic acids from finger millet milling fractions showed antimicrobial activity against *B. cereus* (Varsha et al. 2008).

Scope of the work

The review of the literature on finger millet with special reference to polyphenols clearly indicates that the millet in general and its seed coat in particular, which is a good source of edible polyphenols, could be a new source of nutraceutical. Moreover now-a-days the millet seed coat is readily available as a by-product due to the advent of the millet processing technologies, and exploiting its utilization as a source of food additive or as a pharmaconutrient would be highly remunerative. Hence, investigations on isolation as well as characterization and also the biochemical and nutraceutical qualities of the millet polyphenols were under taken with the following objectives;

1. Localization of the polyphenols in the millet kernels and concentration of the polyphenols rich fraction of the kernels, and also isolation as well as characterization of the constituent phenolics,
2. Evaluation of the biochemical characteristics of the millet polyphenols with special reference to the inhibitory activity on some of the enzymes of plant and animal origin, and
3. Nutraceutical properties of polyphenols with respect to antioxidant and antimicrobial activities.

The research work conducted with these objectives is presented in three chapters, namely, the experimental aspects and the results pertaining to distribution of the polyphenols in the millet kernel and their isolation and characterization; interaction of the millet polyphenols with the millet malt amylases as well as on aldose reducatse extracted from the human eye lenses; and also their antioxidants and the antimicrobial potential.

Introduction

Finger millet contains 0.1 – 3.0 % polyphenols and the polyphenols differ considerably with other plant polyphenols with respect to their solubility. They are very sparingly soluble in water. The reports on the solvents used for extraction of the millet polyphenols, duration of extraction and also the method of extraction differ considerably. Ramachandra et al. (1977), treated the millet meal for 24 h with 1% HCl–methanol with occasional swirling at ambient conditions, whereas Rao and Deosthale (1988) also extracted the millet polyphenols with 1% HCl-methanol at ambient but duration of extraction was shorter. On the other hand, Shankara (1991) refluxed the millet meal with 1 % HCl-methanol for 30 min, whereas Sripriya et al. (1996), refluxed the millet with methanol alone at 60 ºC for 2 h. But Subba Rao and Muralikrishna (2001), used 70 % ethanol and 1 M sodium hydroxide solution containing sodium borohydrate to extract free and bound phenolics respectively. They extracted the polyphenols under nitrogen atmosphere to prevent oxidation of the phenolics.

For assay of the millet polyphenols including tannins, the common methods used are Folin-Dennis method of Swain and Hillis (1959), vanillin-HCl method of Burns (1971) and Folin-Ciocalteau method of Singleton et al. (1995). The vanillin–HCl method involves condensation of the aromatic aldehydes, namely, vanillin with monomeric flavanols and their oligomers to form a red adduct which has absorption maxima of 500 nm, but Folin-Ciocalteau method is based on the oxidation of phenols with a mixture of phosphotungstic acid and phosphomolybdic acids in alkaline conditions leading to the formation of blue colored compounds, the concentration of which is read at 760 nm. The polyphenols content of the millet are reported as equivalents of chlorogenic, catechin, gallic and tannic acids. Ramachandra

et al. (1977), assayed totalphenols by Folin-Dennis method as chlorogenic acid and catechin equivalents. McDonough et al. (1986), used Vanillin-HCl method for the assay of tannin and Folin-Ciocalteau method for total polyphenols. The large variability in the polyphenols content of the millet reported by different studies could be attributed to the different methods followed for the assay.

In view of these, it was felt necessary to identify a suitable solvent system for extraction of millet polyphenols. Accordingly a few polar and non-polar solvents as such and also after acidifying the solvents were examined. Further to that the efforts were made to characterize the constituent phenolics extracted by a suitable solvent system.

Materials and methods

Chemicals

Gallic, vanillic, ferulic, *p*-coumaric, *p*-hydroxybenzoic, syringic, *trans*-cinnamic, *p*-catechuic, trifluoroacetic acids and quercetin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Methanol (HPLC grade) was purchased from Merck (India). The other reagents like polar and non-polar solvents, hydrochloric acid, sulphuric acid used were of analytical grade or HPLC grade and were obtained from Merck (Mumbai, India).

Methods

Sample preparation

A few popularly cultivated high yielding finger millet cultivars (GPU 26, GPU 28, PR 202, HR 911, Indaf 9, Indaf 5 and Indaf 7) procured from the University of Agriculture Sciences, Bangalore, were cleaned to free from the extraneous matter. The cleaned millet were subjected to mild abrassion in Engleburg huller (Sri Ganesha Engineering Works, Chennai, India) to detach the pericarp (glumes) covering the

caryopsis. The glumes were aspirated off and the deglumed millet samples were used for determination of their polyphenols content.

The deglumed millets cultivars (100 g each) were pulverized in a domestic model carborundum disc mill (Sl. No. 95745, Milone, manufactured by M/s. Radhika Industries, Rajkot, India). The meal was sifted through 250 micron (BSS) sieve and the tailings were again pulverized to pass through 250 micron sieve completely. Tailings and flour obtained separately. The tailings were again sieved and the same was mixed thoroughly with the flour obtained from the first stage grinding to prepare whole meal.

The whole meals were defatted with petroleum ether (60 – 80 $^{\circ}$C) using the Soxhlet extractors and the defatted samples were used for assay of the polyphenols.

Assay of polyphenols

Principle

The polyphenols react with Folin-Ciocalteau reagent (phosphotungstic and phosphomolybdic acids) in alkaline conditions and developed blue color. The intensity of the color is read at 760 nm. The intensity of the color is proportional to the concentration of the polyphenols.

Extraction

Only one of the cultivars (GPU 28, popularly cultivated millet) was used for optimizing the procedure for the extraction of the millet polyphenols. Accordingly, (5 g) of the defatted sample was extracted using water, acetone, propanol, ethanol and methanol (100 ml each) as follows;

a. By just mixing the sample in the solvents (100 ml each) and stirring vigorously using a magnetic stirrer for about 3 h at ambient conditions (25 – 30 $^{\circ}$C),

b. By refluxing the samples mixed with the solvents for about 3 h using boiling water bath,

c. By just mixing the samples with the solvents, acidified with HCl (99:1, v/v) at ambient conditions, and stirring vigorously using magnetic stirrer for about 3 h, and

d. By refluxing the samples mixed with acidified solvents (as per 'c') for about 3 h using water bath.

The extracts from each of the solvents and from each of the conditions were centrifuged, and the centrifugate containing the polyphenols were concentrated under reduced pressure at 40 ºC and used for the assay of the polyphenols (Singleton et al 1995).

Preparation of reagents

1. *Saturated sodium carbonate (Na_2CO_3) solution*: Dissolve 35 g anhydrous sodium carbonate in 100 ml water at 80° C, leave the content over night, decant the clear supernatant,
2. *Folin-Ciocalteau reagent*: Dissolve sodium tungstate (100 g), phosphomolybdic acid (20 g) and 50 ml of 85 % phosphoric acid in 750 ml water, reflux for 2 h, and diluted to 1 L with water.
3. *Gallic acid standard*: Dissolve gallic acid (0.1 g) in 1 ml water to prepare standard solution containing 100 µg gallic acid per ml of solution.

Procedure

To 1 ml extract, 5 ml of Folin–Ciocalteau reagent and 10 ml of saturated sodium carbonate solution were added. The contents were mixed well and diluted to 100 ml using distilled water and allowed to stand for 30 min and the optical density of the blue color developed was measured at 760 nm.

A calibration graph prepared using standard gallic acid solution, such that a linear plot forcing through the zero of the axis was prepared and the slope was obtained from the gradient of the linear part.

$$\text{Standard slope for gallic acid} = \frac{\text{Optical density of standard solution}}{\text{Concentration of standard solution of gallic acid (µg)}}$$

Total polyphenols (µg gallic acid equivalents) in the extract of the aliquot (*TP $_{ext}$)*

$$TP_{ext} = \frac{\text{Optical density of sample}}{\text{Slope of standard gallic acid}}$$

Percent total polyphenols in the undiluted sample is calculated as follows

$$\text{Total polyphenols (\%)} = \frac{TP_{ext} \times V_1 \times d}{\text{Wt of the sample taken (g)} \times V_2} \times 100$$

V_1 = Volume (in ml) of extract
d = dilution factor
V_2 = volume taken for analysis (in ml)

Based on the observations of these experiment acidic methanol (99 ml methanol : 1 ml Conc. HCl) was used for further studies and this is referred as acidic methanol in subsequent basis.

Varietal variations

The defatted whole meals (5 g each) from the cultivars (as indicated earlier) were refluxed in a boiling water bath with about 100 ml of acidic-methanol solvent and the polyphenols content were assayed as described in the earlier.

Guided by the outcome of these experiments, GPU 28 variety which contained fairly high levels of polyphenols was used for further studies.

Localization of the polyphenols in the millet kernel

A few kernels of the millet (GPU 28) were soaked in 25 % aqeous solution of propylene glycol, overnight. The softened grain were embedded in paraffin wax containing 5 % bee wax and from that 5 - 8 µ thick sections were prepared using a microtome (American Opticals, USA). The sections were mounted on glass slides using glycerol over which the cover slip was placed and viewed under fluorescence microscope at 280 and 360 nm (Olympus, USA) and the fluorescing locations indicating the presence of polyphenols were photographed. It was observed that, the intensity of the fluorescence was very high in the seed coat tissue revealing their presence but was feeble in the cell walls of the endosperm.

In view of this, the polyphenols rich portion of the millet, namely, the seed coat matter was prepared following special milling protocol.

Preparation of polyphenols rich fraction

The millet was sprayed with 5 % additional water, tempered for about 10 min and pulverized in a comminuting mill fitted with 0.32 mm opening screen (Apex Constructions Ltd., England). Soon after pulverizing, the meal was shifted through a sieve of '180 µ' openings and the tailings ('+ 180 µ' fraction) was again pulverized immediately and sieved through the same sieve. The process was repeated for the third time (**Fig. 4**). The flour samples ('-180 µ' fraction) from 1, 2 and 3 passes were pooled and designated as refined flour fraction (RFF), whereas, the tailings from the 3rd stage pulverization was termed as seed coat fraction (SCF). It was noticed that the SCF contained considerable proportion of starch (tested by iodine staining method), it was washed with water to free from adhering starch. The water extract and the residue were dried. The residue was micropulverized and sifted through a sieve of '180 µ' openings and the overtails ('+ 180 µ') and the throughs ('– 180 µ') were collected

separately. The water extract and the 'over tails' and the 'throughs' were analyzed for their polyphenols content. It was observed that, the '+ 180 µ' fraction contained considerably higher proportion of polyphenols than other fractions and hence, it was termed as polyphenols rich seed coat fraction (PRSCF) and the same was used for subsequent studies for isolation and characteristics of the polyphenols.

Extraction of polyphenols from PRSCF

About 50 g of the PRSCF was mixed with about 500 ml of acidic-methanol and refluxed using water bath for about 30 min, filtered and the residue was re-extracted with fresh 500 ml solvent and the process of extraction was repeated till the extract tested (with F/C reagent) negative to polyphenols. The extracts thus prepared were pooled and assayed for their polyphenols content. For all the subsequent investigations, the same method of extraction of the polyphenols was followed. The extract was examined for the stability of the polyphenols against changes in pH and temperature and also used for identification and characterization of the constituent phenolics.

Temperature and pH stability of the polyphenols in the extract

Aliquots (100 ml) of the extract taken in separate conical flasks were incubated at 30 – 90 ºC with 10 ºC increment for about 48 h. The flasks were fitted with water condensers to prevent evaporation losses of the extract. The polyphenols content of the extracts, maintained at different temperatures was assayed as described earlier.

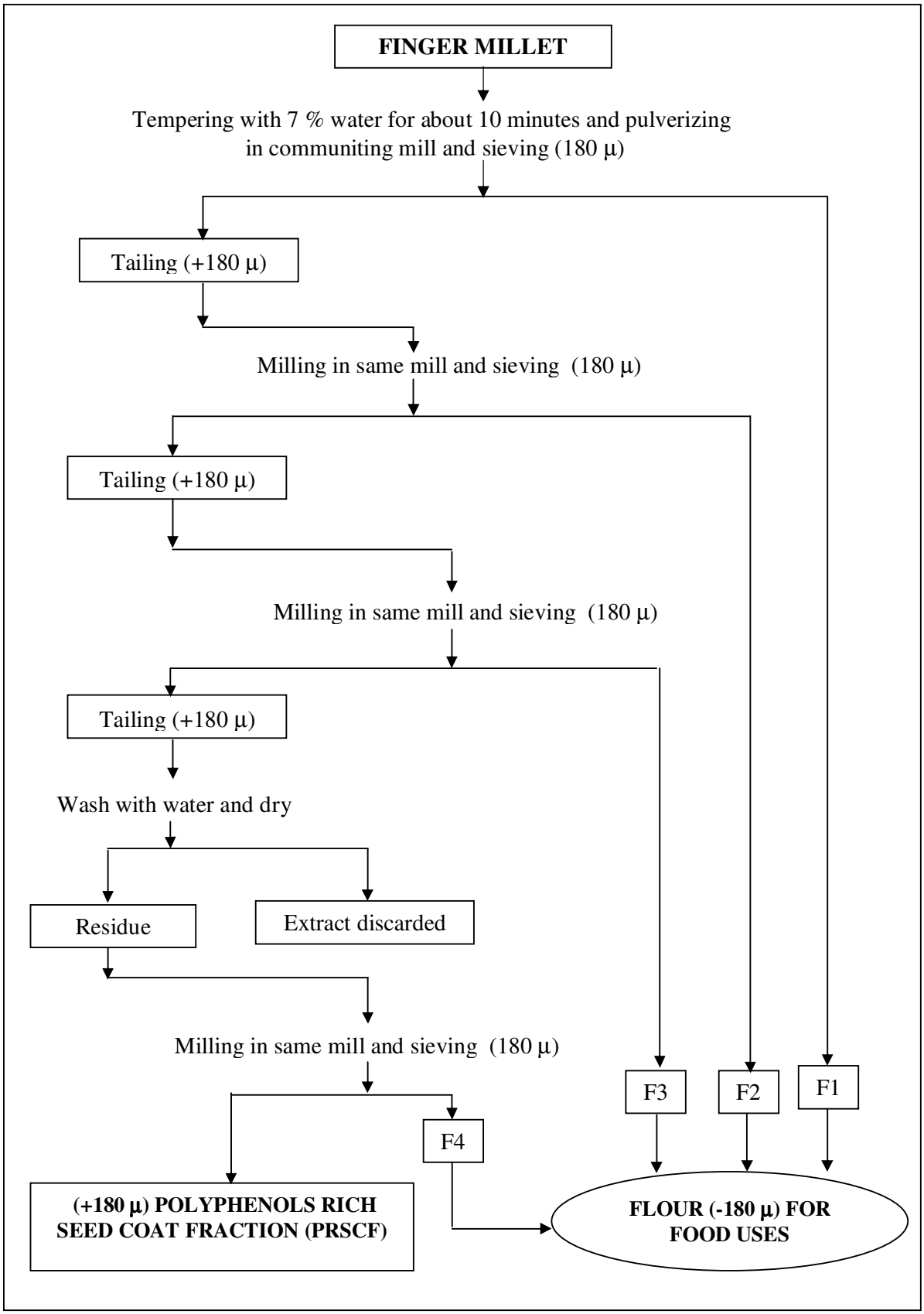


Fig 4: Protocal for the preparation of the polyphenols rich seed coat fraction

In a separate experiment, 200 ml of the extract taken separately in different beakers were mixed with NaOH of suitable strengths (0.1 – 4 N NaOH) depending upon the target pH. Care was taken to minimize quantity of alkali required to raise the pH of the extract up to 12, with 1 unit difference by using concentrated NaOH initially. The contents were allowed to stand for about 30 min. Since, the formation of precipitate was observed in all the samples the contents were centrifuged to separate the precipitated matter. The polyphenols content of the supernatant as well as the precipitated matter from each of the samples was assayed.

The λ_{max} of the supernatants was also recorded in a spectrophotometer in the UV-Visible range, and their color intensity was also measured and recorded in terms of L* a* b* colour indices with Lab Scan XE (Hunter Lab Instruments, USA) with CIE standard observer (10° view angle) and the CIE standard illuminant D_{65}. Chromatic analyses were carried out in terms of CIE (L*a*b*).

In a separate experiment, the supernatant with pH 7 was concentrated at low temperature (40 ºC) and low pressure (30″ Hg) using a rotary flash evaporator and the concentrate was freeze-dried. The precipitated matter was also freeze-dried. Both the supernatant and the precipitated matter were analyzed for the nutrient composition according to AACC (2000), and also for Ca, Mn, K, Fe, Zn, Cu and Pb contents were estimated by atomic absorption spectrophotometer (Model "AA - 670F", Shimadzu, Singapore). The standard solutions of the minerals and the ash solutions of suitable dilutions as required for the instrument were used and the absorption was read at 3 - 4 ranges of standard solutions. The burner was flushed with deionised water between samples. The calibration curves for each of the minerals using the respective standards were prepared.

pH sensitivity of pure phenolics

To compare the behavior of the crude polyphenolic extract of the millet, chlorogenic acid (2.8×10^{-6} M), gallic acid (5.88×10^{-6} M) and *trans*-cinnamic acid (6.0×10^{-6} M) solutions in acidic-methanol solvent in a separate beakers (about 50 ml) were taken and their pH was adjusted to 1, 3, 7 and 10 using suitable concentration of NaOH. The absorption spectra of these samples at different pH were recorded from 190 to 400 nm. The molar extinction coefficients (ε) of the samples was determined by the following equation (Freifelder 1982);

$\varepsilon = A/dc$,

where A = absorbance or optical density;

d = 1 cm, the thickness of the UV cell;

and c = molar concentration of the test compound, moles/L

Fractionation of polyphenols by HPLC

The extract at pH 3, 7 and 10 were centrifuged and the supernatants were concentrated and fractionated into the component phenolics using reverse-phase analytical HPLC (Court 1977), [Shimadzu LC - 10A liquid chromatograph fitted with 250 mm × 4.6 mm ODS 2 C_{18} column and equipped with CBM-10A system controller, SPD - M10 AVP photo diode array detector and a software class 10A]. The mobile phase consisted of a binary solvent system comprising solvent A (water acidified with 0.1 % trifluoroacetic acid) and solvent B (100 % methanol) maintained at a flow rate of 1.0 ml/min. The gradient programme initiated with 80 % eluent A and 20 % eluent B, ramped linearly to 60 % of solvent A and 40 % of solvent B within 40 min. This proportion (60 : 40) was maintained for next 10 min and subsequently, the solvent gradient was reverted to the initial conditions (80 : 20) within next 5 min (total run time was 55 min). Detection and quantification of the

eluted phenolics was performed at 295 nm. Each phenolic compound was expressed with a standard run on similar lines. The constituent phenolics recorded as peaks were identified on the basis of the retention time and the spectra of each peak compared with the known phenolics.

The residue (precipitated matter) at pH 3, 7 and 10 were also dissolved in methanol, centrifuged and the centrifugate comprising of the stabilized polyphenols were fractionated through HPLC as described earlier.

Characterization of phenolics with mass spectrometry

Some of the constituent phenolics eluted in the HPLC were examined further for their purity by mass spectrometry (MS), on a Waters LC-MS/MS Q-Tof Ultima (QTOF – GAA 082), equipped with a Turbo-electron spray ionisation source. The MS conditions were optimised with a few reference compounds by direct infusion. Positive ion mode was used to monitor phenolic compounds with the following settings: capillary voltage 3500 V, N_2 gas as nebulizer and the collision energy (CE) of 10 V. The drying gas was heated to 300 ºC and introduced at a flow rate of 8300 ml min^{-1}. Full scan data were acquired by scanning from 100 to 400 m/z in a profile mode.

Nuclear magnetic resonance

To confirm the identity of some of the phenolics identified by HPLC and also by LC-MS/MS, three of the phenolic acids isolated from the HPLC fractionation experiments were subjected to NMR analysis (Naidu et al. 2008). Accordingly, the ^{1}H and ^{13}C Spectra of the polyphenols were also recorded in deuterated methanol (MeOD) using a Bruker AVANCE 500 NMR spectrometer (Rheinstetten, Germany) with tetra methyl silane (TMS) as an internal standard.

Results and discussion

The polyphenols content from the millet extracted from pure water, acetone, propanol, ethanol and methanol at ambient temperature (25 - 30 $^{\circ}$C) and also after refluxing, presented in **Table 9**, indicates the significant differences in the extractability of the solvents tested and also the method of extraction. Only 7.4, 13.1, 10.0, 13.1 and 19.6 % of the polyphenols content were extracted in water, acetone, propanol, ethanol and methanol at ambient temperature and 10.9, 39.0, 37.4, 44.3 and 53.9 % after refluxing, respectively. On the other hand, acidification of the solvents improved the extractability to 14.8, 21.8, 25.2, 31.3 and 39.5 % of the polyphenols in cold condition and 19.6, 45.2, 53.5, 60.0 and 100 % on refluxing, respectively. This shows that, pure solvents were very poor extractants of the millet polyphenols at ambient temperature, but acidification of the solvents enhances the extractability considerably. Further to that, refluxing seems to be very effective method for extraction of the millet polyphenols. Among the different methods and different solvents tried, refluxing the millet seed coat matter with 1 % HCl-methanol solvent was very effective. This could be due to softening of the seed coat tissues by the acid in elevated temperature resulting in weakening the polyphenol-protein and polyphenol-polysaccharide linkages. Thus the acidic nature of the hot methanol causes migration of the polyphenols into the solvent easily thereby improving the extraction.

Table 9. Finger millet polyphenol (g %) extracted by different solvents under ambient conditions and also after refluxing

Solvents	Extraction at ambient condition		Extraction by refluxing	
	Pure	Acidified with 1% HCl	Pure	Acidified with 1% HCl
Water	0.17 (7.4)	0.34 (14.8)	0.25 (10.9)	0.45 (19.6)
Acetone	0.30 (13.0)	0.50 (21.75)	0.90 (39.0)	1.04 (45.2)
Propanol	0.24 (1.0)	0.58 (25.2)	0.86 (37.3)	1.23 (53.5)
Ethanol	0.30 (13.1)	0.74 (31.3)	1.02 (44.3)	1.38 (60.0)
Methanol	0.45 (19.6)	0.90 (39.5)	1.38 (53.9)	2.30 (100)

Average of two determinants. Values in the parenthesis indicate percentages of total assayable polyphenols.

Many of the phenolic compounds of cereals are reported to be soluble in polar solvents and the choice of solvents depends on the number of hydroxyl groups of the phenolics. Accordingly, use of acidic methanol for extraction of finger millet polyphenols has been favored by most of the workers (Ramachandra et al. 1977; Sripriya et al. 1996). But for extraction of total phenolics of cereals, Naczk and Shahidi (2006) suggested using methanol, ethanol, propanol, acetone, ethyl acetate, dimethylformaamide individually and also in their combinations.

Most of the methods for the assay of total polyphenols of cereals are based on the ability of phenolics to react with oxidizing agents, and among the various reagents, Folin-Ciocaltaeu (FC) reagent (Singleton et al. 1995) is prominent. This reagent preferred to others as it is is non-specific for any particular phenolics and the colour developed depends on hydroxyl groups as well as their position in the polyphenols molecule. In view of this FC reagent was used for assay of the wide range of polyphenols in the millet also in the present study.

The absorption spectra of the polyphenols extracted in methanol, ethanol, acetone and propanol as such and also after acidification presented in **Fig. 5**, reveals that, the λ_{max} of the phenolics extracted by pure solvents was only at 220 nm, whereas the phenolics extracted by acidified solvents exhibited λ_{max} at 220 and 280 nm prominently. This shows that, acidified solvents extract more number of phenolic constituents with different structures compared to the pure solvents.

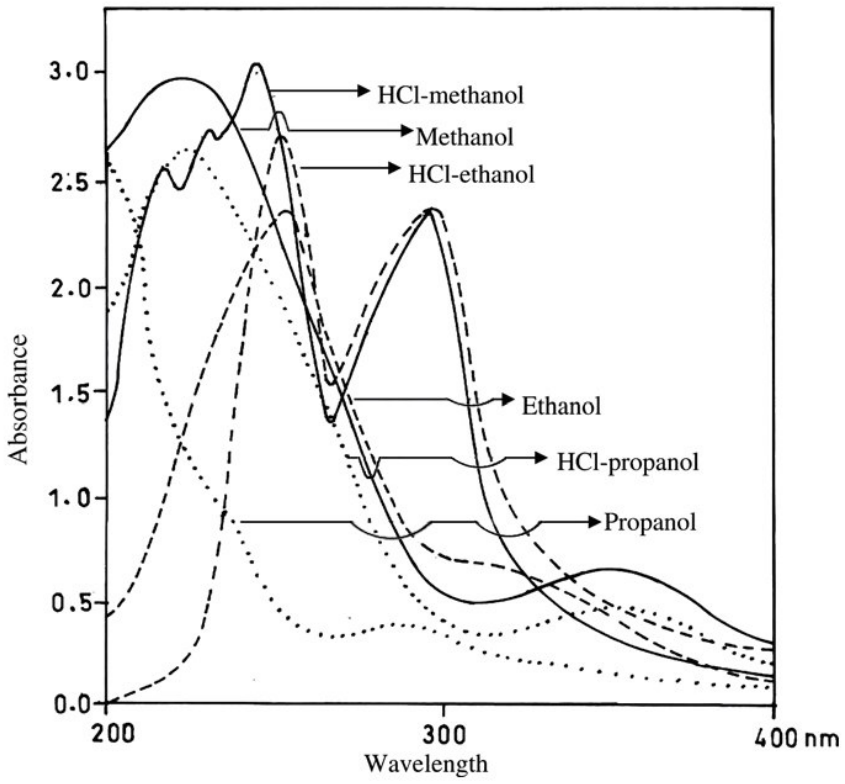


Fig. 5. Absorption spectra of the polyphenols extracted with pure and acidified (1 ml of conc. HCl to 100 ml of respective solvents) solvent.

Varietal variations in polyphenols content

Considerable variations with respect to the polyphenols content among the varieties examined was observed and it was found that, the brown colored varieties contained (1.2 - 2.3 %) slightly higher proportion of polyphenols compared to white (0.3 - 0.5 %) varieties (**Table 10**). The noticeable difference between white and brown varieties could be due to the presence of the colored pigments, such as anthocyanins, which are generally polymerized phenolics present in brown cultivars (**Fig. 6**). The polyphenols content of the millet varieties ranged from 0.3 to 2.3 % and the minimum and maximum values were for Indaf 7 and GPU 28 varieties respectively. The polyphenols extract of GPU 28, GPU 26, PR 202 and Indaf 9, showed the λ_{max} at 4 different wavelengths (240, 280, 460, 530) but for HR 911 it was at 3 (240, 280, 530) and for Indaf 5 and also for Indaf 7 it was only at 2 (240, 280) different wave lengths (**Table 10**). This shows that the millet polyphenols are of complex nature and are variety specific also. Large varietal differences, with respect to the polyphenols content of Indian white (0.06 – 1.0%), as well as brown (0.3 – 1.5%) varieties, and also a few African brown (0.5 – 3.0%) varieties, have been reported (Ramachandra et al. 1977). Rao and Prabhavati (1982), in an unspecified variety of finger millet reported 0.36 % tannin (catechin equivalents). According to McDonough et al. (1986), the millet generally contains 0.55 - 0.59 % total polyphenols and 0.17 - 0.32 % tannins (catechin equivalent). On the contrary Rao and Deosthale (1988), reported 0.35–2.39 % tannins in brown colored varieties (n = 12) but did not detect tannins in white varieties (n = 3). Shankara (1991), analyzed a large number of finger millet varieties (n = 85) from the Indian state of Karnataka and found a wide variability in total polyphenols content assayed and reported as as chlorogenic acid (0.06 – 0.67 %), tannic acid (0.03 – 0.57 %) and catechin (0.03 – 2.37 %)

Table 10. Polyphenol contents and their λ_{max} of a few finger millet varieties (g %)

Variety	Polyphenols** (%)	Wavelength (nm)	Absorbance
Indaf 7*	0.3 ± 0.1	282	2.98
		241	3.1
Indaf 5*	0.5 ± 0.1	280	3.51
		239	2.5
Indaf 9	1.3 ± 0.2	532	0.65
		462	0.52
		280	3.31
		240	3.99
GPU 26	1.7 ± 0.3	532	0.65
		460	0.42
		282	3.31
		236	3.95
PR 202	1.8 ± 0.4	532	0.59
		460	0.51
		286	3.9
		236	3.99
HR 911	2.0 ± 0.4	534	0.68
		284	3.26
		236	3.99
GPU 28	2.3 ± 0.2	532	0.6
		460	0.5
		284	3.9
		246	3.99

* The millet with white seed coat

** Gallic acid equivalent

Fig. 6. Finger millet cultivars with different colored seed coat

equivalents. According to Sripriya et al. (1996), the total polyphenols content of a brown variety of the millet (0.1 %) was higher than the white variety (0.003 %).

This information on the polyphenols content of the millet variety gives an indication that, considerable variations exist among different genotypes of the millet. However, the values have to be taken on their face value, because the methods followed for extraction and also the assay procedure followed besides the standards used vary considerably among different reports.

Distribution of polyphenols in the millet kernel

The photographs of the milling fractions of the millet indicated in **Fig. 7** and the polyphenols of the kernel is as shown in the milling fractions are presented in **Table 11**. The microscopic examination of the millet sections under UV light indicated the fluorescence prominently at the seed coat but the intensity was feeble in the endosperm cell walls (**Fig. 8**). This reveals that, the polyphenols of the millet are concentrated mainly at the seed coat. The actual determination of the polyphenols of the seed coat and the endosperm of the millet confirmed this. Fulcher et al. (1972), observed intense blue auto-fluorescence in the barley cell walls and he attributed the presence of phenolic acids, probably ferulic acid. Similarly, (Earp et al. 1983) reported blue autofluorescence in the cell walls of sorghum endosperm indicating the presence of ferulic and di-ferulic acids. It has been reported that, in the cereals, the testa contains bulk of the polyphenols as well as tannins (Hahn et al. 1984).

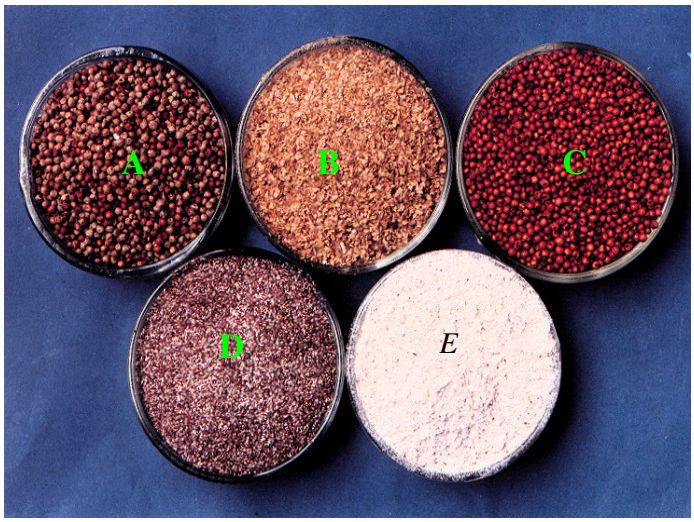


Fig. 7. Finger millet milling fractions : A-Native, B- Glumes, C-Deglumed millet, D-Seed coat, E- Refined flour

Table 11. **Polyphenols content (%) from different milling fractions**

Fractions	Yield (%)	Polyphenols content (%)
Native	100	2.1 ± 0.3
Glumes	2.0	Negligible
Deglumed millet	98	1.9 ± 0.2
Refined flour	82	0.5 ± 0.2
Seed coat	**16**	**6.4 ± 0.7**

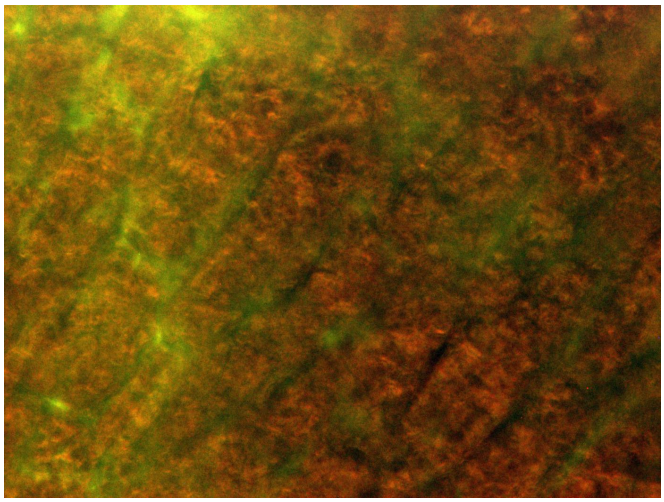


Fig. 8. Section viewed under florescence microscopy showing cell walls fluorescing

McDonough et al. (1986), also substantiated the observation of Fulcher (1982), relating to the distribution of phenolic acids, with the aid of fluorescence microscopy and reported that, the phenolic acids are mainly concentrated in the testa and are also distributed in the cell walls of the endosperm. The polyphenols content of the flour fractions of the millet observed in the present study (about 35 % of the millet polyphenols) could be mainly due to the content of the pulverised seed coat in the flour and also that of the cell wall components of the flour. Plant cell walls are known to contain phenolic acids, mainly ferulic acid which is ester-linked to various cell walls and concentrated in the seed coat. A small proportion of polyphenols are also distributed in the endosperm cell walls and are bound to arabinoxylans (Subba Rao and Muralikrishna 2001). A limited information on the distribution of polyphenols in the millet kernel indicates that, about 65 % of phenolics are concentrated in seed coat and embryo. Ferulic acid traces may be present in the starchy endosperm (Pussaynawin et al. 1988) and it forms the major phenolic acid in many cereals mainly existing predominantly in the seed coat (Watanabe et al. 1997). The polyphenols content in seed coat milling fractions shown in **Table 11**.

According to Ramachandra et al. (1977), dehulling of finger millet removed nearly 80 % of the total phenolics from the high tannin finger millet varieties. Malleshi (2003) reported that, decorticated finger millet after hydrothermal treatment contained 0.067 % of polyphenols as against 0.24% in the native kernel.

Nutrient composition of the milling fractions

The polyphenols rich seed coat fraction contained 13.1 % protein, 3.2 % fat, 5.6 % ash and 43.8 % dietary fibre (**Table 12**). These values are significantly higher than that for the whole meal millet (5, 1 and 8.4 %). This indicates that many of the nutrients are concentrated in the seed coat of the millet. It also contained

Table 12. Proximate composition and polyphenols content of whole meal and the milling fractions of the millet (g %)

	Whole meal	Refined flour fraction ('-180' μ)	Seed coat fraction ('+180' μ)
Yield	100	79.0	21.0
Moisture	11.0	10.1	11.2
Protein	7.0	5.1	13.0
Fat	1.4	0.9	2.0
Ash	2.0	1.1	5.9
Acid insoluble ash	ND	ND	0.7
Dietary fibre (Insoluble)	15.7	8.4	43.8
Dietary fibre (Soluble)	1.4	2.5	13.8
Calcium	0.32	0.22	0.77
Polyphenols*	**2.3**	**0.8**	**6.2**

ND – not detected, Average of two determinants,
* Gallic acid equivalent

Table 13. Polyphenols content of seed coat milling fractions (g %)

Sample	Polyphenols*
a. Whole meal	2.3 ± 0.3
b. Seed coat	6.4 ± 1.5
c. Seed coat after water wash	9.0 ± 2.0
d. Water extractable fraction	0.5 ± 0.02
e. **After pulverizing 'c' and its '+180' µ fraction**	12.8 ± 1.3
f. '-180' µ fraction	2.6 ± 0.4

*Gallic acid equivalent

1.25 % calcium, which forms shows about 50 % of the calcium content of the whole grain. These values are in agreement with the values reported Kurien et al. (1959), who reported the distribution of major nutrients in the millet kernel.

Normally, the seed coat content of the millet accounts for 12 $\pm$ 2 % of the kernel, but the yield of the seed coat fraction prepared by milling the millet in the present study was nearly 20 %. The higher yield was mainly due to the endosperm matter adhering to the seed coat. Hence it was washed with excess water to free the adhering starch. Further, micro pulverizing the washed and dried seed coat matter and removal of finer fraction by sieving through '185 μ' sieve, enriched the coarser ('+ 185 μ') fraction with polyphenols (**Table 13**). The concentrated matter contained (12.8 %) polyphenols as against the original seed coat matter which contained about 6 % polyphenols. From this it may be stated that separation of starchy matter helped to enhance its polyphenols content. This method of preparation of polyphenols rich fraction from the millet appears to be advantageous over method of chemical extraction, because, a large quantity of solvent is required (about 2 L of acidic methanol for 100 g seed coat) to extract the polyphenols, which has to be evaporated subsequently to prepare the polyphenols rich material.

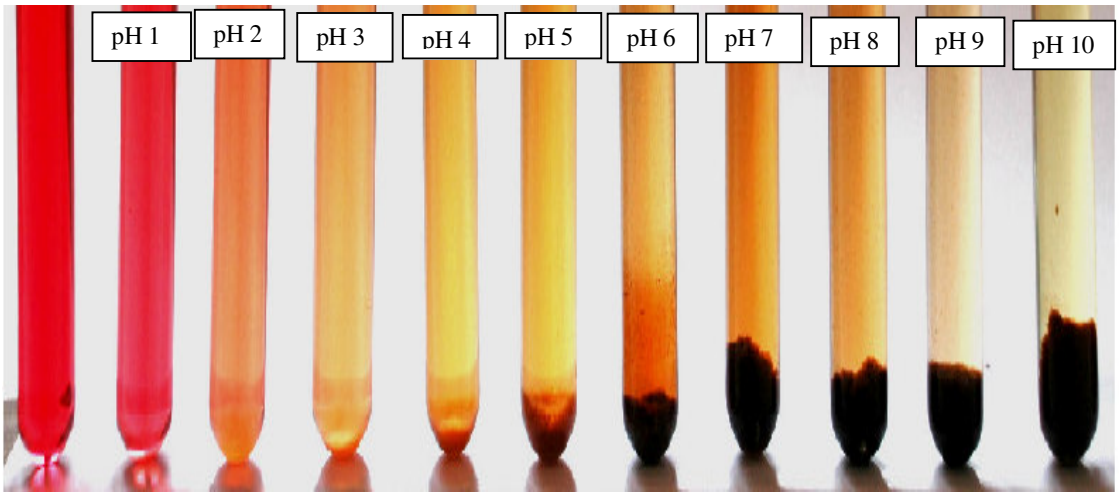
Characteristics of the polyphenols in the extract

The assayable phenolics in the extract remained constant even after 20 days of storage at ambient (30 $\pm$ 2 $^{\circ}$C) conditions and also on heating the same to 90 $^{\circ}$C. This showed that, extraction of the millet polyphenols by refluxing with acidic-methanol is feasible. However, the polyphenols in the extract was highly sensitive to the changes in pH, as it was observed that, even a slight increase in pH resulted in formation of precipitate, and the quantity of precipitate formed increased concurrently with increase in pH (**Fig. 9a**). While, the amount of precipitate formed was about

4.0 % at pH 1, it was about 44 % of the extracted material at pH 10 (**Fig. 9b**). The formation of the precipitate was found to be reversible as it dissolved completely when the acidity of the extract was increased equal to the pH of acidic methanol solvent used for extraction.

It was observed that, the assayable polyphenols content in the extract decreased as the pH increased towards alkalinity and the absorption pattern for the phenolics in the supernatant also changed. The absorption peaks at 220 and 280 nm were prominent at pH 1 but at alkaline pH, only the peak at 220 nm was prominent and the area under second peak at 280 nm was negligible (**Fig. 10**). Polyphenols usually exhibit the absorption maxima between 200 and 360 nm and the band at shorter wavelength is known as B-band and one at longer wavelength as C–band (Dearden and Forbes 1959). Cabrita et al. (1999), reported that, the absorptivities are higher at pH 1 for all anthocyanidin 3-glucosides and decrease as acidity decreases to pH 5. Generally, both the bands get displaced to a longer wavelength depending on the nature of solvents and the presence of electron withdrawing and electron donating substituents in the benzene ring(s) (Mendel and Jurgens 2000).

a.



b.

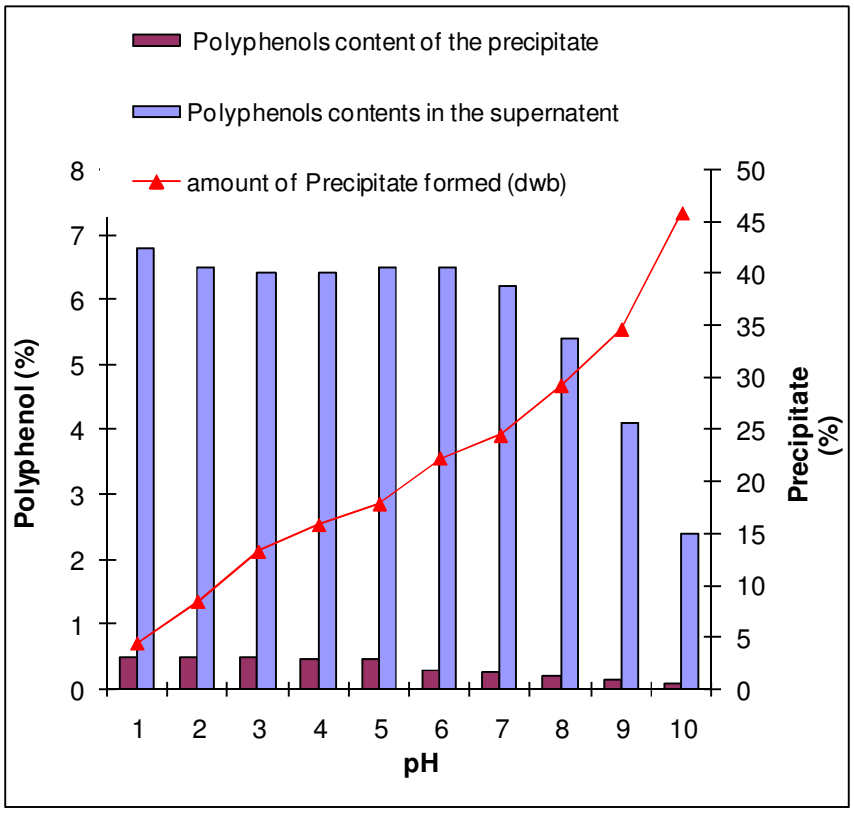


Fig. 9. a. Color of the (1% HCl methanol) extract and precipitate at different pH (1 – 10). b. Polyphenol content of the supernatant and the precipitate formed at different pH values.

Fig. 10. Effect of pH on the absorption spectra of the millet polyphenols extracted with acidic-methanol.

The color values of the supernatant of the extract at 1 - 10 pH recorded in terms of L*a*b* and their synergestic effect is expressed as 'ΔE', [$\Delta E^* = (\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$, which represents the magnitude of the 'total color difference' or deviation from the standard white material with 100 % apparent reflection (**Table 14**). The apparent color intensity (ΔE) of the supernatant was 48.7 at pH 1 and it decreased to 25.7 at pH 10. The decrease in ΔE (decrease in the intensity of color or the increase in lightness of pink color) was concomitant to the increase in the pH. However, a slight deviations at pH 4 (ΔE=31.8) and pH 6 (ΔE=35.4) was noticed, which were lower than the pH 7 (ΔE=37.5). The change in the color of the extract with change in pH probably could be due to the precipitation of the colored pigments or alteration in their chromophoric characteristics at alkaline conditions. The deviations observed at pH 4 and pH 6 may be due to selective solubility characteristics of some of the pigments and the polyphenols of the millet at this pH. In the case of red wine also, the pigment, malvidin 3-glucoside at pH 1 was reported to be red but colorless at pH 6 and 7, but yellow at pH 8 (Lapidot et al. 1999). The trend in changes in the color as a result of change in pH shows the presence of pigments in the acidic methanol extract of the millet.

The chemical composition of the precipitate and the supernatant obtained from the polyphenols extract after adjusting to pH 7, presented in **Table 15** shows that, the precipitate contained 86 % minerals but it did not contain protein and fat at measurable levels. The other matter present in the precipitate could be pigments and some of the unidentified seed coat matter extracted by acidic methanol. It also contained small levels of gallic acid (0.37 %) and *p*-catechuic acid (0.4 %).

Table 14. Colour values of polyphenolic extract at different pH

pH	L*	a*	b*	ΔE
1	42.38	33.35	18.4	48.7
2	48.89	24.87	22.9	41.2
3	55.22	15.03	26.4	34.6
4	59.61	16.1	28.9	31.8
5	50.88	16.6	26.2	37.3
6	50.67	16.3	23.12	35.4
7	48.66	18.3	23.26	37.4
8	56.99	7.5	23.26	27.9
9	58.27	5.18	22.68	27.0
10	59.2	5.0	23.25	25.7

L* = (lightness) axis, indicates '0' is black and '100' is white.
a* = (red-green) axis indicates positive values represent red and '0' is neutral and negative value indicates green.
b* = (yellow-blue) axis indicates positive values they are yellow when positive; negative value represent blue and '0' is neutral.

Table 15. Proximate composition (g %) and some of the mineral contents (mg %) of the supernatant and the precipitate of the extract, at neutral pH (dry weight basis)

	Extract	Precipitate
Protein	ND	ND
Fat	ND	ND
Ash	72.5	86.7
Silica	ND	0.6
Total dietary fibre	1.8	12.6
Calcium	0.29	0.59
Phophorous	0.05	0.4
Minerals (mg %)		
Manganese	5.4	8.0
Calcium	290.0	590.0
Potassium	1.0	1.3
Iron	0.7	1.1
Zinc	0.3	1.3
Copper	0.7	2.4
Lead	0.07	0.09

ND-not detected, Average of two determinants

The possibility of formation of hydroxides by some of the minerals on addition of NaOH and these may form precipitate. This is clear from the high proportion of minerals in the precipitate. Calcium content of the precipitate was highest compared to other minerals (Mn, K, Fe, Zn, Cu, Pb), besides it contained 12.6 % total dietary fibre, which could be the non-starchy polysaccharides extracted form the millet by acidic methanol solvent system.

The absorption maxima of pure gallic acid, chlorogenic acid and trans-cinnamic acid were determined to find similarities with their counterparts from the extract and it was observed that the absorption spectra of gallic acid, in the pH range from 1 to 10, was comparable to that of the polyphenols extract (**Fig. 11**). But, in the case of chlorogenic acid, the K_{max} at 274 nm was comparable to that of the extract at pH 3 only, as at pH 7 it showed two peaks at 218 and 290 nm (**Table 16**). In the case of *trans*-cinnamic acid, the K_{max} values were at 276 nm and 217 nm and there was a substantial change in the K_{max} values with change in pH. These results demonstrate that the susceptibility of different plant phenolics to changes in pH depends on the structure of the phenolics. *Trans*-cinnamic acid is said to be a non-phenolic because it does not have any OH groups located on the benzene ring. This suggests that, conjugated non-phenolic aromatic acids such as *trans*-cinnamic acid are stable under the influence of pH (Mendel and Jurgens 2000). The results of this study demonstrated that the susceptibility of structurally plant phenolics to pH strongly depends on their structure.

The crude polyphenols extracted using acidic methanol were fractionated into component phenolic acids by HPLC (**Fig. 12**). Nine phenolics, namely, the benzoic acid derivatives (gallic, protocatechuic, *p*-hydroxybenzoic, vanillic, ferulic acids),

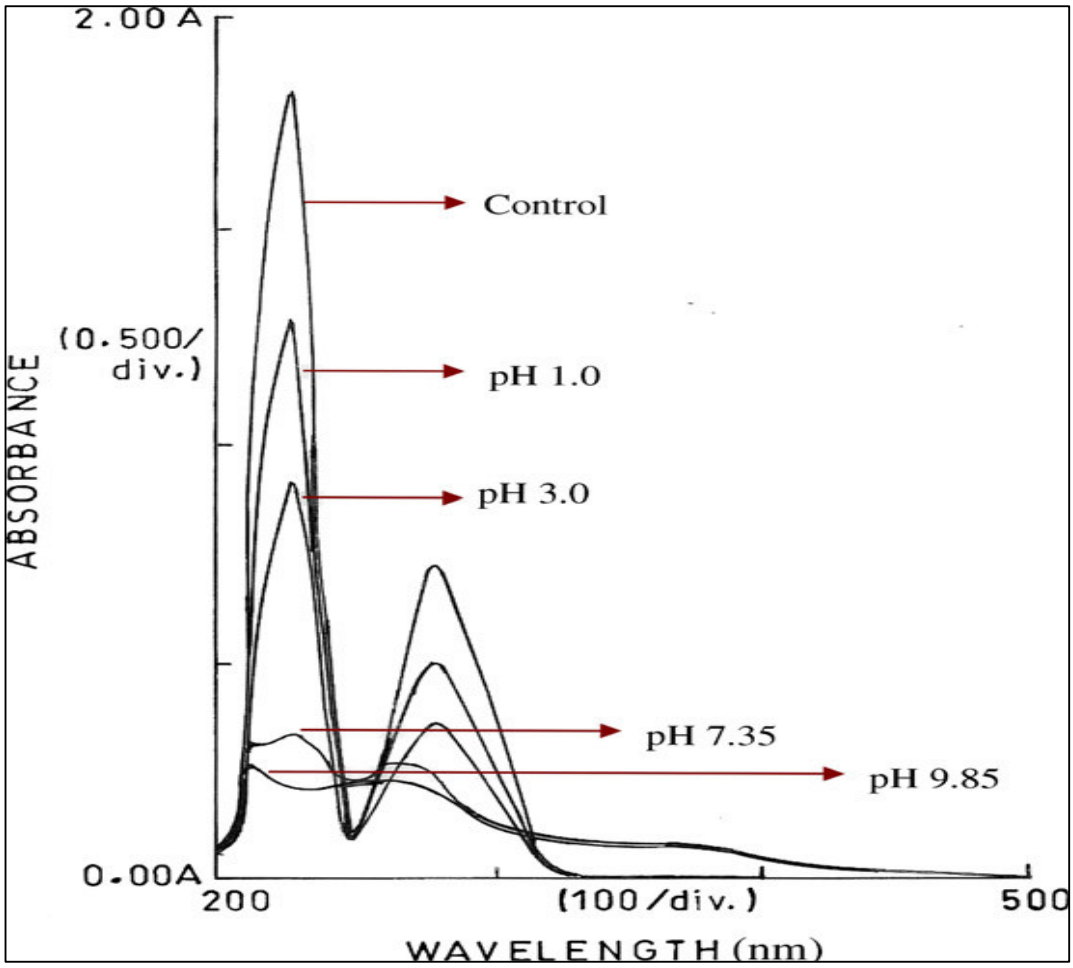


Fig. 11. UV-Visible spectra of 5.8×10^{-6} M gallic acid at different pH values

Table 16. UV absorption maxima (λ_{max}, nm) and molar absorptivity (ε, m^{-1} cm^{-1}) for 5.8×10^{-6} M gallic acid, 2.8×10^{-6} M chlorogenic acid and 6.0×10^{-6} M *trans*-cinnamic acid

pH	Gallic acid		Chlorogenic acid		*Trans*-cinnamic acid	
	λ_{max}	ε	λ_{max}	ε	λ_{max}	ε
Control	275	113758	326	720000	276	252166
	218	289655	244	408035	217	171000
			218	539285		
1.0	274	82413	326	585000	276	563928
	218	213793	242	325714	217	178333
3.0	256	42206	326	490357	276	632142
	220	63965	242	273750	217	202333
7.4	262	47586	322	455357	276	273166
	214	50000	218	42500	217	188333
9.9	258	20344	318	322142	275	268000
	212	36551	290	321428	216	198166

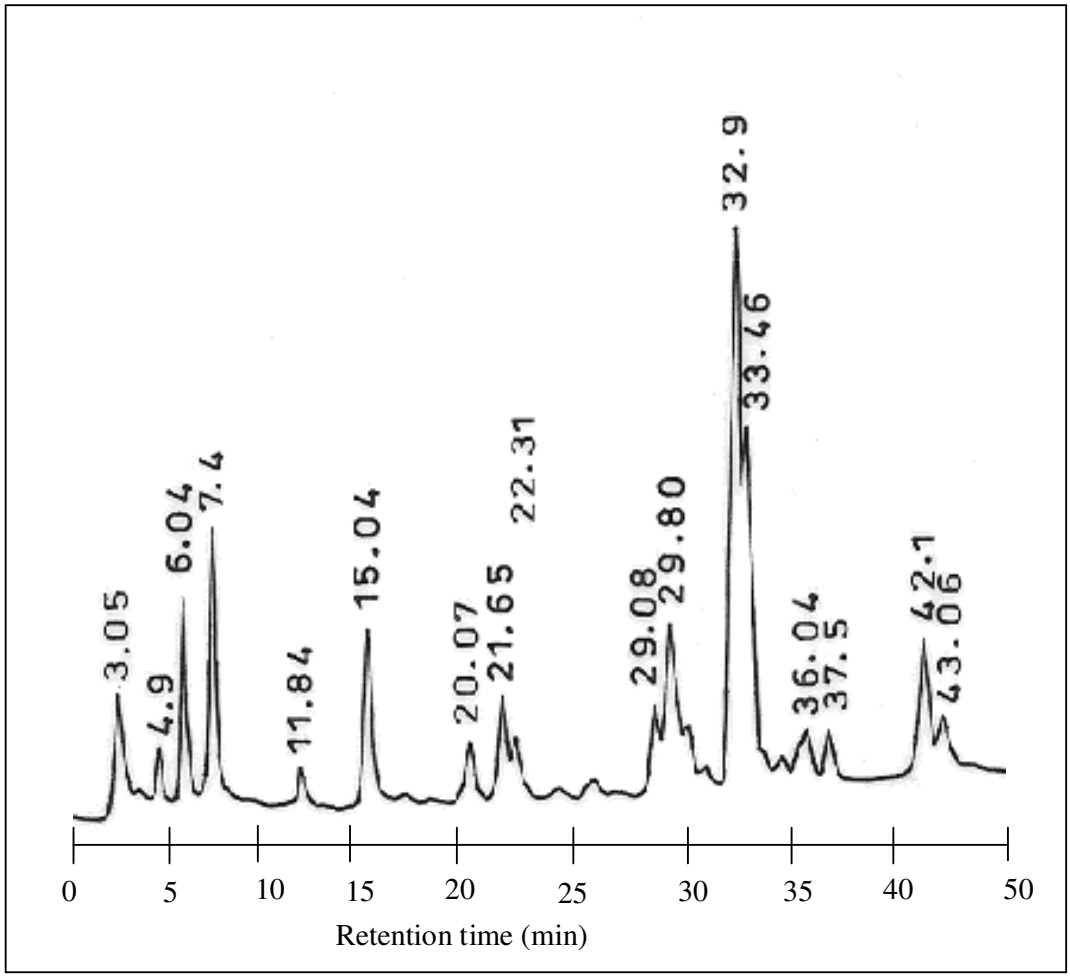


Fig. 12. HPLC chromatogram of native millet polyphenols. Polyphenols were fractionated on µ - Bondapack, C-18 column, using 0.1% tri-fluoroacetic acid: methanol at a flow rate of 1.0ml/min. phenolics identified based on elution time. Accordingly, the retention time in min and the corresponding phenolics are; 3.1:gallic acid; 6.04: proto-catechuic acid; 7.4: *p*- hydroxybenzoic acid; 11.84:vanillic acid; 15.04: *p*-coumaric acid; 20.07: syringic acid; 22.3: ferulic acid; 32.9: *trans*-cinnamic acid; 43.06: quercetin.

and cinnamic acid derivatives (syringic, *trans*-cinnamic, *p*-coumaric acids) and also a flavonoid compound namely, quercetin. The relative proportion of these phenolics was ferulic (32.8 %), *p*-hydroxy benzoic (17.9 %), protocatechuic (15.3 %), gallic (12.6 %), *p*-coumaric (4.4 %), syringic (4.0 %), vanillic (3.8 %), *trans*-cinnamic (3.6 %) acids and quercetin (5.6 %). Benzoic and cinnamic acid derivatives accounted for about 95 % of the total phenolics identified.

In the present study, characterization of the phenolic compounds was based on comparison of their retention time obtained from HPLC and mass spectra with the standards run parallely. Even though molecular masses for the constituent phenolics could be obtained, further ionization and fragmentation could not be obtained under the mass spectral conditions employed (**Fig. 13**). The mass spectra of the crude sample were also matched with that of the standard phenolic compounds (**Table 17**).

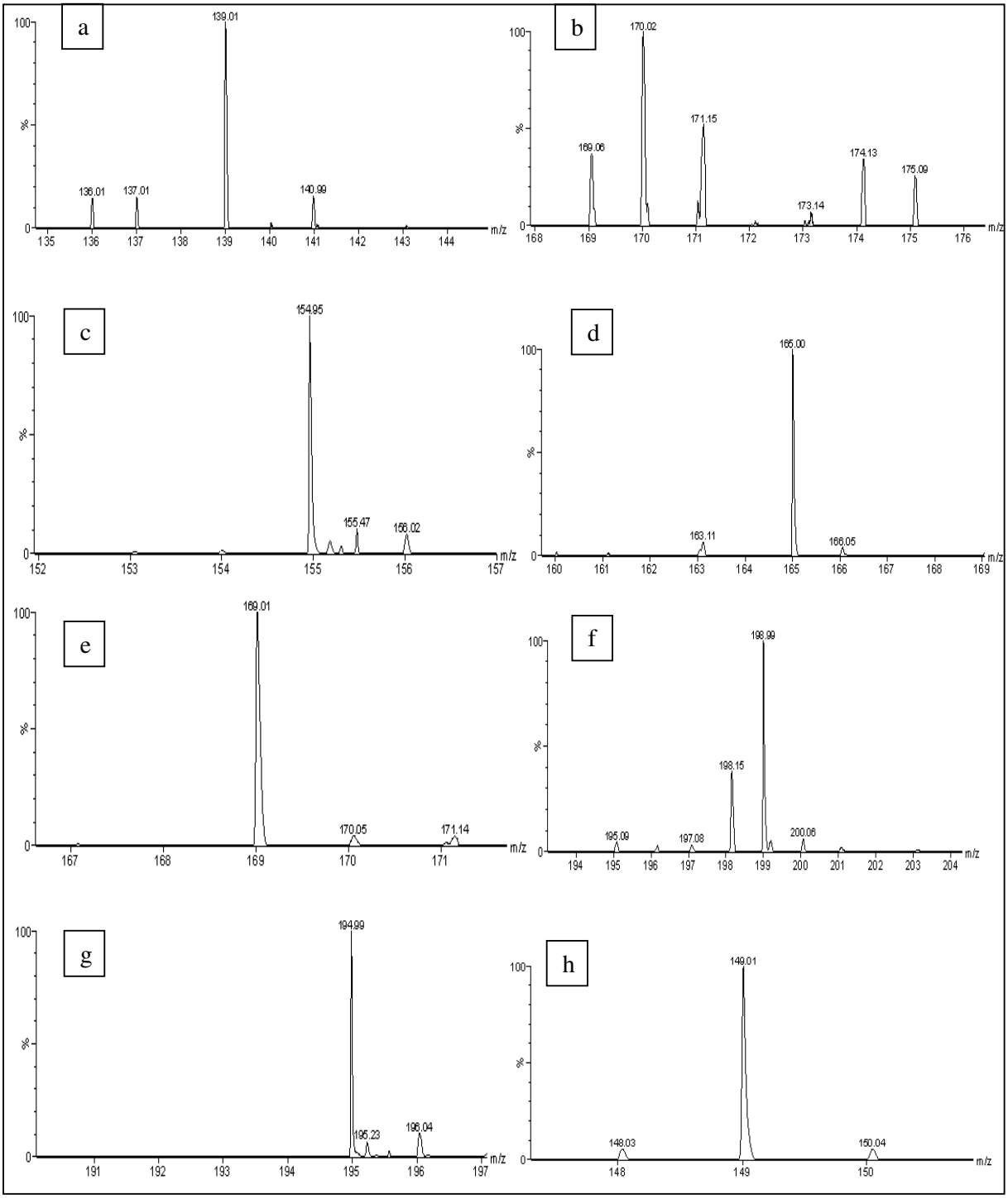
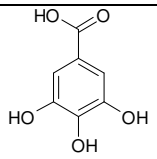
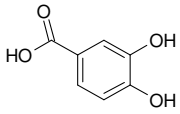
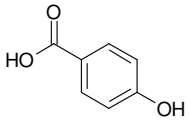
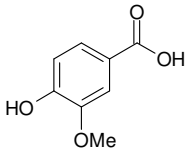
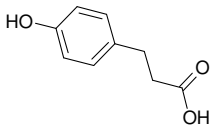
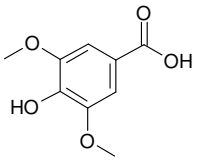
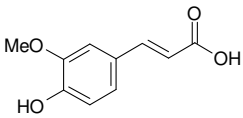
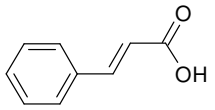
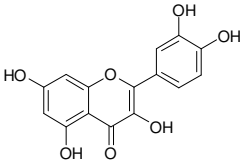


Fig. 13. **Mass (m/Z) spectrometry profile of the identified phenolic compounds. (a). hydroxybenzoic acid, (b). gallic acid, (c). *p*-catechuic acid, (d). *p*-coumaric acid, (e). vanillic acid, (f). syringic acid, (g). ferulic acid and (h). *trans*-cinnamic acid.**

Table 17. Phenolic acids isolated from finger millet

Phenolics	Structure	Retention time (min)	Mass ($m+H^+$)
Gallic acid *3,4,5-trihydroxybenzoic acid*		3.05	171
Protocatechuic acid *3, 4-dihydroxybenzoic acid*		6.046	154.97
***p*-hydroxy benzoic acid** *4-hydroxybenzoic acid*		7.43	139
Vanillic acid *4-hydroxy-3-methoxybenzoic acid*		11.845	169
***p*-coumaric acid** *Trans*-4-hydroxycinnamic acid		15.043	165
Syringic acid *3,5-dimethoxy-4-hydroxybenzoic acid*		20.07	199
Ferulic acid *4-hydroxy-3-methoxycinnamic acid*		22.317	195
***Trans*-cinnamic acid** *3-phenylacrylic acid*		32.9	149
Quercetin *2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-1-Benzopyran-4-one*		43.066	302

Characterization of phenolics

The composition of the mobile phase of the HPLC eluent was optimized to achieve good resolution between the peaks, and it was observed that the best resolution and sharp peaks were obtained with a gradient of 0.1 % trifluoroacetic acid in water as phase A and methanol as phase B. **Fig. 14,** represents LC-DAD chromatograms of the polyphenols from the extract at pH 3, 7 and 10. **Fig. 15,** represents the LC-DAD chromatograms of the precipitated matter at the respective pH. The compositions of the phenolic acids in the extracts at pH 3, identified were benzoic acid derivatives namely gallic, protocatechuic, *p*-hydroxy benzoic acids and also cinnamic acid derivatives namely, *p*-coumaric, syringic, ferulic and *trans*-cinnamic acids, but at pH 7.0 only gallic, syringic, ferulic and *trans*-cinnamic acids were identified. On the other hand, at pH 10.0, only gallic, syringic and *trans*-cinnamic acids could be identified. However, a good number of peaks remained unidentified, could be flavanols. But, in the case of precipitated matter from all the samples, at different pH tested only benzoic acid derivatives, namely gallic and protocatechuic acids were detected. From this it may be inferred that, the millet phenolics are more stable at acidic conditions and are labile at alkaline conditions. The reason for the loss of phenolics in alkaline condition could be due to oxidation of phenolics or chelating with metal ions and their removal as precipitate.

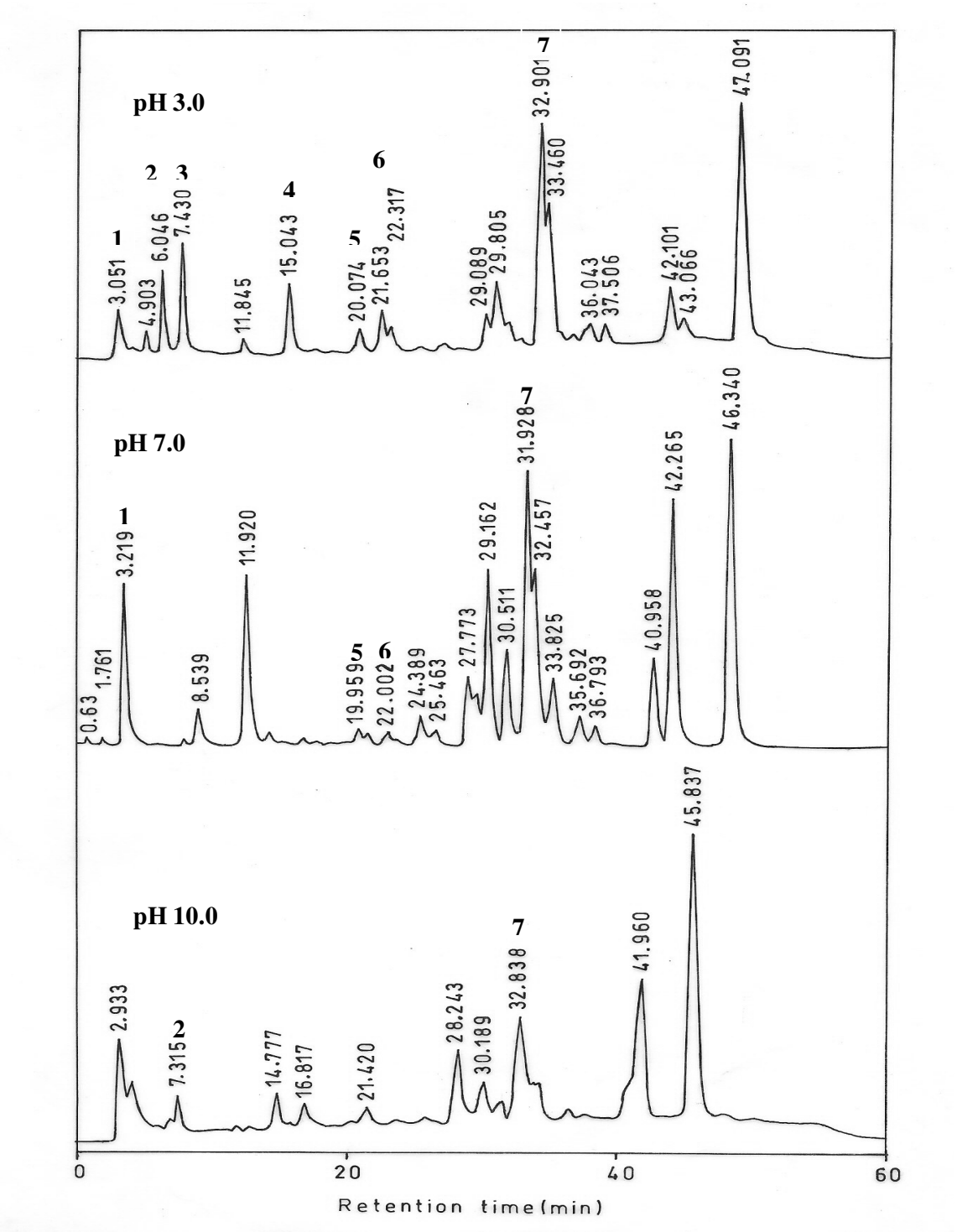


Fig. 14. Fractionation of the phenolics extracted at different pH by HPLC at 295nm. Peak numbers: (1) gallic acid; (2) proto-catechuic acid; (3) *p*-hydroxy benzoic acid; (4) *p*-coumaric acid; (5) syringic acid; (6) ferulic acid; (7) *trans*-cinnamic acid.

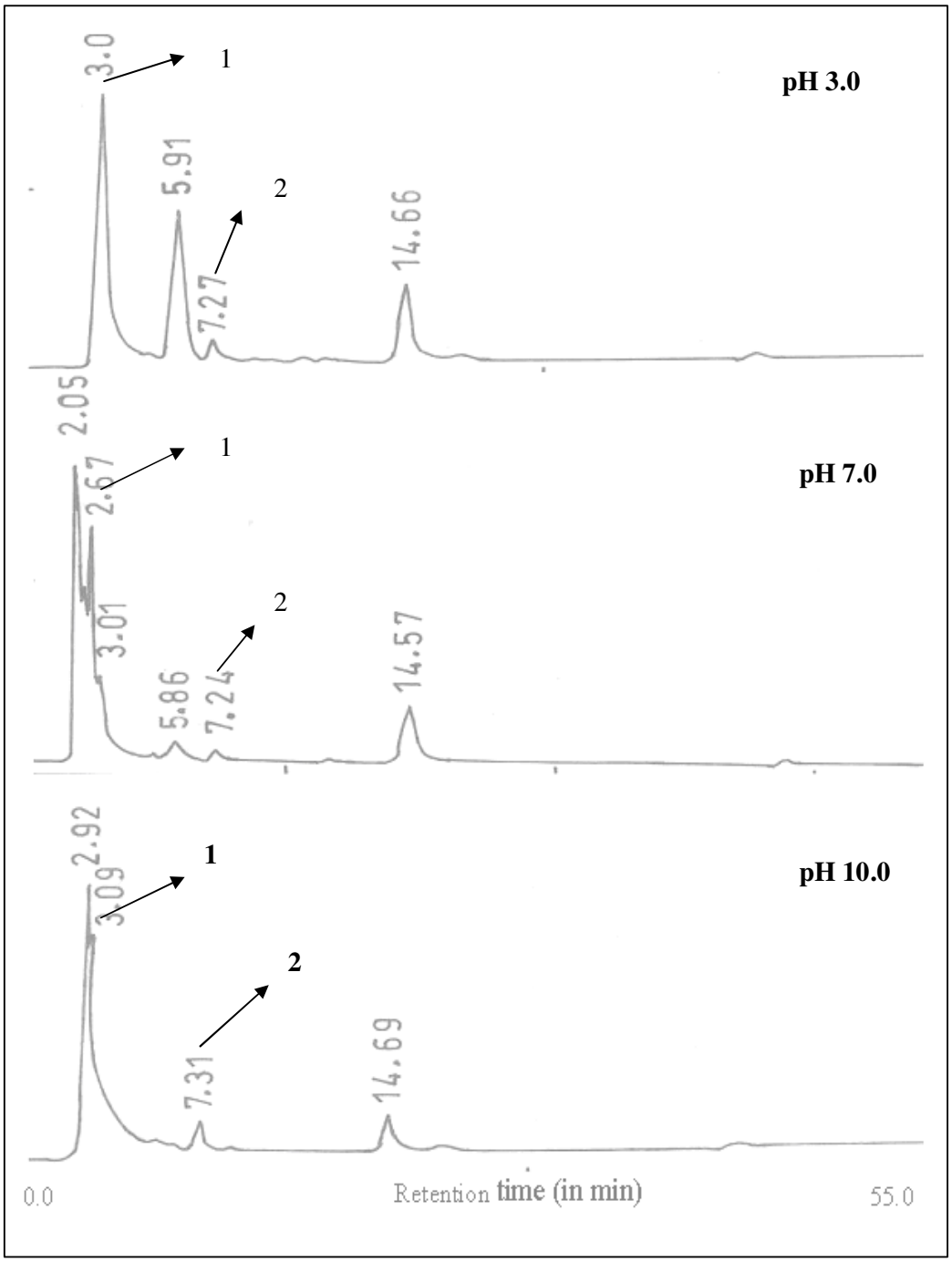


Fig. 15. Fractionation of the phenolics of the precipitate formed at different pH by HPLC at 295nm. Peak numbers (1) gallic acid; (2) proto-catechuic acid.

Phenolic acids may form both ester and ether linkages owing to the bi-functional nature through reactions involving their carboxylic and hydroxyl groups respectively. This allows phenolic acids to form cross-links with the cell wall macromolecules (Yu et al. 2001). Significant amounts of alk(en)yl resorcinols containing non-isoprenoid side chain (15 - 25 carbons in length) attached to the hydroxybenzene ring have also been detected in some cereals (barley, rye, wheat) (Ross et al. 2003).

Characterization of phenolics by NMR

The NMR spectra of *p*-hydroxybenzoic acid [^{1}H: 7.90, 1H, dd, J = 2 and 2.5 Hz; 7.89, 1H, dd, J = 1.5 and 3 Hz; 6.84, 1H, dd, J = 1.5 and 3 Hz; 6.82, 1H, dd, J = 2 and 2.5 Hz; ^{13}C NMR: 114.3, 121.0, 131.3, 161. 6 and 168.3 ppm] (**Fig. 16**), gallic acid [^{1}H: 7.07, s, 2H; ^{13}C NMR: 108.6, 120.3, 144.7, 168.7 ppm] (**Fig. 17**) and *trans*-cinnamic acid [^{1}H: 7.69, 1H, d, J = 16 Hz; 7.59-7.64, m, 2H; 7.39-7.45, m, 3H; 6.50, 1H, d, J = 16 Hz; ^{13}C NMR: and 117.7, 127.4, 128.3, 129.7, 134.2, 144.6 and 168.6 ppm] presented in **Fig. 18,** confirms the identification carried out by HPLC and LC-MS. Based on this it can be conclusively inferred that the gallic, *trans*-cinnamic, vanillic, ferulic, syringic, coumaric, protocatechuic, hydroxybenzoic acids and querectin are constituent of finger millet polyphenols. However, still further investigations needs to be carried out to identify the remaining phenolics obtained on the other individual peaks of the HPLC chromatograms.

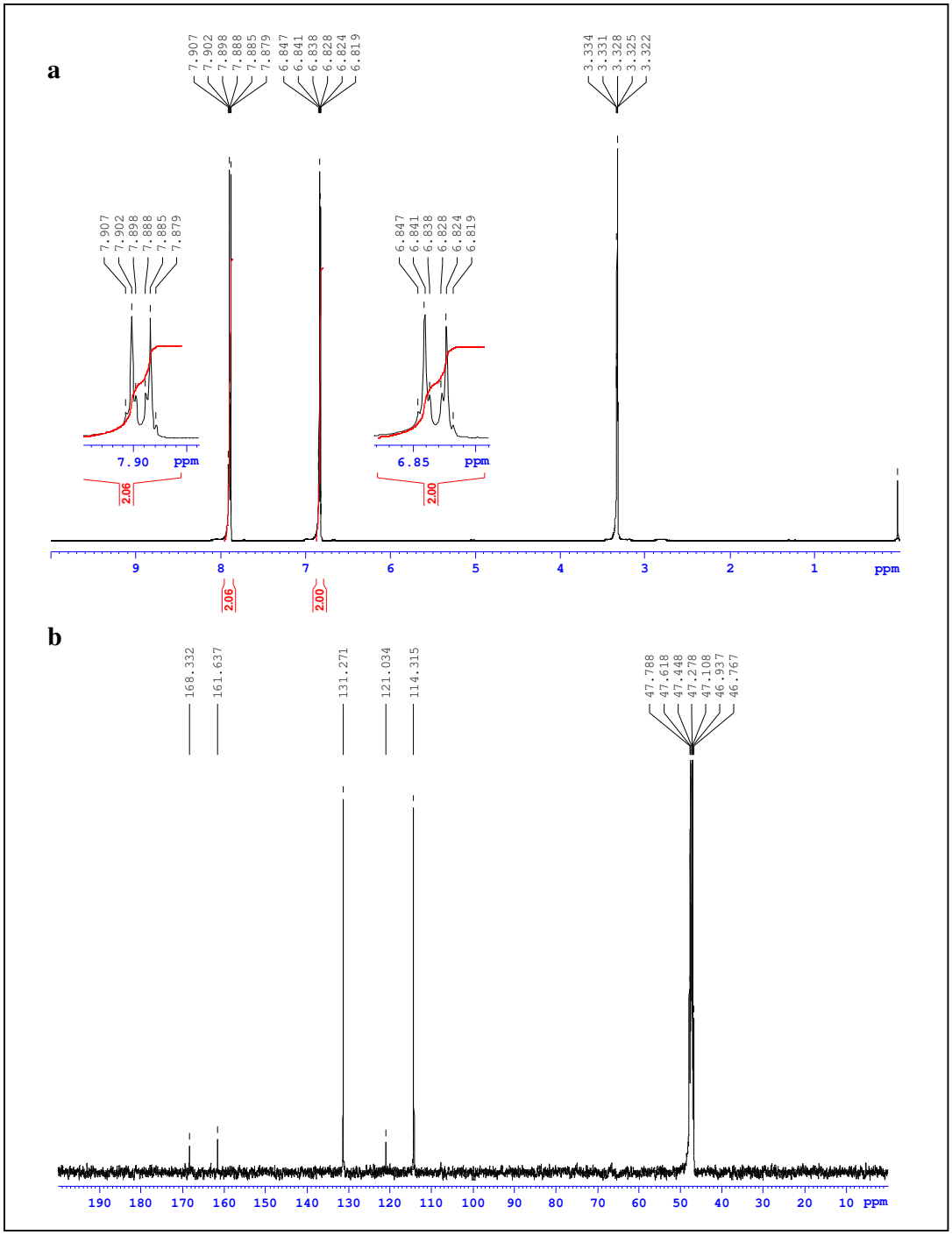


Fig. 16. NMR spectra for *p*-hydroxybenzoic acid. a. ^{1}H proton NMR spectra, b. ^{13}C NMR spectrum for *p*-hydroxybenzoic acid of finger millet.

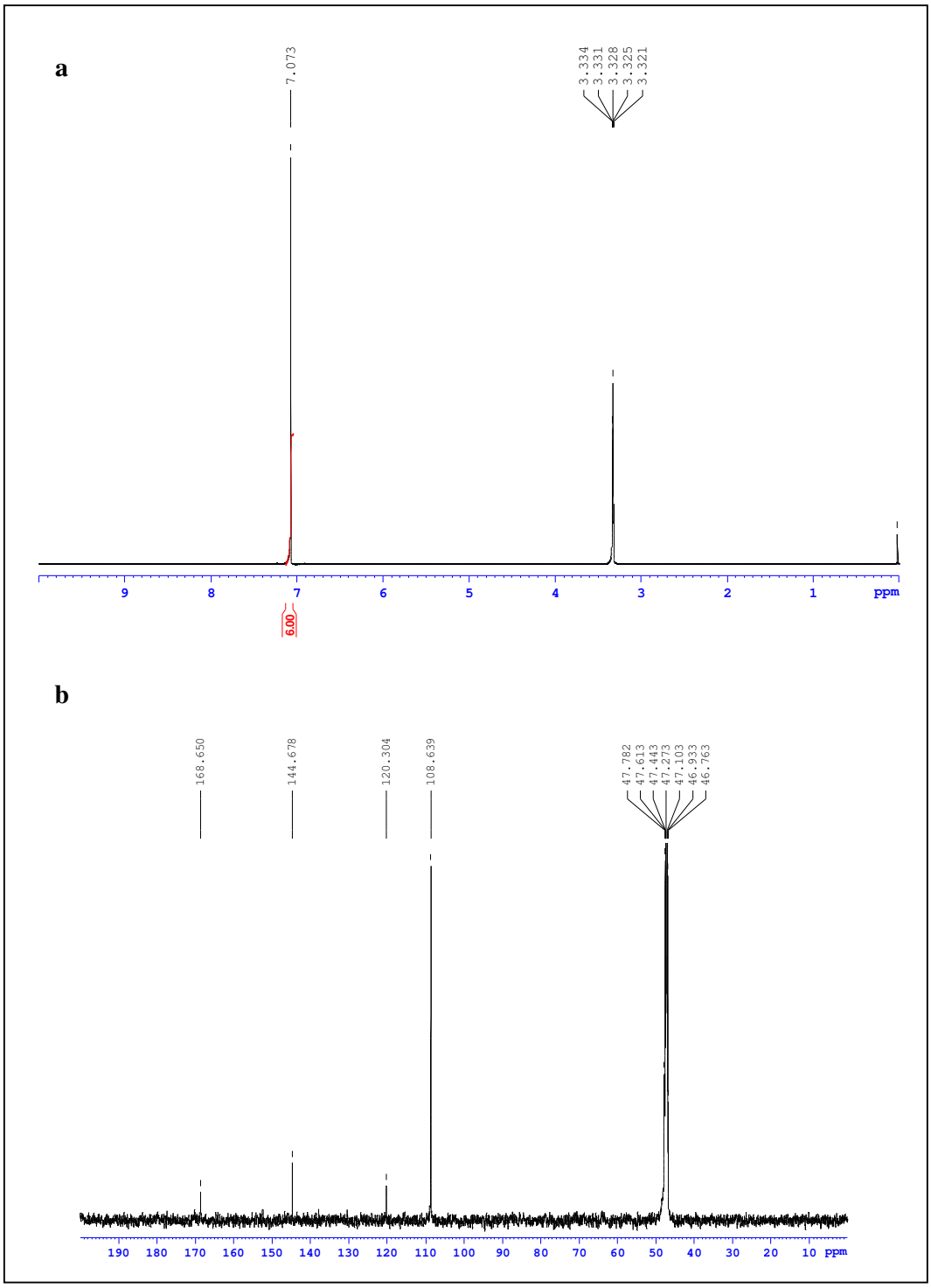


Fig. 17. NMR spectra for gallic acid. a. ^{1}H proton NMR spectra, b. ^{13}C NMR spectra of gallic acid.

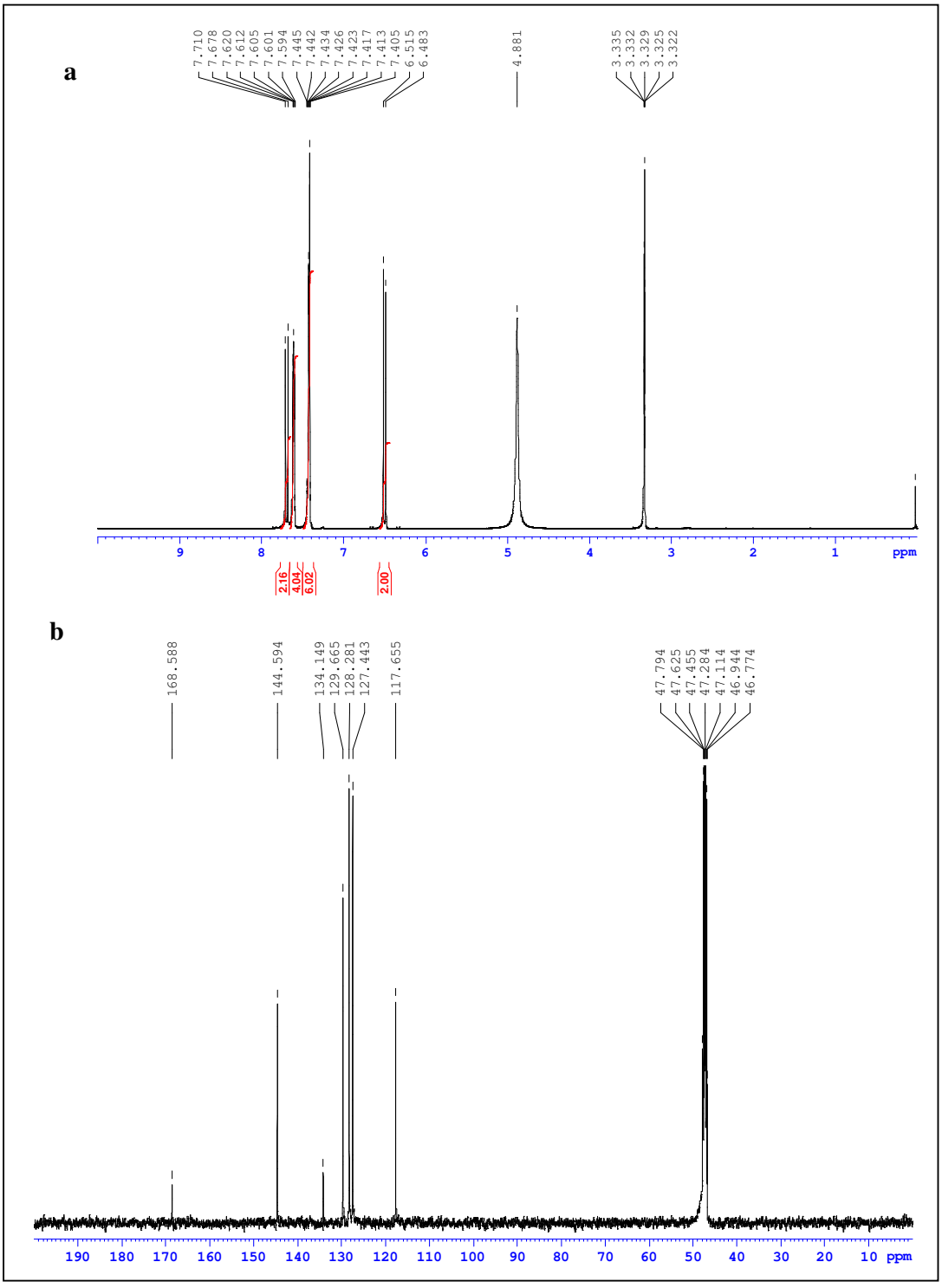


Fig. 18. NMR spectra for *trans*-cinnamic acid. a. ^{1}H proton NMR spectra for *trans*-cinnamic acid, b. ^{13}C NMR spectra for *trans*-cinnamic acid.

Summary and conclusions

- To extract the polyphenols of finger millet, a few polar and non-polar solvents were tried and it was observed that, acidic-methanol (99 ml methanol : 1 ml conc. HCl) solvent system was very effective extractant.
- From the studies on the varietal variations with respect to the polyphenols content of the millet, it was noticed that, varieties with brown seed coat contained (1.2 - 2.3 %) higher proportion of polyphenols than white seed coat (0.3 - 0.5 %) varieties.
- The histochemical and the analysis of the milling fraction of the millet for the polyphenols content indicated that, about 70 % of the polyphenols are concentrated in the seed coat and the remaining is distributed in the endosperm cell walls. Accordingly, a protocol to prepare polyphenols rich seed coat matter was developed.
- The polyphenols of the millet seed coat matter extracted in acidic methanol exhibit very high sensitive to the changes in pH. Neutralizing the extract results in the formation of precipitate, and the quantity of precipitate formed increases concurrently with increase in pH. At high alkaline pH (pH 12) condition, nearly 45 % of the extracted matter formed precipitate. As the pH increases, the polyphenols content in the extract decreases.
- Nine phenolic acids namely, gallic, protocatechuic, *p*-hydroxybenzoic, vanillic and ferulic acids (benzoic acid derivatives) and syringic, *trans*-cinnamic and *p*-coumaric acids (cinnamic acid derivatives) and also a flavonoid, namely, quercetin, were identified by RP-HPLC in the polyphenols extracted.

- The structural analysis of three of the phenolics isolated from the millet, namely gallic, *p*-hydroxybenzoic and *trans*-cinnamic acids, were purified by HPLC, LC-MS and their identity was confirmed by NMR spectroscopy.

From these observations, it may be concluded that, disruption of the millet cell walls by acidic methanol is essential to release the polyphenols for their effective extraction. The millet phenolics are stable in acidic conditions and are denatured in alkaline conditions. Only nine phenolic acids were identified from the complex mixture of the millet polyphenols extracted. The information generated may facilitate isolation of specific phenolics of pharmaco-nutritional importance.

Introduction

Polyphenols are known to inhibit the activity of enzymes and this has relevance with respect to the digestive enzymes such as amylase, glucosidase, pepsin, trypsin and lipases (Rohn et al. 2002). The role in mediating amylase inhibition has the potential to contribute to the management of type 2 *Diabetes mellitus*, which is characterized by high blood glucose levels (Toeller 1994; Shobana et al. 2008). Polyphenols may act as inhibitors of amylase and glucosidase similar to acarbose, miglitol and voglibose leading to decrease in post-prandial hyperglycemia (Saito et al. 1998; Bailey 2001). One of the therapeutic approaches to decrease postprandial hyperglycemia is to retard absorption of glucose through the inhibition of carbohydrate digesting enzymes, e.g., α-amylase and α-glucosidase, in the digestive tract (Kim et al. 2005).

The accumulation of polyols within the human eye lens is a primary contributing factor for cataract formation. Certain tissues of the body, including the eye lens, do not require insulin for glucose or other simple sugars such as galactose to enter into the cells. In diabetes, the concentration of the sugar is high in the aqueous humor and can diffuse passively into the lens. The enzyme, aldose reductase converts glucose to sorbitol or galactose to galactitol in the eye lens. These polyols cannot diffuse passively out of the lens and accumulate or are converted to fructose. The accumulation of polyols results in an osmotic gradient, which encourages diffusion of fluids from the aqueous humor. A number of compounds, both natural and synthetic, have been found to inhibit aldose reductase. These so called aldose reductase inhibitors (ARIs) bind to aldose reductase, inhibiting the polyol production (Zenon et al. 1990). As a group, flavonoids are among the most potent naturally occurring ARIs. Several *in vitro* studies indicate that the flavonoids inhibit aldose

reductase (Varma et al. 1975; Nakai et al. 1985).

Production and release of venom is an evolutionary adaptation in snakes primarily to immobilize the prey and secondarily to defend and also to support digestion. To bring about these effects, each species have unique venom composition with different amounts of hydrolytic enzymes and specific systemic toxins (Chippaux et al. 1991). The common hydrolytic enzymes found in most venom are proteolytic enzymes, phospholipases and hyaluronidases and are responsible for local tissue damage (Shashidharamurthy et al. 2002). Several flavonoids and retinoids (Fawzy et al. 1988) have been characterized as PLA_2 inhibitors. Inhibition of PLA_2, a rate limiting step in inflammatory reaction is a key event and the inhibitor of this enzyme has potential therapeutic relevance in inflammatory disorders.

Types of enzyme inhibitions

Enzyme inhibition can be of two types namely, irreversible and reversible.

Irreversible inhibition

An irreversible inhibitor binds tightly, often covalently to amino acid residues at the active site of the enzyme and permanently inactivates the enzyme. Examples of irreversible inhibitors are diisopropylphosphofluoridate (DIPF), iodoacetamide and penicillin.

Reversible inhibition

Reversible inhibitors bind to the enzymes using weak bonds, similar to those used in binding the substrate. These bonds are formed rapidly, but also break easily. In consequence, the reversible inhibitors are effectively instantaneous in their action, but do not disable the enzyme permanently. Reversible inhibition can be subdivided into competitive, noncompetitive and uncompetitive.

A competitive inhibitor competes with the substrate molecules for binding to the active site of the enzyme.

A noncompetitive inhibitor binds at a site other than the active site of the enzyme and decreases its catalytic rate by causing a conformational change in the three dimensional shape of the enzyme.

The key features of uncompetitive inhibitor are that, they are incapable of binding to free enzyme and can only bind to the enzyme –substrate complex. This could be because the substrate itself is directly involved in binding the inhibitor or because it brings about a conformational change in binding site of an inhibitor which otherwise happen to be incapable of binding the inhibitor. Once the inhibitor binds, it prevents the enzyme from turning the substrate in to the product. This reduction in the effective concentration of the E-S complex increases the enzymes apparent affinity for the substrate (K_m is lowered) and decreases maximum enzyme activity (V_{max}) as it takes longer time for the substrate or product to leave the active site. Uncompetitive inhibition works best when substrate concentration is at high levels.

Amylases of finger millet malt

Malting of cereals is an important food biotechnological process. Essentially malting process consists of three unit operations, namely (a) steeping, (b) germination, and (c) kilning (**Fig. 19)** (Malleshi and Desikachar, 1982; Leloup et al. 1994). During steeping, the grains are hydrated to a level that facilitates uniform germination and normally 10 – 20 h of steeping at ambient temperature is followed in the tropical region for most of the cereals including finger millet. Very short duration of steeping leads to uneven germination whereas a long period of steeping retards germination.

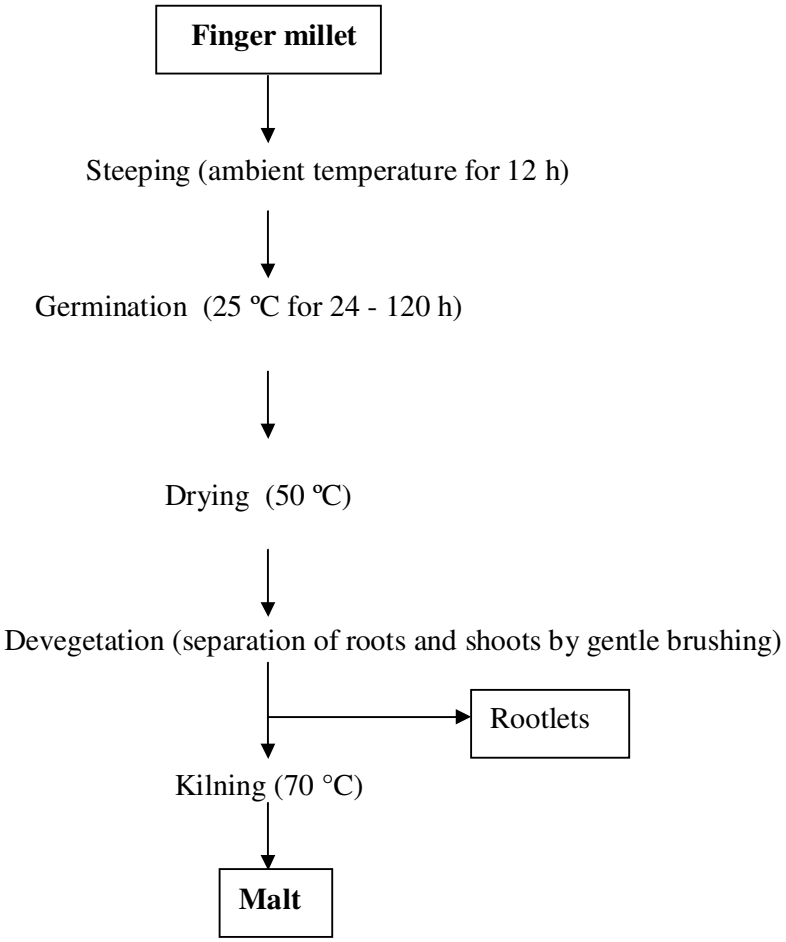


Fig. 19. Flow chart for preparation of finger millet malt

Finger millet, one of the important tropical cereals is malted for food and allied uses. It's malt is used in various specialty foods, especially to prepare low bulk and nutrient dense foods (Desikachar and Malleshi 1985). The amylases developed during germination, remain dormant and hydrolyse the starch on cooking the slurry, thereby reducing the water holding capacity of starch, or in other terms lowering the viscosity of the foods (Malleshi and Desikachar 1982).

Germination is the most crucial step in the malting process where the induction of various hydrolases takes place. These include amylases, proteases, esterases, pentosanases, glucanase etc. However, the level of enzyme development or their activation depends on the variety, the amount of endogenous gibberllic acid present in the seed and also on the temperature and duration of germination (Chavan and Kadam 1989).

Kilning of the sprouted cereals is the process of drying and curing the malt. Kilning dehydrates the sprouts and arrests the process of germination and related biological activity of the germinating grains. Normally the germinated millet is kilned at about 70 °C so that the malt amylases are not affected by this treatment (Meredith and Jenkins 1973).

Amylases are most important enzymes of malt, which have large implications about the end uses of malt. They specifically cleave the *O*-glycosidic bonds in starch, the storage polysaccharide of cereals. The malt amylases are mainly of two groups, namely, endoamylases and exoamylases. The endoamylases (α-amylases) also known as "liquefying" enzymes, which cleave α-1, 4 glycosidic bonds in amylase and amylopectin and also the related polysaccharides. The products of hydrolysis of these enzymes are oligosaccharides of varying chain length, and will have the α-configuration at the C_1 site of the reducing glucose unit. The endoamylases hydrolyze

the bonds located in the inner regions of the substrate resulting in rapid decrease of the viscosity of the starch solution as well as decrease its iodine staining power (Hill and MacGregor 1965).

Exoamylases (β-amylases), also known as "Saccharifying" enzymes and cleave α-1, 4 glucosidic bonds of amylose and amylopectin from the non-reducing end by successive removal of maltose/glucose in a stepwise manner. The products of hydrolysis with the β-amylase are maltose, glucose and β-limit dextrins (Bird and Hopkins 1954). In contrast to the action of endoamylases, this results in a slow decrease in the viscosity or iodine staining power of starch. In the case of finger millet, α-amylase is prominent although, B-amylase contributes for the hydrolytic capacity of the malt.

A number of analytical techniques are available for the assay of amylase activity. The most common procedures followed are reducing of carbohydrates by (a) alkaline copper method of Nelson (1944), (b) alkaline 3, 5, dinitrosalicylic acid (DNS) method of Bernfeld (1955), and (c) alkaline ferricyanide method of Robyt et al. (1972). Besides these, the semi-quantitative determination of starch hydrolysis by α-amylase involves the measurement of the decrease in the intensity of blue color produced by starch when complexed with iodine solution (VanDyk and Caldwell 1956). Among these methods, DNS method is the most commonly used because of its simplicity and reliability. These methods measure the new reducing groups formed upon the amyloytic hydrolysis of starch. The enzyme activity is normally represented as the micromoles of products formed or substrate transformed per minute under defined conditions of incubation time and temperature.

Active site of amylase

The active site of the α-amylase is situated in a long cleft of about 3 nm, located between the carboxyl end of the A domain and the B domain and consists of 5–11 sub sites (A-K). The catalytic site is situated between sub sites F and G, and both sites will be occupied by Asp-206 or Glu–230 or Asp-297 and the reducing end of the glucose chain is located towards K sub-site. Difference in the specificity of α-amylase i.e. in the detailed way in which they hydrolyze a polysaccharide has been explained in terms of sub sites at the active site. Interaction of the enzyme with the primary hydroxyl group of a glucose ring unmodified at C-6 is an important requirement for binding at that sub- site.

Two mechanisms have been proposed for the action of α-amylase on amylose in solution, i.e. multiple attack and multichain attack (Banks and Greenwood 1977; Mazur 1984). In the multiple attack, the encounter between enzyme and substrate is a chance factor and all the bonds are equally liable for hydrolysis. After hydrolysis, only one molecule is released, the other retained by the enzyme, slides along the active site and undergoes another hydrolysis. The multiple attacks lead to the formation of small saccharides during early stages of amylose hydrolysis whereas multichain attack doesn't. In the multichain attack mechanism, the encounter between enzyme and the substrate leads to a single hydrolysis, both molecules being released after the catalytic event.

Inhibitors of amylases

All the metal chelators are strong inhibitors of amylases they are metalloenzymes (Muralikrishna and Nirmala 2005). EDTA was one of the most important inhibitor of α-amylases, since they remove loosely bounded calcium which is important for their activity (Bush et al. 1989). Polyphenols are known to inhibit the

activity of enzymes such as amylase, glucosidase, pepsin, trypsin and lipases (Rohn et al. 2002). Acarbose is a natural product which inhibits amylases (Pohl 2005).

Aldose reductase from cataractous human eye lenses.

Blindness in diabetics is largely due to retinopathy and/or cataract. Cataract is the opacification of the lens, which interferes with the transmission of light on to the retina. Diabetes is the major risk factor for cataract and cataractogenesis is a severe metabolic disorder characterized by hyperglycemia. The prevalence of blindness in Indian population is 15 per 1000, while cataract alone accounts for 80 % of this blindness. Despite controlling blood glucose levels, chronic exposure to elevated levels of glucose due to prolonged diabetes can cause hyperglycemic injury to tissues that are insensitive to insulin such as eye lens.

Activation of polyol pathway due to increased activity of aldose reductase (AR) has been implicated in the diabetic cataract. The polyol pathway is a two-step metabolic pathway in which glucose is reduced to sorbitol, which is then converted to fructose. Aldose reductase (alditol: $NADP^+$ 1-oxidoreductase, EC 1.1.1.21) has been implicated in the etiology of complications of diabetes such as neuropathy, nephropathy and retinopathy including cataractogenesis (**Fig. 20**). This enzyme reduces glucose to sorbitol in the presence of nicotinamide adenine di-nucleotide phosphate (NADPH).

The major cause of the complications of diabetes could be the change in the osmotic pressure due to the accumulation of sorbitol, which is relatively impermeable through biological membranes. Increased levels of sorbitol in the tissues of diabetic subjects and also in diabetic animals have indeed been reported (Kubo et al. 1999). It has been reported that, the cataract progression can be slowed or prevented by the use of natural therapies, particularly with those plants having high flavonoid contents

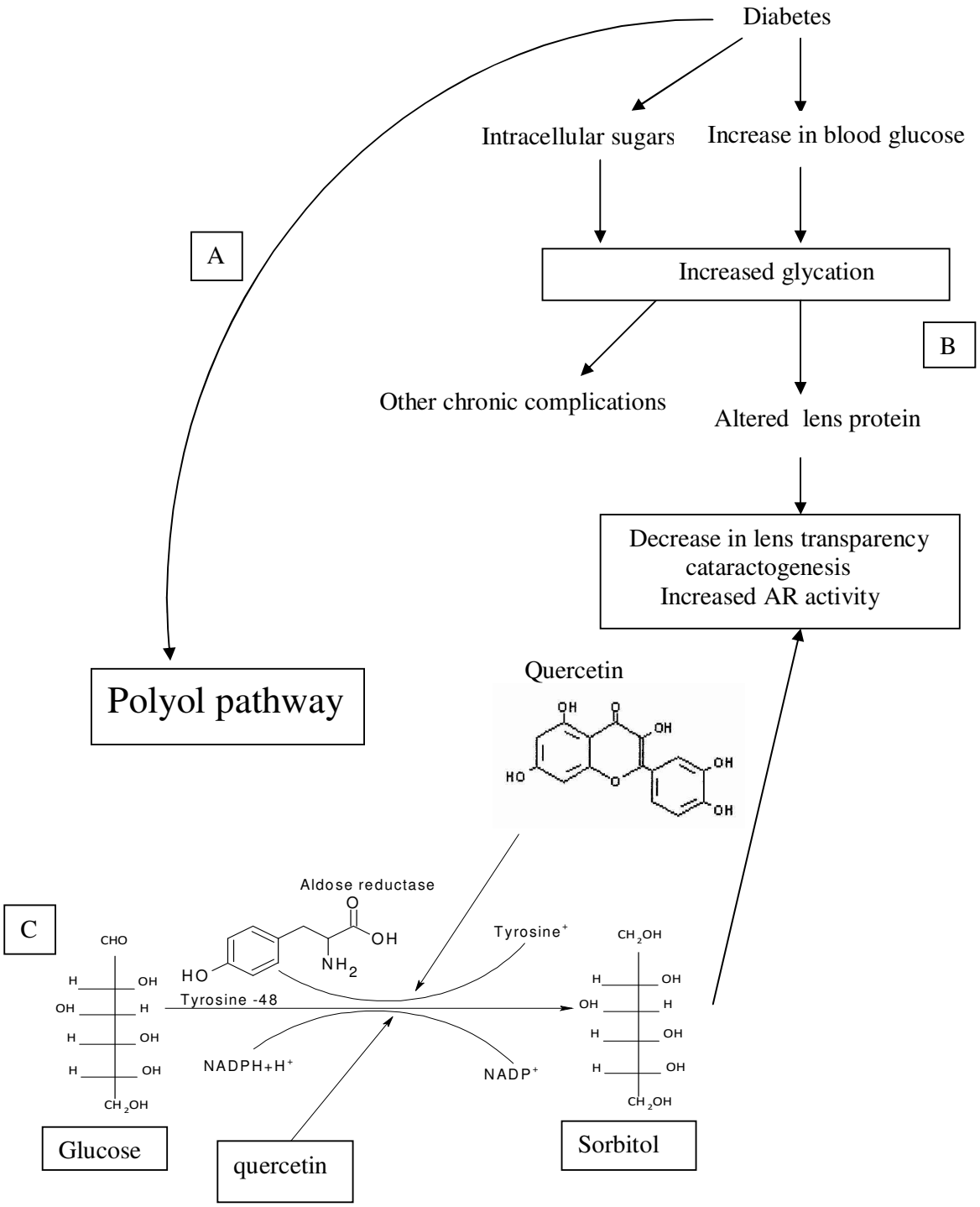


Fig. 20. Etiology of complications of diabetes, (A) complications leading to polyol pathway, (B) complications leading to decrease in lens transparency and formation of cataract and (C) action of quercetin inhibiting polyol pathway in two modes.

mainly by AR inhibitory effect (Lim et al. 2001). Hence, inhibition (or ablation) of aldose reductase, the first and rate-limiting enzyme in the pathway, reproducibly prevents diabetic retinopathy.

Phospholipases (PLA_2) from snake venom

Snake venom PLA_2's cause for a wide range of pharmacological pathogenesis like neurotoxicity, myotoxicity, cardiotoxicity, cytotoxicity, anticoagulant activity, edema, hemolytic activity, hypotensive activity, apoptosis and hemorrhagic activity. A very common localized effect seen on snakebite is swelling and edema of the bitten region. Although inflammation is a complex process involving many factors like enzymes, hormones, toxins and trauma, regulation of PLA_2 activity decreases inflammation significantly than altering any other factors that could contribute to inflammatory reaction (Touqui and Alaoui-El-Azar 2001).

General mechanism of PLA_2 catalysed reaction comprises the substrate phospholipid, involved in polar interaction (hydrogen bonding), specifically His-48 of the enzyme with oxygen atom carbonyl group of the ester at Sn-2 position of phospholipid. The orientation of a water molecule by hydrogen bonding to the active site of the histidine, dictates the pH dependence of 7–9 for all PLA_2 with a catalytic histidine. Furthermore, located adjacent to the catalytic histidine is a conserved aspartate, forming the so-called His/Asp dyad. This Asp is essential for calcium, which forms the positively charged transition state of the PLA_2 reaction. This is the origin of the calcium dependence of the histidine PLA_2 enzymes. One more polar interaction was between hydrogen of NH group of the Lys 69 residue with the carbonyl group of ester at Sn-1 position of the phospholipid. Water acts as nucleophilic after hydrogen has been abstracted from His 48 of the enzyme. This reaction releases the hydroxyl group (OH), which attacks the electrophilic carbon

of the carbonyl group of the ester at Sn-2 position. This attack results in the hydrolysis of phospholipid at Sn-2 position releasing proinflammatory fatty acid (arachidonic acid) and lysophospholipid (Davidson et al. 1987). Therefore, treatment of inflammation using nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit either cyclooxygenase or lipoxygenase enzymes, which are involved in inflammatory reactions. Thus, inhibition of PLA_2, a rate-limiting step in inflammatory reaction is a key event and the inhibitor of this enzyme has potential therapeutic relevance in inflammatory disorders. In many of the snake venoms, the principle toxic component is a PLA_2 enzyme and its pharmacological actions overlap with catalytic activity of the enzyme (Rosenberg 1997). Thus specific PLA_2 inhibitor to these groups of enzymes has a greater therapeutic application in reducing the venom toxicity (Chandra et al. 2002).

In the present study, the inhibitory effects of the millet polyphenols on the millet malt amylases, aldose reductase of human eye lenses and also the snake venom phospholipases (PLA_2) have been investigated. Besides, the possible nature of inhibition and mode of action of the millet polyphenols on the millet malt amylases and also on aldose reductase from cataractous human eye lenses were also studied through kinetic studies, using Michaelis-Menten and Lineweaver-Burk (LB) equations.

Materials and methods

Chemicals

Escherichia coli [lyophilized cells of strain W (ATCC 9637)], Reduced Nicotinamide adenine dinucleotide phosphate, CM-sephadex and sephadex G-50 from Sigma Chemical Co. (St. Louis, MO, USA), Methanol (HPLC grade), lithium sulphate (Li_2SO_4), Starch, phosphate buffer saline and β-mercaptoethanol from Merck (India). ^{14}C-oleic acid was obtained from Perkin Elmer Life Sciences Inc. (Boston, MA, USA), fatty acid free bovine serum albumin (BSA) fraction V was purchased from PAA Laboratories (Haidmannweg, Austria). 3, 5-dinitrosalicylic acid (DNS) from Loba Chem., Bombay, India. Agar agar, beef extract, peptone and lactose were obtained from Himedia Laboratories Private Limited (Mumbai, India), Scintillation cocktail (Ultima Gold) from Packard Bioscience (USA). All reagents were of analytical and HPLC grade.

Materials

Finger millet: GPU 28 variety of the millet was used for malting and extraction of amylases.

Snake venom: Lyophilized powder of *Naja naja* snake venoms from Hindustan Park (Kolkata, India) and Irula Co-operative Society Ltd. (Chennai, India) were procured.

Cataractous human eye lenses were collected from a local eye hospital (Mysore Race Course Eye Hospital, Mysore, India).

Mice

Swiss Wistar *albino* mice weighing 20-25 gms, were obtained from Central Animal House Facility, Department of Studies in Zoology, University of Mysore, Mysore, India. The animal care and handling were conducted in compliance with

National Regulations for Animal Research. The animal experiments were carried out after reviewing, the protocols by the Animal Ethical Committee of the University of Mysore, Mysore, India vide sanction order no. MGZ/1041/08-09.

Methods

Preparation of finger millet malt

The millet seeds (1 kg) and soaked in fresh water (steeped) for 12 h. The steep water was changed once in 6 h and finally the excess water was drained off and the seeds were couched on moist cloth and germinated up to 5 days 25 °C in BOD incubator. Water was sprayed during germination to keep the grains moist, the sprouts were withdrawn from the germination bed at an interval of about 24 h and dried at 50 °C in an air oven for 6 – 8 h and the vegetative growth was removed by gentle brushing. Devegetated seeds were powdered in a laboratory cyclone mill (Udy Company, Boulder, CO. USA) and the whole meals were used for extraction of the amylases (Malleshi and Desikachar 1982).

Extraction of amylases

The whole meal malt samples were suspended in 3 volumes (w/v) of 0.1 M sodium acetate buffer (pH 4.8), the contents were agitated by continuous shaking for 2 h at 4 °C, centrifuged for 20 min at 6,500 × g at 4 °C using refrigerated centrifuge. The supernatant containing the enzymes was dialyzed against the extraction buffer and its activity was assayed. The polyphenols content of the malt samples was also extracted and assayed as described earlier.

Assay of amylase activity

Reagents

a. DNS reagent: 3, 5-dinitrosalicylic acid (DNS) (1 g) was dissolved in 80 ml of 30 % sodium potassium tartarate to which 20 ml of 2 N sodium hydroxide was also added. The contents were mixed well and filtered.

b. Buffer (0.02 M): Sodium acetate (4.8 g) was dissolved in double distilled water, pH of this solution was adjusted to 4.8 with 0.1 M acetic acid and the volume made up to 1 L with distilled water.

c. Soluble starch (1 %): Starch (1 g) dispersed in ≈ 70 ml of double distilled water. The starch solution was boiled for 10 min with stirring to gelatinize the starch completely and diluted to 100 ml with distilled water.

d. Standard maltose: Maltose (2 mg/ml) was prepared in double distilled water.

Procedure

Amylase activity was assayed according to Bernfeld (1955). The starch solution in sodium acetate buffer (50 mM, pH 4.8) was incubated with appropriate aliquots of the enzyme extracts at 37 $^{\circ}$C for 30 min. The reaction was terminated by adding adequate quantity of 3, 5-dinitrosalicylic acid reagent. The amount of reducing sugars released were quantified by measuring the optical density (OD) at 540 nm. The amount of reducing sugars were calculated from a standard curve of maltose. One unit of enzyme activity was defined as micro mole maltose equivalent released min^{-1} under the assay conditions.

$$\text{Amylase activity (micro mole)} = \frac{\text{maltose (µg) released}}{\text{Molecular weight of maltose}}$$

Amylase inhibitory activity

The assay of the amylase activity of the malt samples revealed that, the malt sample prepared from 96 h germinated millet contained highest level of amylase activity. Hence, the enzyme extracted from 96 h germinated sample was used for the inhibitory studies. The crude polyphenols extracted from native millet seed coat matter was evaluated for its inhibitory activity on the malt amylases according to Maeda et al. (1985). An aliquot of the enzyme extract was pre-incubated with different levels of polyphenols (0 - 200 µg/ml) at 37 °C for 10 min and the residual amylase activity was quantified as described above.

Similarly, the inhibitory activity of individual phenolic compounds obtained by HPLC fractionation was also determined as described above by pre-incubating a single concentration of phenolic compound (50 µg/ml).

Amylase activity staining

Polyacrylamide gel electrophoresis under native condition was carried out according to the method of Davis (1964).

Polyacrylamide gel electrophoresis

Vertical slab electrophoresis was carried out on a mini slab gel electrophoresis (Broviga) unit at ambient temperature.

SDS Polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE at pH 8.3, was carried out according to the method of Laemmli (1970).

Preparation of reagents

a. Acrlyamide (14.6 g) and bis-acrylamide (0.4 g) were dissolved in water and volume made up to 50 ml, filtered and stored in a brown bottle at 4°C.

b. TRIS (18.5 g) was dissolved in water and volume made up to 100 ml and the pH of the solution was adjusted to 8.8 with HCl (6 N) and stored at 4°C.

c. TRIS (3g) was dissolved in water and the pH of the solution was adjusted to 6.8 with HCl (6 N), volume made up to 50 ml and stored at 4°C.

d. Sodium dodecyl sulfate (SDS) (10 g) was dissolved in water and volume made up to 100 ml.

e. TRIS (0.3g), glycine (1.44 g) and SDS (0.1 g) were dissolved in water and volume made up to 100 ml.

f. Coomassie brilliant blue (0.2 g) was dissolved in a mixture of methanol: acetic acid: water (25:15:60, v/v)

g. Destaining reagent: Methanol: acetic acid: water (25:15:60, v/v).

Running gel (12 %) was prepared by mixing solutions 'a' (4 ml) and 'b' (2.5 ml) with water (3.4 ml). The mixture was degassed and to that was added the solution 'd' (0.1 ml), N,N,N′N′- tetramethyl ethylene diamine (TEMED, 20 µl) and ammonium persulphate (6 mg). The contents were mixed and poured between the assembled glass plates with edges sealed with agar (2 %). Gels were allowed to polymerise at room temperature for 30 min.

Stacking gel (5 %) was prepared by mixing solutions 'a' (0.83 ml) and 'c' (1.25 ml) with water (3.0 ml) and degassed. To the mixture solution 'd' (0.05 ml), TEMED (10 µl) and ammonium persulfate (2 mg) and poured above the polymerized running gel. The polymerization of the stacking gel was facilitated at room temperature for 30 min. The gels thus prepared were of the size 105 × 90 mm.

Samples were prepared by dissolving protein (10 - 25 µg) in solution 'c' (0.1 ml) containing SDS (4 %), mercaptoethanol (10 %) and glycerol (20 %). To the above mixture, bromophenol blue was added and heated in a boiling water bath for

5 min. Samples on cooling were loaded onto the wells immersed in solution 'e' and were run at constant voltage (50 V) for 3 – 4 min 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 standard mixture was used as molecular weight markers. The lyophilized marker mixture 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 Coomassie blue reagent for 6 h at room temperature followed by destaining in reagent 'g'

Ten percent gels were prepared without the addition of SDS. The amylases containing about 1 – 2 µg of protein was loaded on to the gel. After running the electrophoresis, the gel was immersed in gelatinized and cooled starch solution (2 %) containing 50 mM sodium acetate buffer at pH 5.0 and incubated at 45 $^{\circ}$C for 30 min. The gel was then rinsed with distilled water and stained in a solution containing 3 % iodine and allowed to stain for 10 min. The bands representing amylase were visualized as transparent bands on a dark blue background. Similarly, for the determination of inhibitory effects of polyphenols on the amylase activity, the gel after electrophoresis was pre-incubated with the inhibitor (polyphenol solution) and then with the substrate and incubated at 45 $^{\circ}$C for 30 min, and thereafter, the gel was stained with iodine reagent. The amylase activity staining showed clear bands against dark blue background.

Kinetics of amylase inhibition

The inhibitory activity of polyphenols against amylases was measured with increasing concentrations of starch as a substrate (0.5 – 2.0 %) in the presence of

phenolics at different concentrations (30 – 700 ηM). Type of inhibition was determined by Lineweaver-Burk (LB) plot analysis of the data, which was calculated from the results according to Michaelis-Menten kinetics. The inhibitory constants (K_i) and dissociation constants of enzyme-substrate inhibitor complexes (K_i') were also determined (Dixon 1953; Bowden 1974). All the experiments were repeated three to four times and the average values were used for plotting the graphs.

Isolation of aldose reductase from cataracted human eye lenses

Fresh cataracted human eye lenses were collected from a local hospital (Mysore Race Course Eye Hospital). The lenses were washed with phosphate buffer saline and their fresh weights were recorded. The lenses were pooled and homogenized in (1:2 w/v) sodium phosphate buffer (135 mM, pH 7.0) containing 0.5 mM phenylmethanesulphonyl fluoride (PMSF) and 10 mM β-mercaptoethanol, centrifuged at 8000 × *g* for 30 min at 4 ºC to isolate the aldose reductase (Halder et al. 2003). The protein content of the suspension was determined by Bradford (1976) method.

Aldose reductase (AR) activity

Reagents

a. Sodium phosphate buffer

   Solution A: Di-sodium hydrogen phosphate (50 mM): Na_2HPO_4 (0.889 g) dissolved in 100 ml double distilled water.

   Solution B: Sodium di-hydrogen phosphate (50 mM): NaH_2PO_4 (0.78 g) dissolved in 100 ml double distilled water.

   The pH of solution A was adjusted to 7.0 with solution B and made up to 200 ml with distilled water.

b. Reduced Nicotinamide adenine dinucleotide phosphate (1 mM): NADPH (83.3 mg) dissolved in 10 ml of sodium phosphate buffer (pH 7.0).

c. Lithium sulphate (100 mM): Li_2SO_4 (0.13 g) dissolved in 50 ml of sodium phosphate buffer (pH 7.0).

d. *DL* - glyceraldehyde (100 mM): Glyceraldehyde (90 mg) was dissolved in 10 ml sodium phosphate buffer (pH 7.0).

Procedure

The AR activity of the lenses was determined as described by Srivastava et al. (1984). The reaction mixture containing 50 mM sodium potassium phosphate buffer (pH 7.0), 0.1 mM NADPH, 0.4 M Li_2SO_4, 10 mM glyceraldehyde (substrate) and appropriate amount of enzyme (0 – 3000 µg protein) was taken in 1 ml cuvette. The reaction was started by the addition of glyceraldehyde and the decrease in the optical density at 340 nm was recorded. The activity was calculated as per the equation;

$$\text{Activity (unit/ml)} = \frac{\Delta\text{A test sample/min} - \Delta\text{A control sample/min}}{6.2 \times \text{vol taken for analysis}} \times \text{total volume (ml)}$$

The specific activity of the AR was calculated as

$$\text{Specific activity (unit/mg protein)} = \frac{\text{Activity (unit/ml)}}{\text{Protein conc. (mg/ml)}}$$

AR inhibitory activity

To 1 ml cuvette containing enzyme (750 µg/ml protein), varying concentration of polyphenol extract (1 - 200 µg/ml) in 50 mM sodium potassium phosphate buffer (pH 7.0), 0.7 µM NADPH, 0.4 M Li_2SO_4 and 2.5 mM of glyceraldehyde (substrate) were added. The reaction was initiated by the addition of glyceraldehyde and the

decrease in the optical density at 340 nm was recorded. The IC_{50} of the polyphenol extract was calculated using Graphpad software (ver 5.0, USA).

The same conditions were used for determining the inhibitory activity of the phenolics obtained by HPLC fractionation (1–200 µg/ml). Guided by this, the phenolic compound that showed higher inhibitory activity was taken further for kinetic studies.

Kinetics of AR inhibition

The inhibition was measured in the presence of commercially available quercetin at different concentrations (0 – 15 µM) and increasing concentrations of glyceraldehyde (0.05 – 2.5 mM). Mode of inhibition was determined following LB plot analysis of the data and calculated according to Michaelis–Menten kinetics in order to understand the probable mode of action.

Purification of PLA_2 from Naja naja venom

PLA_2 from *Naja naja* venom was purified to homogeneity as described by Rudrammaji and Gowda (1998). Briefly, *Naja naja* venom (110 mg) was fractionated on CM-Sephadex C-25 column (1.4 × 120 cm) using phosphate buffer of different molarities (0.02 - 0.4) and pH (7.0 - 8.5). This was eluted into thirteen major fractions. Fraction PLA_2 was chosen for further purification. The lyophilized fraction (16 mg) was rechromatographed on CM - Sephadex C-25 column (1.6 × 30 cm) which was pre- equilibrated with 0.1 M phosphate buffer of pH 8.0. The protein was eluted using phosphate buffers of 0.1 M, pH 8.0 and 0.15 M, pH 8.0 respectively. This was eluted into two fractions. These 2 fractions were dissolved separately in 0.1 M NaCl and loaded on to Sephadex G-50 column (1.4 × 92 cm) which was pre-equilibrated with 0.1 M NaCl and also eluted with the same solvent. Both the peaks

were homogenous and showed PLA_2 activity. Homogeneity was checked by SDS-PAGE (Laemmli, 1970) and by HPLC.

Preparation of ^{14}C-oleate labeled E. coli (substrate)

^{14}C-oleate labeled *E. coli* substrate was prepared according to the protocol developed by Patriarca et al. (1972). A single colony of *E. coli* was picked to prepare a mini culture using 10 ml of lactose bile broth (LBB). The culture was grown overnight at 37 ^{o}C in rotating (90 rpm) water bath. This mini culture was diluted 100 times with LBB medium and continued incubation at 37 ^{o}C in rotating water bath. The growth of the bacteria was monitored by measuring the absorbance of the culture. An absorbance of 0.6 - 0.9 at 520 nm indicates that the bacteria were in log phase of their growth. These cells were harvested by centrifugation at 1500 *g* for 5 min. The cell pellet was resuspended in 100 ml of LBB. In a separate conical flask 125 µCi of ^{14}C-oleate was evaporated and was re-suspended in 5 ml of 20 % fatty acid free BSA. The prepared *E. coli* culture was mixed with ^{14}C-oleate and incubation was continued at 37 ^{o}C in rotating shaking water bath (90 rpm) for 4 h. The labeled *E. coli* were washed 3 - 4 times with phosphate buffer saline (PBS) and autoclaved to inactivate endogenous PLA_2 activity. The labeled *E. coli* cells were diluted with PBS and similarly prepared autoclaved cold *E. coli* cells to get 10,000 counts per minute (cpm) in 30 µl of diluted sample. This preparation was used further as substrate for assay PLA_2 enzyme activity.

Phospholipase assay

The PLA_2 activity of *Naja naja* venom was measured using ^{14}C-oleate labeled autoclaved *E. coli* (Vishwanath et al. 1993).

Reagents

a. Tris–HCl buffer

   Solution A: Tris (100 mM, pH 7.4): Tris (1.21 g) dissolved in 100 ml double distilled water.

   Solution B: HCl (100 mM, pH 7.4): HCl (0.88 ml) in 100 ml double distilled water.

   Mix 50 ml of solution A and 41.4 ml of solution B and make up to 200 ml with double distilled water.

b. HCl (2N) : HCl (17.48 ml) diluted to 100 ml with double distilled water.

c. Fatty acid free BSA (100 g/L): BSA free from fatty acid (100 g) dissolved in 1 litre of double distilled water.

Procedure

The reaction mixture 350 µl contained 100 mM Tris-HCl buffer pH 7.4, 5 mM calcium and 3.18×10^9 autoclaved *E. coli* cells (corresponds to 10,000 cpm and 60 ηmole lipid phosphorus). The reaction components were mixed in the following order; buffer, calcium, enzyme and water. The reaction was initiated by adding 30 µl of *E. coli* substrate and incubated at 37 ^oC for 60 min. The reaction was terminated by adding 100 µl of 2 N HCl and to that 100 µl of fatty acid free BSA (100 g/L) was added to entrap free fatty acids released. The tubes were vortexed and centrifuged at 20,000 *g* for 5 min. An aliquot (140 µl) of supernatant containing released ^{14}C-oleic acid was mixed with scintillation cocktail (Ultima Gold, Packard Bioscience, USA) and ^{14}C radiation was measured in Packard Scintillation Analyzer (TRI CARB-2100 TR, Hewlett Packard, USA). Enzyme activity was expressed as ηmole of free fatty acids released min^{-1} mg^{-1} protein at 37 ^oC and the activity was calculated as follows;

$$\text{Activity} = \frac{\text{Sample (cpm)} \times 60}{2500}$$

2500 cpm = 60 ηmoles of free fatty acid released

Inhibition of PLA_2 activity

For the assay of PLA_2, the amount of protein was chosen from the purified *Naja naja* venom PLA_2 such that 60 - 70 % hydrolysis of substrate was obtained at 37 °C for 60 min. The crude polyphenols (25-200 µg/ml) and the HPLC fractionated pure phenolics (100 µg/ml each) were used to screen for PLA_2 inhibition.

$$\text{Inhibition (\%)} = \frac{\eta\text{moles of control} - \eta\text{moles of test sample}}{\eta \text{ moles of control}} \times 100$$

The most potent HPLC fraction was chosen to determine the IC_{50} value, which was calculated using Graphpad software (Ver 5.0, USA).

Phospholipase A_2 inducced hind paw mouse edema

Mouse paw edema was carried out according the method (Yamakawa et al. 1976). To induce edema six different PLA_2's were used. The PLA_2 enzyme (50 µl) in saline was injected into the right hind paw (1 µg of PLA_2 from *Naja naja*) with and without fraction 1. In all the cases the left paw received the same volume of saline, which served as control. After 45 min, mice were sacrificed by cervical dislocation and both legs were removed at ankle joints and weighed individually. The increase in weight due to edema was calculated as the edema ratio.

$$\text{Edema ratio} = \frac{\text{weight of edematous leg}}{\text{weight of the normal leg}} \text{ x } 100$$

Results and discussion

Polyphenols content in the millet malt

The changes in the polyphenols content of the millet as a function of germination up to 120 h is presented in **Fig. 21**. The native millet contained about 2.1 % polyphenols and as germination progressed there was reduction in its content. Nearly 44 % of it decreased during the first 24 h of germination and the decreasing trend continued as germination progressed. The polyphenols in the millet are present both in free and bound forms and the initial decrease could be due to leaching during steeping and its hydrolysis during germination. It may also be due to the action of induced esterase activity on bound phenolics, which act on various phenolic acid esters linked either to arabinoxylans or other non-starch polysaccharides (Maillard et al. 1996). A decrease in the polyphenols content during malting of cereals has also been reported earlier (Chukwura and Muller 1982).

Amylase activity in the millet malt

Millet malt, is a rich source of α-amylase, besides being a good source of β-amylases. Various malting conditions including the duration of germination are important factors with respect to the amylase activity of cereals, as it is well known that in all the cereals, the amylase activity increases with the period of germination up to certain point and then decreases (Muralikrishna and Nirmala 2005). In the case of finger millet, the amylase activity increases rapidly during germination up to 96 h and decreases subsequently (**Fig. 21**). The amylases are known to exist in multiple isoforms in different cereals (Beleia and Marston 1981) and the genetic diversity is one of the main reasons for the multiple isoforms (Ainsworth and Gale 1987; Muthukrishnan and Chandra 1988). The Michealis-Menten constant (K_m) and maximum velocity (V_{max}) for the millet amylases were calculated from double

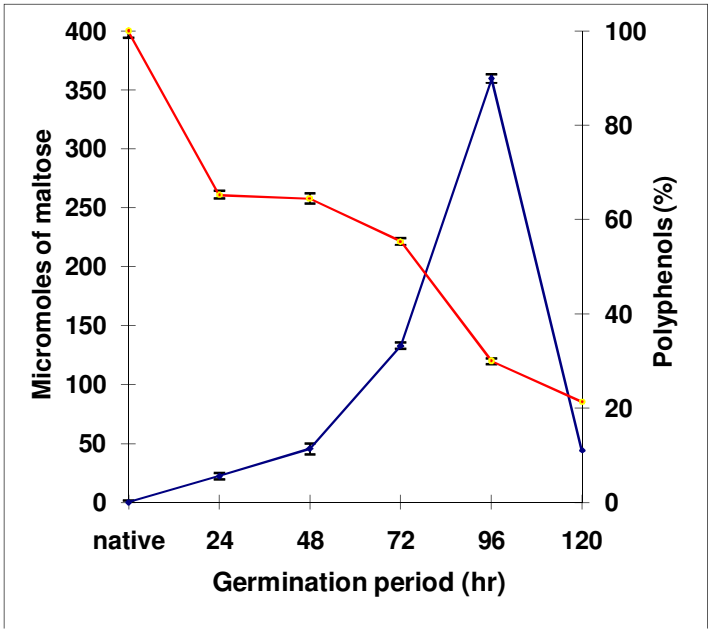


Fig. 21. Total phenolic contents (red) and amylase activity (blue) during the course of finger millet malting. Results are expressed as mean ± standard deviation.

reciprocal plots. The K_m and V_{max} were found to be 1.6 % and 201 units/g respectively, when rice starch was used as a substrate. The amylases of 96 h germinated millet was quantitatively extracted and fractionated on non-denaturing polyacrylamide gel electrophoresis (PAGE). The activity stain assay indicated a single isoform of amylase (**Fig. 22**), in contrast three isoforms reported for the Indaf 15 variety of finger millet by Nirmala and Muralikrishna (2003).

The K_m for barley amylase is reported to be 0.21, 79.3 and 213 μM for amylose, maltodextrin and maltoheptaose respectively (Oudjeriouat et al. 2003). Millet malt amylases exhibit higher apparent affinity for the starch compared to sorghum malt amylases whereas, wheat amylases exhibit high affinity for starch compared to other cereal amylase (Kumar et al. 2005). The kinetic constants for amylases vary with different substrates. The millet malt amylases exhibited V_{max} comparable to other cereal amylases.

Inhibition of the amylase activity

The extent of inhibition of the millet amylases by individual phenolic acids of the millet is presented in **Table 18**. Among the phenolic acids tested, gallic acid (67.7 %), vanillic acid (71.9 %), the flavonoid quercetin (73.5 %) and *trans*-cinnamic acid (79.2 %) were potent inhibitors of the millet amylases. *Trans*-cinnamic acid exhibited a higher degree of inhibition as compared to other phenolic compounds and syringic acid was found to be a weaker inhibitor (~ 56 % inhibition). Even though proto-catechuic acid and *p*-hydroxy benzoic acid were detected as the main derivatives of benzoic acid of the millet polyphenol extract, they were less effective at inhibiting the amylase activity. Depending on the structure, the phenolics react with the enzymes and alter their activity depending on the concentration as well as the number and position of hydroxyl groups of the phenolics (Rohn et al. 2002).

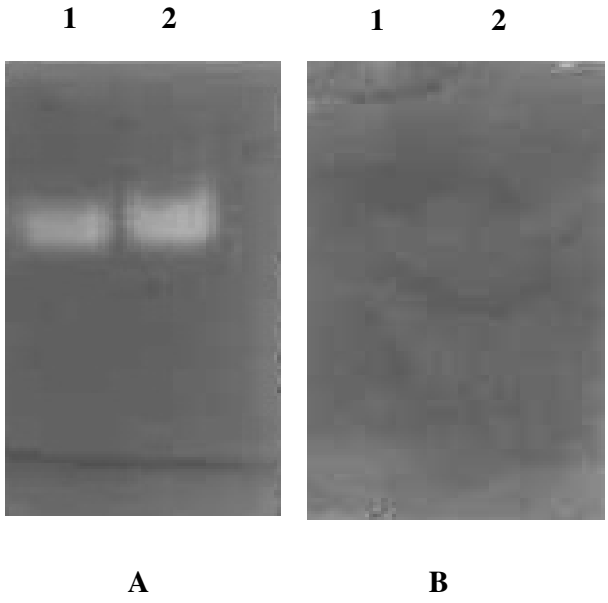


Fig. 22. **Native PAGE of the crude polyphenol extract of FMSCP and malted finger millet amylase stained for amylase activity. (A) without inhibitor and (B) with inhibitor. Lanes 1 and 2 are extracts from 72 h and 96 h malted finger millet, respectively (~ 1 µg of protein was loaded in each lane). The amylase activity staining shows clear bands against dark blue background.**

Table 18. The inhibitory activity of some of phenolics of the millet by HPLC on millet malt amylases

Phenolic compounds	HPLC retention time (min)	Concentration present in finger millet (g %)	(%) Inhibition of amylases @ 50 µg/ml
Gallic acid	3.1	12.6	67.7
Protocatechuic acid	6.1	15.3	64.4
p-hydroxy benzoic acid	7.4	17.9	61.3
p-coumaric acid	11.9	4.4	62.5
Vanillic acid	15.0	3.8	71.9
Syringic acid	20.1	4.0	55.8
Ferulic acid	22.3	32.8	65.6
Trans-cinnamic acid	32.9	3.6	79.2
Quercetin	43.1	5.6	73.5

Average of three determinants

Mode of inhibition of polyphenols on malt amylases

The millet polyphenols may affect the activity of the amylases in several ways, namely by competing with the substrate to bind to the active site of the enzyme or by disrupting irreversibly the catalytic process. The mode of inhibition is also dependent on the substrate specificity of the enzymes. Acarbose, a synthetic amylase inhibitor has been reported to exhibit uncompetitive, mixed and noncompetitive types of inhibition when amylose, maltodextrin and maltoheptaose were used as respective substrates (Oudjeriouat et al. 2003). Kinetic studies were performed using the Michaelis–Menten and LB derivations to identify the mode of inhibition of millet phenolics. In the presence of crude phenolic extract, slope of the straight lines in double reciprocal plot increased with increasing concentrations of polyphenols. The straight lines were intercepted at a single point in the second quadrant indicating mixed non-competitive inhibition (**Fig. 23**). The heterogeneity of phenolics having different structural features in crude extract may be the reason for the observed mode of inhibition. However, the concentrations of the millet polyphenols affect both the slope and the vertical axis intercept of LB plot. The crude extract exhibited an inhibitory constant K_i value of 66.7 µg (**Fig. 24**).The mode of inhibition of *trans*-cinnamic acid, quercetin, vanillic acid and gallic acid were investigated by Michaelis-Menten and LB equations as these were found to be highly potent inhibitors. The presence of inhibitor in the reaction mixture resulted in no intersection of the straight lines (i.e., lines are parallel) in the LB plot (**Fig. 25**). In this case, the inhibitor only binds to the enzyme substrate complex resulting in a change in K_m and V_{max} values.

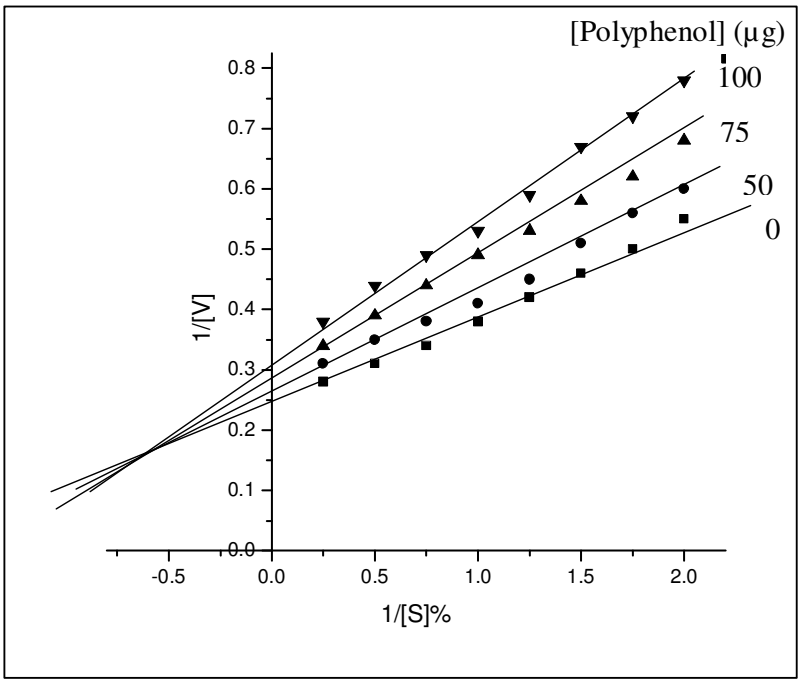


Fig. 23. Lineweaver - Burk plot with starch as the substrate. Reciprocal plots obtained at variable starch concentrations (0.25 – 2.0 %) and fixed polyphenol concentrations (crude polyphenol extract), as indicated on the right part of the graph. These plots were calculated from three to four experiments. The values plotted are from one set of experimental data.

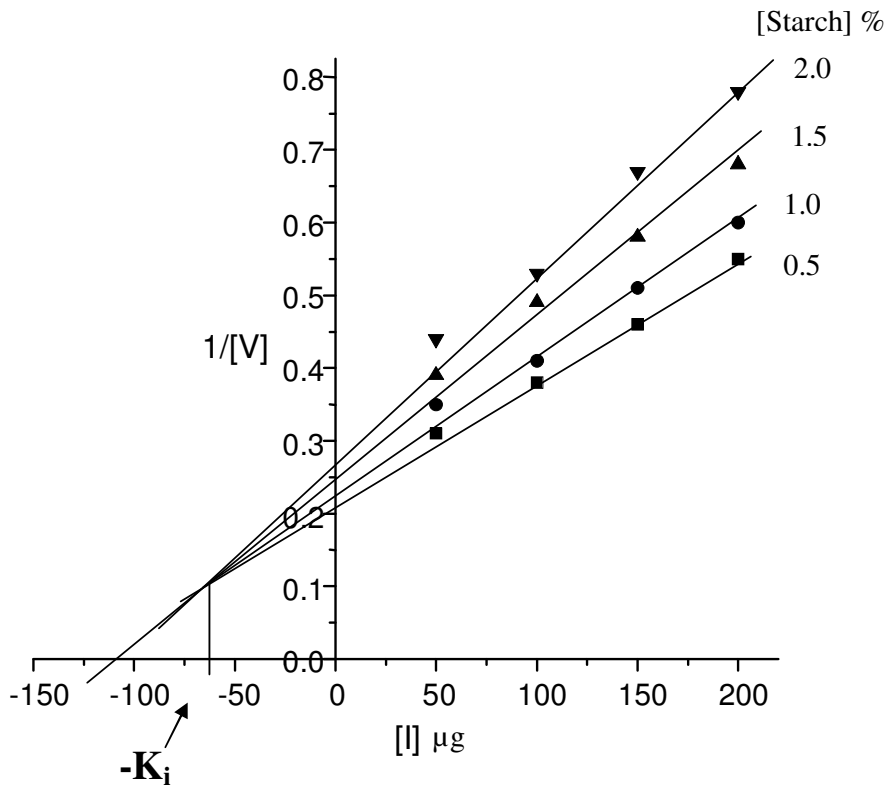


Fig. 24. **Dixon plot of amylase – catalysed hydrolysis reaction at fixed starch concentrations as indicated on the right part of the graph and at variable polyphenol concentrations (50 – 200 µg).**

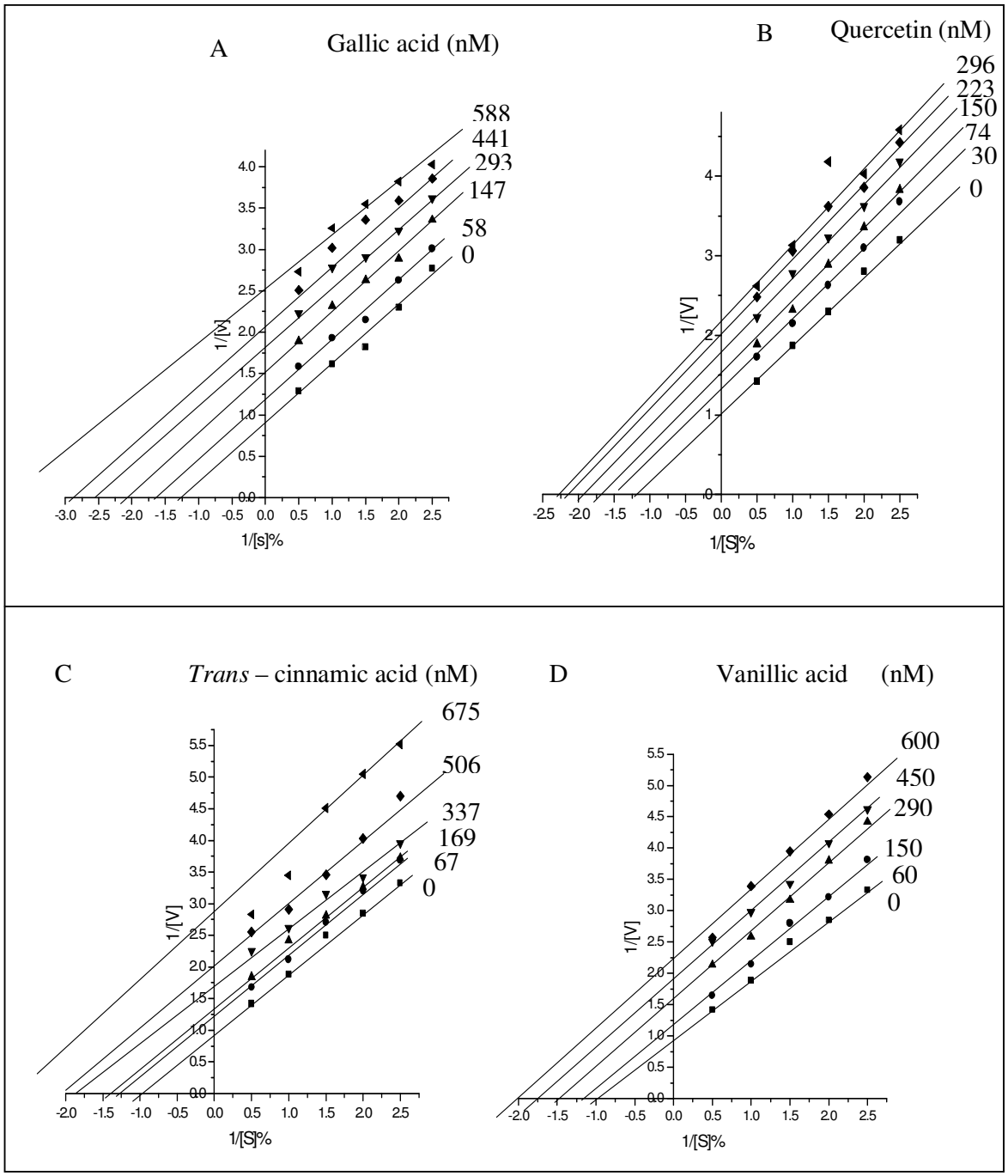


Fig. 25. Lineweaver – Burk plot with starch as the substrate. Reciprocal plots obtained at variable starch concentrations (0.5% - 2.0%) and fixed polyphenol concentrations (fractionated phenolics), as indicated on the right part of the graph. These plots were calculated from three to four experiments. The values plotted are from one set of experimental data.

The mixed non-competitive inhibition exerted by crude polyphenols extract on the malted millet amylases indicates that the phenolics can bind to enzyme or to the enzyme-substrate complex other than catalytic site, decreasing the V_{max} proportionate to the concentration of phenolic compounds. The kinetic data indicated production of two abortive complexes namely, the amylase-phenolic complex (Enzyme-Inhibitor) resulting from binding of the phenolic compound to the active site and the amylase-starch-phenolic complex (Enzyme-Ssubstrate-Inhibitor) in which the phenolic compound is bound at a secondary binding site other than the active center, which is accessible only after starch binding occurrs at the active center.

In contrast, the individual phenolic compounds purified by HPLC showed uncompetitive inhibition. In this case, binding of starch at the catalytic site may have modified the confirmation of amylase, making the putative inhibitor-binding site available. The kinetic constants K_m and V_{max} decreased upon binding of phenolic compounds. It has long been a practice to determine the inhibition constants of competitive inhibitors by the Dixon plot method. The Dixon plot does not distinguish unambiguously between competitive and mixed inhibitors (Schlamowitz et al. 1969) and, for mixed or uncompetitive inhibitors, it provides no measure on the dissociation constant of the ESI complex (K_i'). Therefore, a new plot, which is similar to the Dixon plot but complementary to it, was used since it provides a simple way of measuring K_i' rather K_i (Bowden 1974). The dissociation constant, K_i' derived from the plots of S/V vs I for gallic acid, *trans*-cinnamic acid, quercetin and vanillic acid are shown in **Fig. 26**. Gallic acid and quercetin showed the highest affinity for the enzyme-substrate complex with K_i' of 4.6×10^{-7} M (**Table 19**). However, *trans*-cinnamic acid had a low affinity for the ES complex of finger millet amylases (K_i' of 7.3×10^{-7} M).

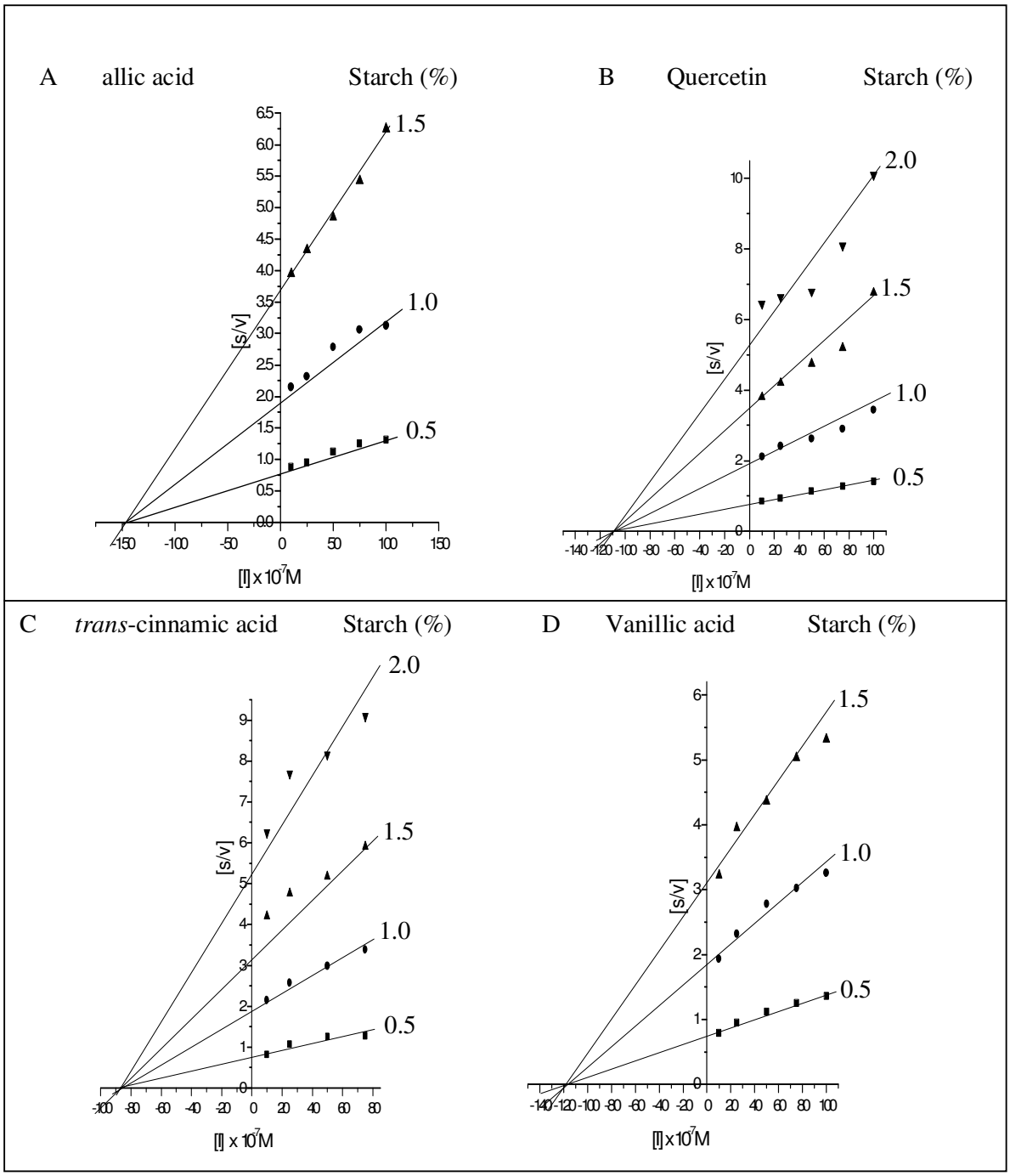


Fig. 26. Determination of dissociation constants for amylase – catalysed reaction at fixed starch concentrations as indicated on the right part of the graph and at variable polyphenol concentrations (10 – 80 × 10^{-7} M).

Table 19. **Dissociation constants (K_i') of phenolic compounds against finger millet malt amylases**

Phenolic compound	K_i'
Gallic acid	4.60×10^{-7} M
Vanillic acid	5.06×10^{-7} M
Quercetin	4.63×10^{-7} M
Trans-cinnamic acid	7.30×10^{-7} M

Based on the observations on the kinetics of amylases in the presence of inhibitors, it may be inferred that finger millet malt amylases have more than one binding site similar to other amylases and the X-ray crystallographic studies of amylases by Weselake (1983) also supports this. The kinetics as well as different spectral studies on porcine pancreatic α-amylase inhibition by α, β and γ-cyclodextrins indicated the involvement of secondary binding site including a tryptophan residue (Koukiekolo et al. 2001). The inhibitory effects of cyclohepta amylose on starch granule hydrolysis exerted by cereal α-amylases suggest the presence of a non-catalytic binding site in the enzyme molecule (Koukiekolo et al. 2001).

From the studies it may be inferred that the inhibition of millet polyphenols on millet malt amylases is of mixed non-competitive type. Hence, whenever the millet malt, which contains polyphenols as well as amylases is heated or warmed in to aqueous slurry the activity of the enzyme is not fully inhibited, probably in view of this the malt amylases hydrolyse the millet starch and reduces its water holding capacity. There by yielding a slurry like product of low-viscosity and high density in a given unit volume.

Aldose reductase activity

The AR activity in cataracted eye lenses was 0.66 unit/mg protein (**Fig. 27**) and the K_m (app) of the substrate gylceraldehyde and NADPH was 1.8 mM (**Fig. 28A**) and 67.56 μM (**Fig. 28B**) respectively. Varma and Kinoshita (1976) also reported K_m value (1.1 mM) for glyceraldehydes.

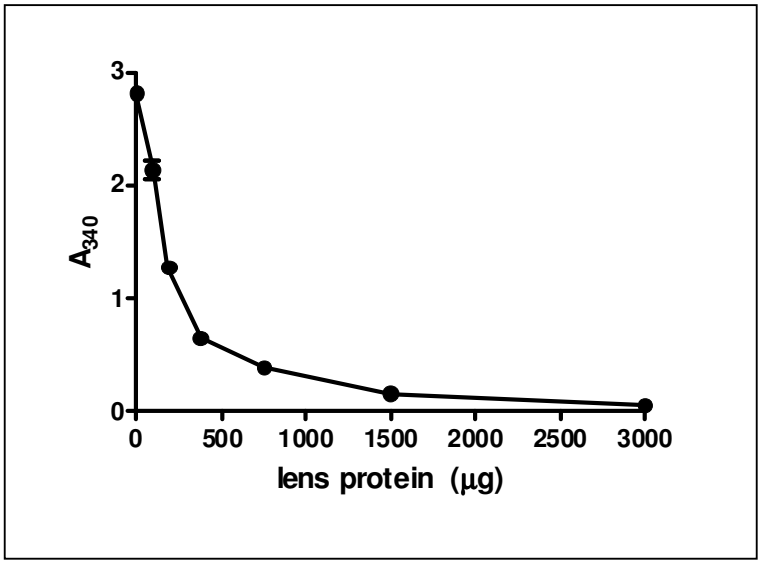


Fig. 27. Aldose reductase activity at different concentrations of lens protein measured by oxidation of NADPH at 340 nm.

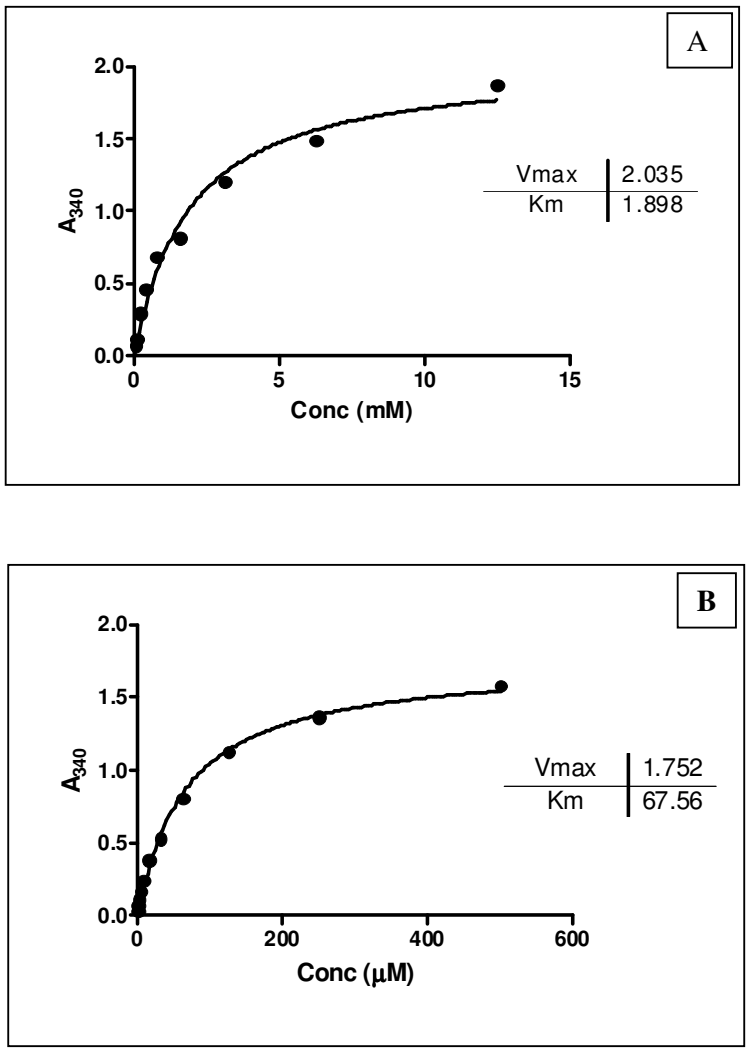


Fig. 28. Optimization conditions for aldose reductase activity A. Michealis-menten constant at varying concentration of D - glyceraldehyde and at saturation concentration of NADPH (0.1 mM) and at optimized enzyme (AR). B. Michealis-menten constant at varying concentration of NADPH at saturation concentration D - glyceraldehyde (10 mM) and optimized enzyme (AR).

Inhibition of aldose reductase

The inhibitory effects of polyphenols on the activity of the AR isolated from cataracted eye lenses was dose dependent and the IC_{50} value was 60 µg/ml (**Fig. 29**). The inhibition of AR by the millet polyphenols could be due to prevention of the enzymatic conversion of glyceraldehydes to glycitol, thereby replenishing the depletion of NADPH levels. It is important to notify here that NADPH is used for several critical reductive metabolic steps, such as the detoxification of reactive oxygen species and hydroperoxides etc. Indeed a large supply of NADPH pool has been shown to envisage cytoprotection against oxidative stress (Harrison et al. 1994; Sun et al. 2003; Ramana et al. 2004; Srivastava et al. 2005). Among the phenolic acids quercetin, protocatechuic and *trans*-cinnamic acids showed 4 - 5 fold inhibitory activity with IC_{50} values of 25, 43 & 68 µg/ml respectively than syringic (172 µg/ml) and *p*-coumaric (162 µg/ml) acids (**Table 20**). *p*-hydroxy benzoic, vanillic and ferulic acids showed negligible or no inhibitory effect against AR.

Data thus indicates that quercetin is the most potent AR inhibitory component among finger millet polyphenolic constituents. The activity was correlated with antioxidant potency with the correlation coefficient 'r~0.99' between antioxidant and AR inhibitory effect of phenolic constituents. This suggests that the antioxidant potency, particularly proton abstracting ability is responsible for AR inhibitory effect. Significant reduction in the activity by *trans*-cinnamic acid (*trans*-3-phenylacrylic acid) to ferulic acid (4-hydroxy-3-methoxycinnamic acid) and gallic acid (3, 4, 5-trihydroxy benzoic) to syringic acid (3, 5-dimethoxy-4-hydroxybenzoic acid) suggest that the presence of the methoxy group may be abolishing the AR inhibition, which could be due to interruption in the redox flow between phenolic acids and the enzyme.

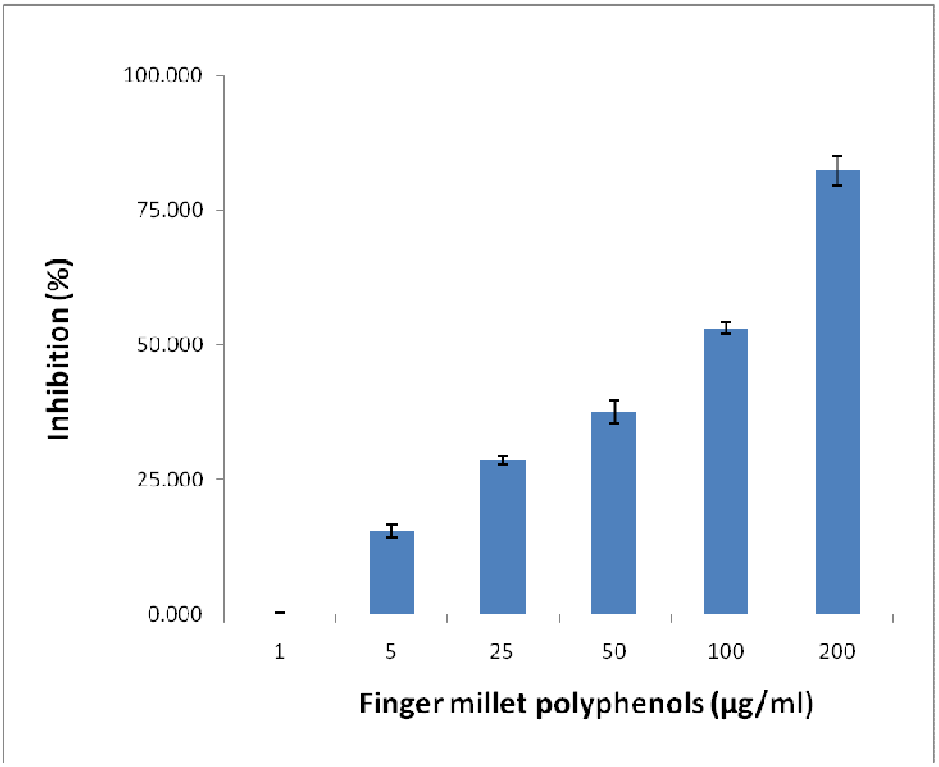


Fig. 29. Inhibitory effects of crude finger millet polyphenols extract at varying concentrations on aldose reductase activity using optimized glyceraldehyde, NADPH and enzyme.

Table 20. The inhibitory activity of the millet seed coat phenolics and its constituent on aldose reductase activity

Phenolics	**AR Inhibition** IC_{50} = µg/ml
Gallic acid 3,4,5-trihydroxybenzoic acid	97 ± 2.5
Protocatechuic acid 3, 4-dihydroxybenzoic acid	43 ± 3.2
p-hydroxy benzoic acid 4-hydroxybenzoic acid	-
p-coumaric acid *Trans*-4-hydroxycinnamic acid	162 ± 12.6
Vanillic acid 4-hydroxy-3-methoxybenzoic acid	-
Syringic acid 3,5-dimethoxy-4-hydroxybenzoic acid	172 ± 8.2
Ferulic acid 4-hydroxy-3-methoxycinnamic acid	-
Trans-cinnamic acid 3-phenylacrylic acid	68 ± 4.8
*Quercetin 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-1-Benzopyran-4-one	25 ± 2.2
Crude phenolic extract	60 ± 3.4

Finger millet seed coat phenolics and its constituents were identified by HPLC and fractionated by preparative HPLC. Fractions were examined for antioxidant and aldose reductase inhibitory property.
*Quercetin showed highly potent activity.
Average of five determinants

Eventhough ferulic and syringic acids have 4-hydroxyl group as part of their structure, they show very poor inhibitory activity against AR. However, it has been reported that flavonoids, benzopyrans, spirohydantoins, alkaloids, non-steroidal anti-inflammatory agents, and quinones have also been shown to inhibit the enzyme with varied degree (Raskin and Rosenstock 1987; Bhatnagar and Srivastava 1992).

The results on AR inhibitory activity are in line wit the previous observation (Matsuda et al. 2002; Jung et al. 2007) that the hydroxylation in the 4' position is crucial for AR inhibition. Systematic designing of related molecules and examination of AR inhibitory activity may further throw some light on the structure-activity relationship.

Quercetin, therefore is a potent AR inhibitor for sorbitol accumulation in polyol pathway at step C in **Fig. 20**. This was further substantiated by examining the potency of pure quercetin on AR activity. The inhibition is similar to that observed in colored rice (Matsuda et al. 2002). The mode of action of quercetin was investigated by determining Michaelis-Menten constant and LB plot equation. Data indicated a noncompetitive inhibition of AR by quercetin, since it did not alter the K_m but altered the V_{max} (**Fig. 30**). Besides, the IC_{50} at a very low range 14.8 ηM by quercetin similar to that of a regulatory endogenous inhibitor may suggest the possibility of efficient inhibition of AR by *in vivo* methods also (Kim et al. 2001). The noncompetitive inhibitors, in general, are those substances that form strong covalent bonds with enzymes and consequently are not displaced by the addition of excess substrate and hence form irreversible reactions. Since, quercetin being a non-competitive inhibitor, it may render irreversible inhibition of AR by successfully blocking the polyol pathway that leads to cataractogenesis.

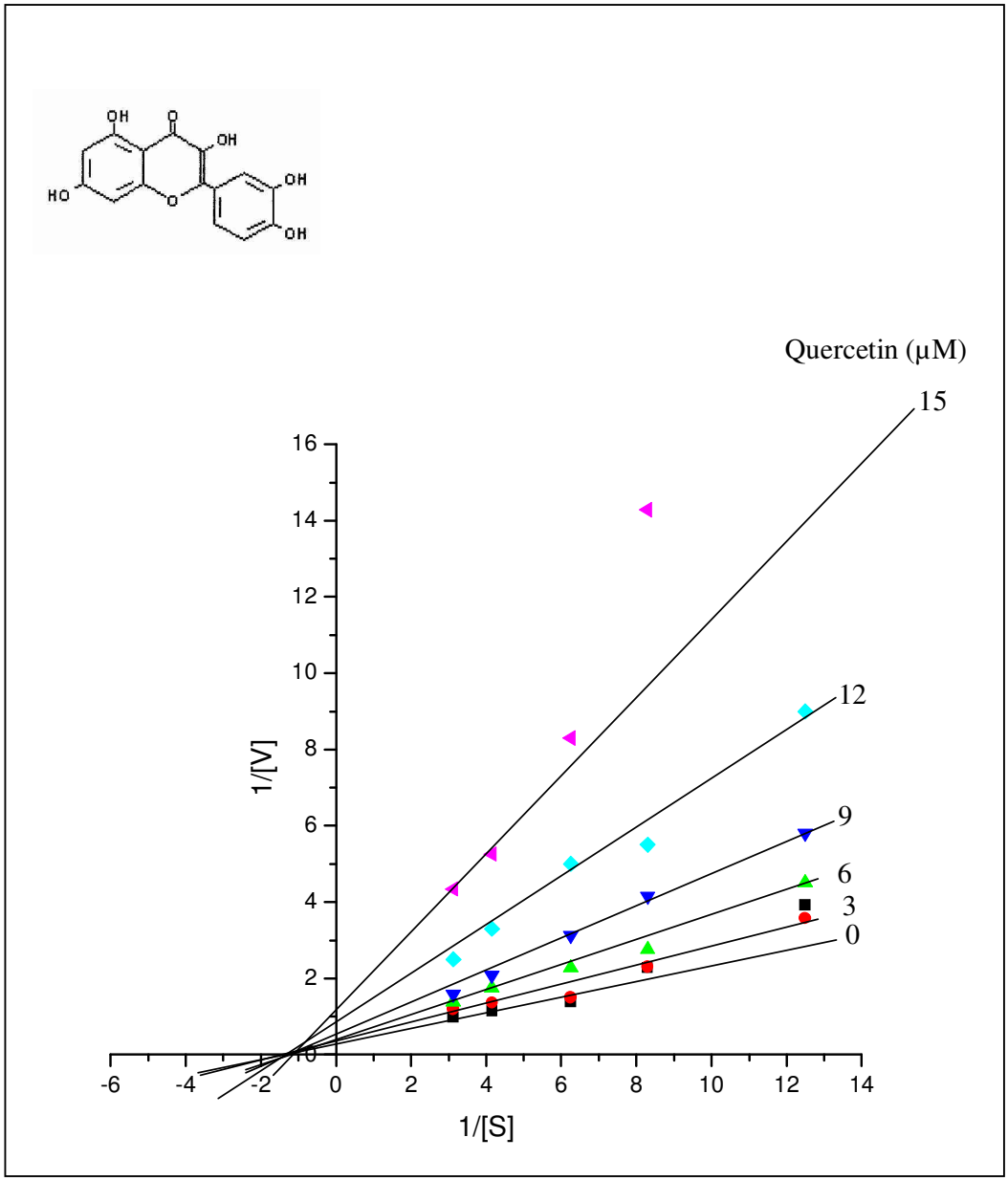


Fig. 30. LB plot of the aldose reductase hydrolysis reaction with variable substrate concentrations (0.05 – 2.5 mM) and at fixed concentration of quercetin (0 – 15 µM) as indicated on the graph. These plots were calculated from three to four experiments. The values plotted are from one set of experimental data.

Quercetin acts as a noncompetitive inhibitor, may bind at a site, near, or remote from the active site. In pure noncompetitive inhibition, however, the inhibitor is assumed to bind to the free enzyme but not to the enzyme-substrate (ES) complex. Thus the strong proton abstracting ability of quercetin may replace the proton donation from AR-Histidine-110/Tyrosine–48, which is a key step in the NADPH regenerating potential substantiating the effective AR blockade activity. The AR inhibition results in preventing the accumulation of sorbitol *in vivo* which is beneficial to anti-cataractogenesis, since sorbitol mediating osmotic pressure may add to the deleterious effect of the eye lenses.

Thus the polyphenols, particularly quercetin present in the finger millet seed coat matter showing noncompetitive inhibition on AR, may serve as potential alternatives to synthetic inhibitors against aldose reductase (Kim et al. 2001; Lee et al. 2001). This approach may circumvent the toxic effects of clinically tested AR inhibitors such as sorbinil, statil, epalrestat, tolrestat and alrestatin.

Snake venom PLA_2 inhibition

The millet polyphenols exhibited considerable inhibition on the PLA_2. The specific activity of *Naja naja* PLA_2 was 139.8 ηmol/mg/min at 37 $^{\circ}$C. The PLA_2 inhibition of some of the phenolics identified from the millet are summarized in **Table 21**. The crude polyphenolic extract was a potent inhibitor and among the phenolics, gallic acid and quercetin were highly potent. The IC_{50} values for the crude polyphenols extract, gallic acid and quercetin were 83.2, 62.3 and 16.8 µg/ml respectively (**Fig. 31**). One of the major groups of polyphenolic compounds is the flavonoids, which are C-15 compounds composed of two phenolic rings connected by a 3-C unit. The A ring has a characteristic hydroxylation pattern at the 5^{th} and 7^{th} position. The B-ring is usually 4′3′4′ or 3′4′5′- hydroxylated.

Table 21. Inhibitory activity of fractionated phenolic compounds by HPLC on snake venom PLA_2 from *Naja naja*

Samples	(%) Inhibition on PLA_2 (100 µg/ml)
Crude extract	62 ± 2.3
Gallic acid	78 ± 1.28
p-hydroxybenzoic acid	11 ± 2.1
p-coumaric acid	31 ± 2.7
p-catechuic acid	15 ± 0.87
Vanillic acid	29 ± 4.2
Ferulic acid	25 ± 1.7
Syringic acid	12 ± 0.91
Trans-cinnamic acid	38 ± 1.72
Quercetin	81 ± 5.6

Average of three determinants

Fig. 31. Inhibition of snake venom *N. naja* PLA_2 by crude polyphenols extract (25 – 200 µg/ml).

Due to the various activities of flavonoids such as anti-inflammatory, anti-allergic, anti-platelet, anti-bacterial, and immune-stimulating (Gil et al. 1994), are of great interest in therapeutic applications. Flavonoids inhibit the enzymes responsible for superoxide radical production, such as xanthine oxidase (Hanasaki et al. 1994) and protein kinase C (Ursini et al. 1994). Some of the known properties of the flavonoids include: free radical scavenging as well as, strong antioxidant activity, inhibition of hydrolytic and oxidative enzymes (PLA_2, COX, LOX) and also anti-inflammatory action (Frankel 1995). Flavonoids also inhibit microsomal monooxygenase, GSH-S-transferase, mitochondrial succino-oxidase and NADH oxidase which are all involved in ROS generation (Korkino et al. 1997). A number of flavonoids efficiently chelate trace metals, which play an important role in oxygen metabolism. Free iron and copper are potential enhancers of ROS formation, by the reduction of hydrogen peroxide with the generation of highly aggressive hydroxyl radical. The proposed binding sites for the trace metals to flavonoids are the catechol moiety in ring B, 3-hydroxyl, 4- oxo groups in the heterocyclic ring and the 4-oxo, 5- hydroxyl groups between the heterocyclic and the A ring. The catechol moiety in ring B apart from chelating metals also has better electron donating property and targets the free radicals. A 2,3 –double bond conjugated with the 4-oxo group, in ring C is responsible for electron delocalization during antioxidant activity. Moreover, the 3-OH in the C- ring and the presence of 2 adjacent hydroxyl groups in B ring are also important for the antioxidant activity (radical scavenging). While additional hydroxyl or methoxyl groups at positions 3, 5 and 7 of rings A and C seem to be less important. These structural features contribute to increase the stability of the aroxyl radical. Flavonoids reduce highly oxidizing free radicals such as superoxide,

peroxyl, alkoxyl and hydroxyl radicals generated during eicosanoid pathway by hydrogen atom donation. The aroxyl radical (Fl-$O^{\cdot}$) may react with second radical, acquiring a stable quinone structure. Besides scavenging, flavonoids may stabilize free radicals involved in the oxidative process by complexing with them. The inhibition of the enzymes by some flavonoids could also be due to a reaction with free radicals generated at the active site of the enzymes as in lipoxygenase (LOX) catalysed reactions. In general, more the $OH^{\cdot}$ substitutions, stronger are the antioxidant activity. Weak antioxidant activity was observed for flavones with single OH substitutions at 3, 6, 2' and 4' position and in flavanones with single OH substitution at the 7, 2′, 3′, 4′ or 7′ position. Kaempferol, quercetin and myricetin exhibited antioxidant activity in increasing order respectively. This is consistent with the inhibitory effects of these flavones on the platelet aggregation induced by ADP, collagen and platelet aggregation factor (PAF).

The current data clearly indicates the therapeutic importance of the millet phenolics as an anti-inflammatory compound. It may be noted that many powerful anti-inflammatory drugs like salicylates (Guerra 1946), indomethacin (Szary et al. 1975) and drugs, which suppresses allergic reaction like disodium cromoglycate inhibits the hyaluronidase enzyme activity (Yingprasertchai et al. 2003) and hence the millet polyphenols my play a positive role similar to these. In ethno-pharmacology several traditional compounds possessing anti-hyaluronidase activity are used against snakebite and also to promote wound healing. These include flavonoids, tannins, curcumins, cinnamic acid and glycyrrhizin from licorice (Kuppusamy and Das 1991; Fluruya et al. 1997). In folk medicine also some plant extract are also used as anti-inflammatory drugs suggesting that many anti-inflammatory drugs inhibit both PLA_2 enzyme and hyaluronidase activity. Similarly, the crude extract of the millet

polyphenols inhibits inflammatory PLA_2 activity indicating its usefulness in regulating the local tissue damage. Another beneficial factor with polyphenols is its preferential inhibition of serine proteases over cysteine proteases, which are the major proteases of all snake venoms responsible for local tissue necrosis.

Neutralization of PLA_2 induced hind paw mice edema

It is very well established that injection of PLA_2 into the mouse footpad induces edema Snake venom PLA_2 (*Naja naja*) induces maximum edema of 180 % (**Fig. 32**). The purified gallic acid (fraction 1) on administration with the PLA_2 enzyme neutralizes the edema inducing activity. The neutralization by gallic acid is dose-dependent and neutralized completely the edema activity of the enzyme studied (**Fig. 33**).

The complex mixture venom toxicity is a multiple poisoning due to the combined action of hydrolytic enzymes and specific toxins and becomes a medical emergency (Ohsaka 1979). In snakes, production and release of venom is an evolutionary adaptation primarily to immobilize the prey and secondary to defense and to support digestion. To bring about these effects, each species have unique venom composition with different amounts of hydrolytic enzymes and specific systemic toxins (Chippaux et al. 1991; Sasa 1999). The common hydrolytic enzymes found in most venom are proteolytic enzymes, phospholipases and hyaluronidases and are responsible for local tissue damage (Shashidharamurthy et al. 2002). The lethality of systemic toxins depends upon the amount and also on the rate at which these toxins diffuse into the systemic circulation for transport to their sites of action (Girish et al. 2004a; 2004b). An early neutralization of local tissue damage is a prerequisite in the better management of snake bite.

Fig. 32. Effect of gallic acid from HPLC on PLA_2 induced paw edema. Different volumes of gallic acid was mixed with different PLA_2 enzyme in a mixture of 50 µl saline and injected into mouse footpad. Values of edema ratio are expressed as mean ± S.D. (*n*=6).

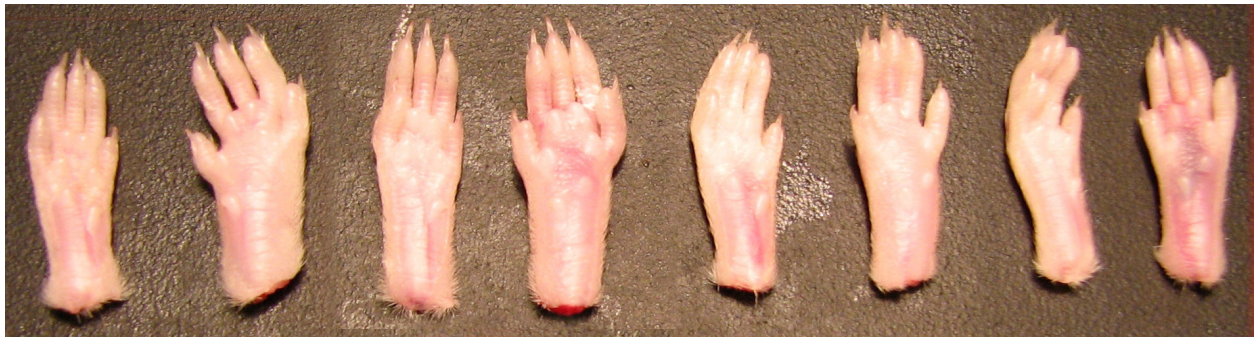


Fig. 33. ***In-vivo*** **anti-inflammatory effect of fraction 1 on PLA_2 induced paw edema. Different volumes of fraction 1 (Gallic acid) was mixed with PLA_2 enzyme in a mixture of 50 µl saline and injected into mouse footpad. A′. PLA_2 without the inhibitor showing high inflammation, B′. PLA_2 with 2 µl of gallic acid showing less inflammation, C′. PLA_2 with 5 µl of gallic acid showing much effect on inflammation, D′. PLA_2 with 10 µl of gallic acid showing reduction on inflammation. Controls for each leg injected only with saline (A-D).**

Summary and conclusions

- The millet polyphenols exhibit inhibitory activity against its malt amylases, aldose reductase of cataracted human eye lenses and also on snake venom phospholipases (PLA_2).
- Considerable differences in the inhibitory activity were observed between crude polyphenols extract and pure phenolics isolated from crude extract. The inhibition by the crude polyphenols extract on the millet malt amylase was of mixed non-competitive type whereas it was uncompetitive type for gallic, vanillic, *trans*-cinnamic acids and quercetin, isolated from the crude extract.
- The K_m and V_{max} of the millet amylases against rice starch were 0.62 % and 201 units/g. The pure phenolics, namely, *trans*-cinnamic acid (79.2 %), quercetin (73.5 %), vanillic acid (71.9 %) and gallic acid (67.7 %) were potent inhibitors of the millet malt amylases. Among the various phenolic acids isolated and purified from the millet seed coat matter, cinnamic acid derivatives exhibited higher activity than the benzoic acid derivatives. The crude polyphenols extract with an inhibitor constant (K_i) of 67 µg was of mixed non-competitive type, whereas the inhibition by the pure phenolics acids was uncompetitive type. Gallic acid and quercetin showed highest affinity for enzyme-substrate complex with K_i' of 4.6×10^{-7} M. However, *trans*-cinnamic acid exhibited low affinity for ES complex of finger millet amylases with K_i' of 7.3×10^{-7} M.

- The inhibition of the millet polyphenols on the AR extracted from the cataracted human eye lenses was of reversible and non-competitive type. Among the phenolic constituents of the millet seed coat polyphenols, quercetin inhibited aldose reductase effectively with an IC_{50} value of 14.8 ηM.
- The millet polyphenols were also effective in inhibiting the activity of snake venom PLA_2. Crude polyphenols inhibited the PLA_2 activity with an IC_{50} value of 83.2 µg/ml. Gallic acid and quercetin were found to be potent inhibitors of snake venom PLA_2 with an IC_{50} values of 62.3 and 16.67 µg/ml, respectively.

The investigations on the mode of inhibition of millet polyphenols on millet malt amylases, aldose reductase of cataracted human eye lenses and also on snake venom PLA_2, revealed that the millet polyphenols play positive role towards the inhibition of amylases and aldose reductase. Thus, the polyphenols may minimize the incidence of cataractogenesis and also act as anti-inflammatory agents by neutralizing PLA_2. The multiple nutraceutical actions of millet polyphenols offer several health benefits, and these results also provide some kind of scientific rationale for the use of finger millet for management of chronic pathologies.

Introduction

The antioxidants are defined as molecules that can delay or prevent oxidation of an oxidizable substrate and have multiple functions in biological systems, including providing defense against oxidative damage and exerting beneficial effects in the major signaling pathways of cells. The important role of antioxidants is to prevent damage caused by the action of reactive oxygen species (ROS) in cells, which include hydrogen peroxide (H_2O_2), the superoxide anion (O_2^-) and free radicals, such as the hydroxyl radical (OH^-). ROS are endogenous intermediates constantly produced in the human body. These are unstable and highly reactive components, generated continuously in signaling the cascade involved in cellular functions such as cellular proliferation and inflammation. Imbalance of cellular oxido/redox status, results in pathological manifestation (Frlich and Riederer 1995). They can damage cells by chain reactions, such as lipid peroxidation or formation of DNA adducts that could cause cancer-promoting mutations or cell death (Shahidi 1997). Hence, for proper functioning of various biochemical and physiological activities of the human body, formation and denaturing the ROS has to be balanced. The production of ROS is an unavoidable consequence of aerobic respiration. When the terminal oxidases, cytochrome-*c*-oxidase and the alternative oxidases, react with the oxygen, four electrons are transferred and water is the product. However, occasionally oxygen can react with other electron transport components, where in only one electron is transferred, the result is the superoxide anion O_2^-. In mammalian cells, it has been estimated that 1 – 2 % of the oxygen consumption leads to superoxide formation. Through a variety of reactions, superoxide leads to the formation of hydrogen peroxide, hydroxyl radical, and other ROS, and these can cause damage in various ways such as breaking the backbone of proteins and modifying the side chains.

Unsaturated fatty acids, for instance in lipids of membrane, are attacked and the peroxide derivatives are formed, eventually leading to breakage of the fatty acid backbone also. Finally, ROS can react with DNA and cause mutations. Hence, it is clear that there is a need to limit the production of ROS, to detoxify ROS once formed, and also to repair damage caused by the ROS. However, when plants are stressed, the steady state level of ROS usually increases, and it has been hypothesized that ROS (specifically hydrogen peroxide) might also act as messengers turning on stress-related genes.

In nature, there are a large number of different types of anti-microbial compounds and among them polyphenols, the group of highly hydroxylated phenolic compounds are potential inhibitors on the growth of microorganisms. The use of anti-microbial agents for preservation of foods is followed universally and most of them are synthetic chemicals. Since, there is greater interest in natural products for food preservation, interest on identifying edible sources for anti-microbial agents is receiving attention. Phenolics are secondary metabolites synthesized by plants and these compounds are largely diversified group of phytochemicals derived from phenylalanine and tyrosine.

Dietary polyphenols have attracted much interest recently because they have a variety of beneficial biological properties, such as free radical scavenging, metal chelation and inhibition of lipid peroxidation. Polyphenols also play a role on inhibition of tooth decay, amelioration of allergy and prevention of gout (Sakanaka et al. 1989). Naturally occurring edible compounds with anti-microbial activity may have medicinal value and may serve as a low cost pharmaconutrients and also as components of drugs (Middleton and Kandaswami 1994). Polyphenolic compounds generally occur as glycosylated derivatives, which are dietary compounds and widely

known as antioxidants that inhibit the oxidation of low density lipoproteins and reduce thrombotic tendencies (Hertog et al. 1995). Phenolic compounds are said to hinder the proliferation of microorganisms also (Alberto et al. 2001).

Hence, investigations towards health beneficial effects of finger millet polyphenols like antioxidant potential and antimicrobial activity were undertaken.

Materials and methods

Material

Polyphenols of the finger millet seed coat matter were extracted with polar and non-polar solvents as indicated earlier and used for the studies.

Chemicals

Phenolic standards like ferulic acid, gallic acid, *p*-coumaric acid, *p*-catechuic acid, quercetin, *trans*-cinnamic acid, vanillc acid, syringic acid; 1, 1-diphenyl-2-picrylhydrazyl (DPPH); butylated hydroxyanisole (BHA); phenazine methosulphate (PMS); hydrogen peroxide (H_2O_2); thiobarbituric acid (TBA); ethylenediamine tetra acetic acid (EDTA); deoxyribose; ammonium persulphate (APS); ABTS [2, 2'-azinobis (3 – ethylbenzothiazoline – 6 – sulfonate)] were purchased from Sigma – Aldrich Chemicals Co. (St. Louis, Missouri, USA). Triple distilled and degassed water was generated by the Milli Q system (Millipore, Bedford, MA). The other reagents like polar and non-polar solvents, hydrochloric acid, sulphuric acid used were of analytical grade or HPLC grade and were obtained from Merck (Mumbai, India). Ascorbic acid, ferric chloride, anhydrous sodium carbonate, potassium ferrricyanide, trichloro acetic acid (TCA); sodium phosphate and ammonium molybdate were from Ranbaxy Laboratories Ltd. (New Delhi, India).

Bacterial strains and culture conditions

The bacterial strains used as test organism *Escherichia coli* (ATCC 25922*),* *Bacillus cereus* (MTCC 441), *Staphylococcus aureus* (ATCC 25923), *Listeria monocytogenes* (MTCC 2451), *Streptococcus pyogenes* (MTCC 1061)*, Proteus vulgaris* (MTCC 1771), *Pseudomonas aeruginosa* (ATCC 27853)*, Serratia marcescen* (ATCC 25471), *Klebsiella pneumoniae* (ATCC 15380) and *Yersinia enterocolitica* (MTCC 986) were obtained from the Department of Food Microbiology, CFTRI, Mysore, India. All the bacteria were cultured aerobically at 37 $^{\circ}$C in nutrient broth and agar medium. Before experimental use, cultures from agar slants were sub-cultured in nutrient broth and incubated for 24 h and used as the inoculums for each experiment.

Assay of antioxidant activities

Reducing power

An aliquot of the extracts from different solvents were mixed with 5.0 ml phosphate buffer (2.0 M, pH 6.6) and 5 ml 1 % potassium ferricyanide and incubated at 50°C for 20 min and to that 5 ml of 10 % trichloroacetic acid was added, and the mixture was centrifuged at 2500 rpm for 10 min. Five ml of the upper layer of the centrifugate was mixed with 5 ml water and 1 ml of 0.1 % ferric chloride, and the color developed was measured at 700 nm and the reducing power was determined according to Yen and Duh (1993).

Total antioxidant potentiality

To an aliquot of the polyphenols extract, 1.2 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) was added and the reaction mixture was incubated at 90 °C for 90 min, cooled to ambient temperature and the absorbance was measured at 695 nm. The antioxidant capacity

was expressed as gallic acid (moles per 100 g extract) equivalent, as per Prieto et al. (1999).

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

The DPPH radical-scavenging activity of the extracts was assayed following the method of Blois (1958). To an aliquot of the extract containing 10 – 50 µg/ml polyphenols, 0.5 ml of 0.5 mM DPPH solution and 100 mM Tris - HCl buffer (pH 7. 4) were added and the volume was made up to 1 ml, incubated at room temperature for 30 min in dark and the absorbance was measured at 517 nm. The radical scavenging activity was quantified following the formula;

$$\text{Radical scavenging activity (\%)} = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \times 100$$

Where, $\text{Abs}_{\text{control}}$ = absorbance of DPPH radical

$\text{Abs}_{\text{sample}}$ = absorbance of DPPH radical of sample

The scavenging activity was expressed as µmol DPPH radical scavenged per gram of sample.

Hydroxyl radical scavenging assay

Deoxyriboses, the non site-specific hydroxyl radical scavenging activities of different extracts were determined according to the method of Halliwell et al. (1987). To 1 ml of reaction mixture containing 832 µl of sample solution of various concentration, 2.5 mM phosphate buffer (pH 7.4), 31.7 µl of freshly prepared 88.3 mM H_20_2, 37.2 µl of 2.68 mM EDTA, 6.5 µl of 6.152 mM $FeCl_3$, 17.6 µl of 5.68 mM ascorbic acid and 75 µl of 74.65 mM deoxyribose were added and incubated at 37 °C under dark for 90 min. To that 1 ml of 49 mM TBA and 1 ml of 153 mM TCA were added and left in boiling water bath for 10 min, cooled rapidly to room

temperature and the absorbance was measured at 532 nm. The hydroxyl radical scavenging activity was calculated as follows:

$$\text{Hydroxyl radical scavenging activity (\%)} = \frac{A_0 - (A_1 - A_2)}{A_0} \times 100$$

Where, A_0 indicates the absorbance of blank; A_1 is the absorbance of the mixture in the presence of sample; A_2 is the absorbance of the mixture in the absence of sample.

ABTS [(2, 2'- azinobis (3 – ethylbenzothiazoline – 6 – sulfonate)] radical scavenging assay

Radical scavenging capacity of the polyphenols was evaluated against the ABTS according to the improved protocol of Auddy et al. (2003). The ABTS radical was prepared by oxidizing 10 ml of 7 mM aqueous solution of ABTS with 10 ml of 2.45 mM ammonium persulphate (APS), mixed well and incubated at 23 °C in dark for 16 hr. Then 680 µl of 3.5 mM ABTS radical solution was diluted to 10 ml with 10 mM phosphate buffer saline (PBS) at pH 7.4, so as to obtain an absorbance of 0.70 ± 0.005 at 734 nm. The polyphenols dissolved in 10 mM PBS (pH 7.4) at various concentrations to have them fit in the range of values in the standard curve. To 20 µl of the test sample solution, in 10 mM PBS (pH 7.4), 230 µl ABTS radical solution (0.238 mM) was added and mixed vigorously and after 6 min standing, the absorbance was measured at 734 nm. The decrease in absorbance after addition of a test compound was recorded. The radical scavenging activity was calculated as follows:

$$\text{ABTS radical scavenging activity (\%)} = \frac{\text{Absorbance}_{(\text{control})} - \text{Absorbance}_{(\text{sample})}}{\text{Absorbance}_{(\text{control})}} \times 100$$

Antibacterial activity

The agar diffusion assay was used to investigate the antibacterial effects of phenolic compounds. The assay was carried out according to the method of Hufford et al. (1975) using Mueller Hinton agar. Twenty milliliters of the specified molten agar (45° C) was aseptically mixed with 100 µl of a bacterial suspension (10^4 CFU/ml) and poured into sterile petri dishes. For the preparation of the inocula, colonies of bacteria were suspended in Mueller Hinton broth. The bacterial suspensions were adjusted turbidometrically to McFarland 1. The total colony-forming unit of bacterial suspension was estimated by serial dilution followed by plate count method. An aliquot (50 µl) of the extracts were placed into 6 - 9 mm wells burrowed using cork borer and the plates were incubated for 24 hr at 37 °C. The tests were carried out in triplicate. The anti-bacterial activity was measured as clear zone of diameter (mm) formed due to inhibition of the growth of the microflora. Solvent control (Methanol) was included in every experiment as negative control. 50 µl of oxacillin (20 µg/ml) and norfloxacin (200 µg/ml) were used as positive controls for gram-positive and gram-negative bacteria respectively. The phenolic acids (nine fractions) obtained by HPLC fractionation were also assessed for their anti-bacterial property individually.

Results and discussion

Reducing power activity

The reducing power of millet polyphenols extracts from different solvents assayed following potassium ferricyanide method at various concentrations are presented in **Table 22**. From that, it could be said that, at any given concentration (between 1.25 to 10 mg) the methanol extract shows the highest activity followed by acetone and ethanol extracts among the polar solvents. On the other hand, among the non - polar solvents, ethyl acetate shows the highest, and diethyl ether showed the least activity. Therefore, the marked antioxidative activity in methanol extract may be associated with its higher reducing power.

Gordon (1990), reported that the antioxidant activity is believed to break the radical chains by donation of a hydrogen atom, indicating that the antioxidative properties are concomitant with the development of the reducing power. Reducing power of any compound is often used as an indicator of its electron-donating ability, which is an important mechanism for testing its antioxidative action; and accordingly, the reducing power of the polyphenols may be used as an indicator of their potential antioxidant activity. Dose dependency of antioxidative activities was also investigated as a function of reducing power, which increased with increasing concentration in all the samples. This property is associated with the presence of reductones that are reported to be the terminators of free radical chain reaction (Duh and Yen 1997).

Table 22. Reducing power assay of the polyphenols at different concentrations

Solvents	Concentration (mg/ml)				
	00	**1.25**	**2.5**	**5.0**	**10.0**
Methanol	0.01±0.00	0.45±0.02	1.1±0.02	1.7±0.15	2.13±0.8
Ethanol	0.01±0.00	0.31±0.04	0.87±0.02	1.3±0.15	1.74±0.5
Propanol	0.01±0.00	0.14±0.01	0.36±0.07	0.73±0.02	1.02±0.02
Butanol	0.01±0.00	0.05±0.00	0.12±0.05	0.23±0.05	0.42±0.06
Acetone	0.01±0.00	0.27±0.02	0.64±0.02	0.91±0.1	1.55±0.41
Water	0.01±0.00	0.08±0.00	0.2±0.06	0.47±0.02	0.9±0.02
HCl–methanol	0.01±0.00	1.24±0.22	1.95±0.5	2.52±0.3	3.21±0.2
HCl – ethanol	0.01±0.00	1.21±0.3	1.77±0.25	2.44±0.35	2.47±0.56
HCl – propanol	0.01±0.00	0.26±0.07	0.79±0.1	1.42±0.1	1.96±0.08
HCl – butanol	0.01±0.00	0.15±0.03	0.25±0.07	0.41±0.02	1.15±0.36
HCl – acetone	0.01±0.00	0.47±0.02	1.14±0.02	1.86±0.5	2.48±0.72
HCl – water	0.01±0.00	0.31±0.05	0.67±0.15	1.13±0.17	1.66±0.5
Diethyl ether	0.01±0.00	0.09±0.01	0.26±0.05	0.47±0.02	1.88±0.5
Ethyl acetate	0.03±0.00	0.15±0.05	0.01±0.00	1.1±0.05	2.54±0.00
Benzene	0.01±0.00	0.01±0.00	0.05±0.01	0.09±0.01	0.12±0.02
Chloroform	0.01±0.00	0.04±0.00	0.06±0.01	0.1±0.02	0.14±0.02

Mean values ± standard deviations (n = 3)

Total antioxidant activity

Among the various extracts, the total antioxidant activity of the methanolic extract was higher (25.6 mmol/g) than ethanol (21.3 mmol/g) and acetone (20.4 mmol/g) extracts (**Fig. 34**). The millet extract in acidified polar solvents showed higher antioxidant activities (between 14.1 – 25.6 mmol/g seed coat) compared to non–acidified polar solvents (between 3.5 – 15.4 mmol/ seed coat). On the other hand, the phenolics extracted by the non-polar solvents showed very little antioxidant activities (1.05 to 9.1 mmol/ g) and among them ethyl acetate extract showed the highest activity (9.1 mmol/g) and diethyl ether showed the least (1.05 mmol/g). Variations in the antioxidant capacity of different extracts may be attributed to the differences in their phenolic contents and, it has also been reported that the solvents used for the extraction also exert influence on the chemical species (Yuan et al. 2005). The antioxidant activity exhibited by the methanolic extracts may be due to the presence of gallic acid, ferulic acid, hydroxybenzoic acids and their derivatives.

DPPH radical scavenging activity

The DPPH radical-scavenging activity ($P<0.05$) of the methanolic extract at all the concentrations was higher than the other extracts (**Table 23**). The millet polyphenols exhibited a strong ability to quench DPPH radicals and it was observed that, the scavenging ability increased with increasing concentrations of polyphenols and at any given concentration between 10 – 200 µg/ml. The IC_{50} values for acidified methanolic, ethanolic and acetone extracts were 63, 92 and 112 µg/ml respectively. Very little activity was observed in the case of extracts from non-polar solvents and among them the activity of diethyl ether was least (4 mg/ml). The DPPH radical-scavenging activity of methanolic extract was comparable ($P < 0.05$) with that of butylated hydroxy anisole (BHA), the commonly used antioxidant in the food

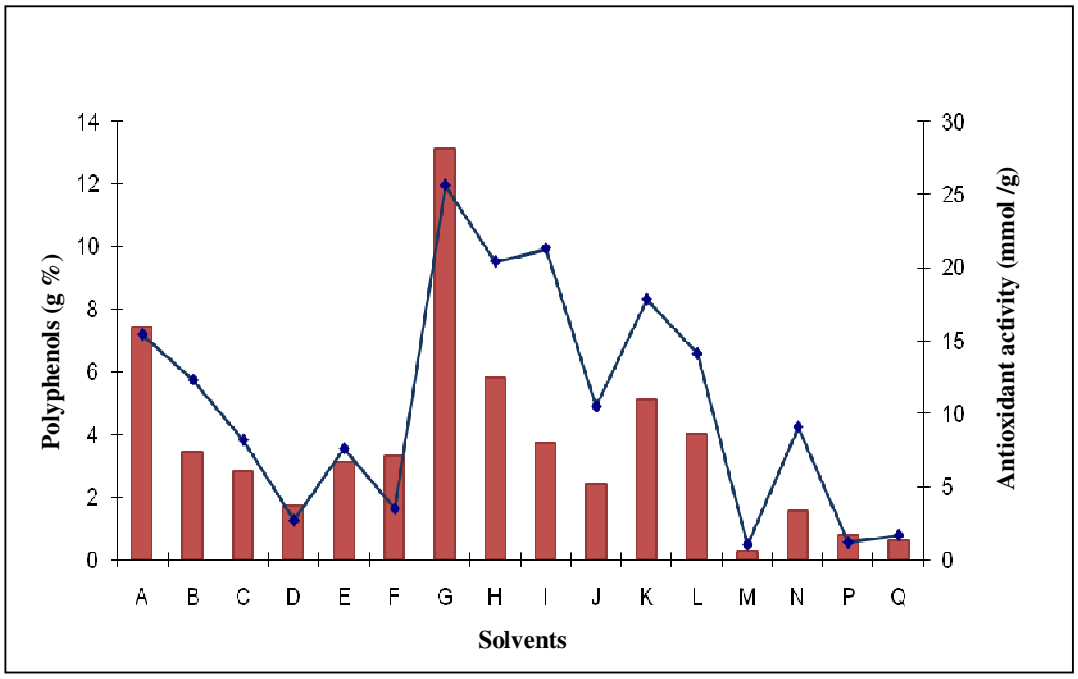


Fig. 34. Total polyphenols (g %) content extracted in different solvents and its total antioxidant activity. (A) methanol; (B) ethanol; (C) propanol; (D) butanol; (E) acetone; (F) water; (G) HCl–methanol; (H) HCl–ethanol; (I) HCl–propanol; (J) HCl–butanol; (K) HCl–acetone; (L) HCl–water; (M) diethyl ether; (N) ethyl acetate; (P) benzene; and (Q) chloroform.

Table 23. Antioxidant potentials (IC_{50} value; µg/ml) of millet polyphenols extract in different solvents

Solvents	Hydroxyl	DPPH	ABTS
Methanol	190.1 ± 2.1	102 ± 1.1	181.7 ± 1.3
Ethanol	454.9 ± 2.6	130.4 ± 1.4	276.5 ± 1.5
Propanol	600.9 ± 1.8	379.5 ± 2.3	1397.5 ± 4.6
Butanol	1234.4 ± 2.5	795.5 ± 2.5	4625 ± 3.2
Acetone	436.7 ± 1.4	159.0 ± 1.3	505.1 ± 3.7
Water	508.6 ±1.9	310.0 ± 1.8	771.0 ± 2.4
HCl-methanol	109.1 ± 1.3	62.7 ± 0.8	114.3 ± 1.8
HCl–ethanol	196.4 ± 1.5	91.6 ± 1.0	169.7 ± 1.5
HCl-propanol	310.5 ± 1.1	183.4 ± 1.1	670 ± 2.5
HCl-butanol	728.8 ± 2.2	330.0 ± 1.7	1799.4 ± 3.1
HCl-acetone	213.1 ± 1.4	112.3 ± 1.3	204.8 ± 2.6
HCl–water	359.3 ± 1.7	141.3 ± 1.7	335.8 ± 1.6
Diethyl ether	5619.9 ± 3.8	4066.1 ± 3.7	20328.7 ± 5.7
Ethyl acetate	967.4 ± 2.1	542.0 ± 2.1	4407.7 ± 3.6
Benzene	5445.2 ± 3.2	5161.0 ± 4.1	17728.9 ± 4.3
Chloroform	4247.3 ± 2.6	2861.6 ± 3.4	12070.7 ± 2.1
BHA	NA	51.8 ± 0.6	NA

Mean values ± standard deviations (n = 3)

industry. This indicates that, the millet polyphenols possess significant levels of antioxidant activity with strong DPPH radical scavenging activity.

According to Villano et al. (2007), nitrogen centered radicals such as $DPPH^{\bullet}$ react with phenols via two different mechanisms: (i) a direct abstraction of H-atom of the phenol and (ii) an electron transfer process from hydroxyl group of phenols (OH) or its phenoxide anion (O^{-}) to $DPPH^{\bullet}$

(i) $OH + DPPH^{\bullet} \rightarrow O^{\bullet} + H\text{-}DPPH$

(ii) $O^{\bullet} + DPPH^{\bullet} \rightarrow$ products

The contribution of one or the other pathway for free radical scavenging activity depends on the nature of the solvents and/or the redox potentials of the species involved. Generally in non-polar solvents, the reduction of phenols and abstraction of phenol-H atom mechanism are predominant, but in polar solvents such as methanol or ethanol, the electron transfer mechanism becomes important as it forms strong hydrogen bonds with the phenolic molecules. In the present study also, it was found that the polar solvents are better for extraction of the radical scavenging activity from the millet seed coat matter. The DPPH radical scavenging activity of the extract has been attributed to the ability of these extracts in pairing with the odd electron of DPPH radical (Park et al. 2004). The DPPH has been used extensively as a substrate to evaluate antioxidant activity of biological as well as chemical substance as it is a useful reagent for investigating the free radical scavenging activities of many compounds (Duan et al. 2006).

Hydroxyl radical scavenging activity

The hydroxyl radical is an extremely reactive free radical formed in biological systems and has been implicated for its damaging activity of very high order in the free radical pathology. It can conjugate with nucleotides in DNA and cause strand

breakage, leading to carcinogenesis, mutagenesis and cytotoxicity besides capable of damaging almost every molecule in living cells (Hochestein and Atallah 1988).

Hydroxyl radical scavenging potential of the polyphenols extracted from different solvents determined in terms of deoxyribose scavenging activities indicate quite similar results compared to those of in DPPH reaction. Among the polar solvents (**Table 23**), the inhibition of methanol extract was the highest having IC_{50} value of 190 μg/ml and least in butanol extract with an IC_{50} value of 1234 μg/ml where as, the acidified polar solvents at the same concentration showed higher antioxidant activities. The non-polar solvents showed very low free radical scavenging activity compared to polar solvents and among them ethyl acetate showed highest activity (967 μg/ml, IC_{50} value). The acidified methanol extract showed higher antioxidant properties (109 μg/ml, IC_{50} value) than pure methanol extract (190 μg/ml, IC_{50} value) which showed that the acid could break the intermolecular bonds and enhance extraction of phenolics. The ability of the millet polyphenols extract to quench reactive hydroxyl radical species has potential application to extend shelf life of food products also (Yuan et al. 2005).

ABTS scavenging activity

The ABTS scavenging activity (IC_{50}) of millet seed coat polyphenols extract in different solvents presented in **Table 23** indicates that the acidified methanolic extract showed the highest scavenging capacity (114.3 ± 1.8 μg/ml) in quenching $ABTS^{\bullet+}$ followed by ethanol (169.7 ± 1.5 μg/ml) and acetone (204.8 ± 2.6 μg/ml). The $ABTS^{\bullet+}$ scavenging activity of the extracts of the millet with non-polar solvents were less compared to polar solvents (4407.7 ± 3.6 to 20328.7 ± 5.7 μg/ml).

Significant correlations were observed between total phenolic contents and ABTS scavenging activity indicating the role of phenolic compounds in inhibiting

free radicals and radical cat ions under these systems. The results suggest that phenolic compounds in the millet may be able to inhibit free radicals formed in the human body. Hagerman et al. (1998), have reported that the high molecular weight phenolics have high ability to quench free radicals ($ABTS^{\cdot+}$) and their effectiveness depends on the molecular weight, the number of aromatic rings and the nature of hydroxyl group's substitution than the specific functional groups.

Correlation between antioxidant activity and phenolic content

The *in vitro* antioxidant capacity of the millet polyphenols, mainly is due to the presence of hydroxycinnamic acid and benzoic acid derivatives, as well as other flavonoids and such other compounds, present mainly as soluble esters and also due to the presence of a small proportion of free acids; mostly extracted in polar solvents (Andreasen et al. 2001). Normally, the antioxidant activity of any compound is system dependent (Adom and Liu 2002) and this is true with millet polyphenols also.

It has been shown that, esterases in the human gut can cleave esterified hydroxycinnamates and form free acids in the small intestine (Andreasen et al. 2001) and both the ester - linked and the free soluble phenolic acids may exert some antioxidant effect in the luminal side of the intestinal tract. Since, the insoluble covalently bound hydroxycinnamates and diferulates arrive almost intact in the large intestine, these compounds can be released in the gut by microbial esterases (Andreasen et al. 2001). The free compounds show antioxidant effect in the colon or undergo further modifications by the action of bacterial enzymes.

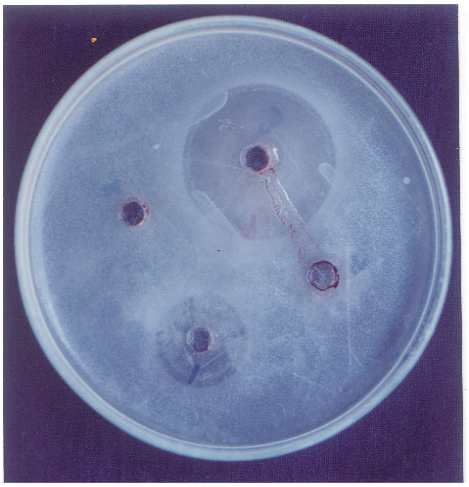
The antioxidant activity of polyphenols depends on the structure and substitution pattern of hydroxyl groups. The presence of the C_2-C_3 double bond configured with a keto-arrangement is known to be responsible for electron delocalization from the ring and it increases the radical scavenging activity.

Reducing power of the millet polyphenols was concentration dependent and increased with increasing concentration in all the extracts (**Table 23**). Same trend has also been reported by Kumaran and Karunakaran (2007) in methanolic extracts of higher plants. Also, it was observed that at any given concentration, acidified methanolic extracts of the millet phenolics exhibited higher reducing power than other solvents. The effect of concentration of antioxidant compounds on the DPPH radical as well as the deoxyribose scavenging activity and also the ABTS radical scavenging potentials was observed even at a very low concentration (10 to 200 µg/ ml) (**Table 23**). The free radical scavenging activity (IC_{50} values) was found to increase with increasing concentration of extract in all the experimental parameters indicating the concentration dependency of the polyphenols for the antioxidant activity. However, the methanolic extracts showed less radical scavenging activity (**Table 23**) as compared to standard antioxidant (BHA). Thus the present finding corroborates well with earlier reports on other higher plants and their enzymatic extracts (Park et al. 2004; Kumaran and Karunakaran 2007). A number of flavonoids efficiently chelate trace metals, which play an important role in oxygen metabolism. Free iron and copper are potential enhancers of ROS formation, by the reduction of hydrogen peroxide with the generation of highly aggressive hydroxyl radical. In general, more the $OH^{\cdot}$ substitutions, stronger are the antioxidant activity. Weak antioxidant activity was observed for flavones with single OH substitutions at 3, 6, 2' and 4' position and in flavanones with single –OH substitution at the 7, 2', 3', 4' or 7' position. Kaempferol, quercetin and myricetin exhibited antioxidant activity in increasing order respectively. The flavonoids that contain multiple OH substitutions have very strong antioxidant activities against peroxyl radicals. The proposed binding sites for the trace metals to flavonoids are the catechol moiety in

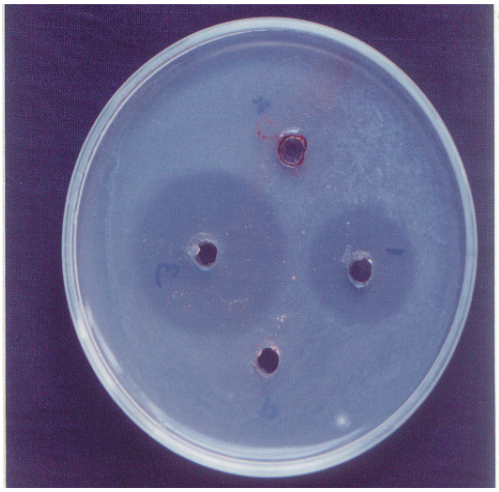
ring B, 3-hydroxyl, 4-oxo groups in the heterocyclic ring and the 4-oxo, 5- hydroxyl groups between the heterocyclic and the A ring. The catechol moiety in ring B apart from chelating metals also has better electron donating property and is a radical target. A 2,3 double bond conjugated with the 4-oxo group, in ring C is responsible for electron delocalization during antioxidant activity. Moreover, the 3 OH in the C- ring and the presence of 2 adjacent hydroxyl groups in B ring are also important for the antioxidant activity (radical scavenging). While additional hydroxyl or methoxyl groups at positions 3, 5 and 7 of rings A and C seem to be less important. These structural features contribute to increase the stability of the aroxyl radical. Flavonoids reduce highly oxidizing free radicals such as superoxide, peroxyl, alkoxyl and hydroxyl radicals generated during eicosanoid pathway by hydrogen atom donation. The aroxyl radical ($Fl\text{-}O^{\cdot}$) may react with the second radical, acquiring a stable quinone structure. Besides, scavenging, the flavonoids may stabilize free radicals involved in the oxidative process by complexing with them (Nanda. et al 2007).

Antibacterial activity of fractionated phenolics

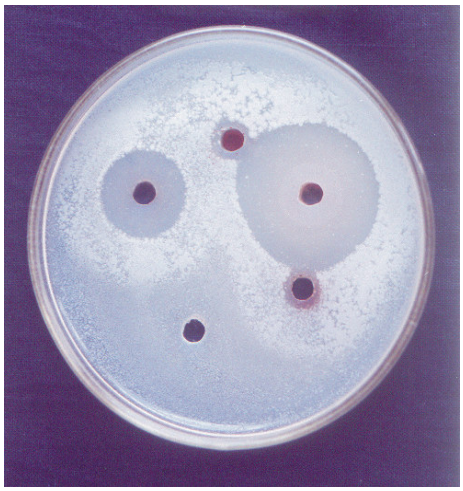
The agar diffusion bioassay relating to antimicrobial activity of the millet polyphenols showed very high levels of activity against all gram-positive bacteria compared to gram-negative bacteria (**Fig. 35**). The anti-bacterial screening of the isolated compounds which has been identified as gallic acid, protocatechuic, *p*-hydroxybenzoic, vanillic, *p*-coumaric, syringic, ferulic, *trans*-cinnamic acids and quercetin are presented in **Table 24**. Quercetin inhibited the growth of all the pathogenic bacteria screened whereas, gallic, ferulic, proto-catechuic, *p*-hydroxy benzoic acids showed their activity restricted only to a few bacterial strains, namely *E. coli*, *B. cereus*, *S. aureus*, *Y. enterocolitica* and *L. monocytogenes*. Phenolic acids



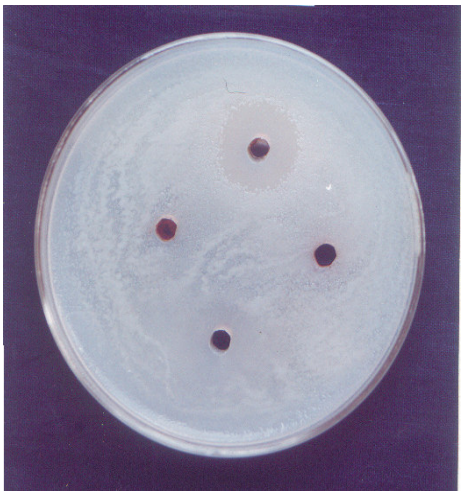
E.coli



S.aureus



B.cereus



L.monocytogenes

Fig. 35. Crude polyphenol extract showing anti-microbial activity. The clear next to the well is the inhibitory zone where polyphenols has inhibited the proliferation of cell growth.

Table 24. Antimicrobial activity of phenolic compounds against pathogenic bacteria

Phenolic compounds	Zone of inhibition (in mm)										
	ppm	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Crude polyphenol extract	250	5	-	6	6	5	5	-	-	-	-
Gallic acid	30	4	-	6	5	3	3	-	-	-	-
Quercetin	3	8	6	9	16	16	11	7	6	4	6
Protocatechuic acid	405	-	-	-	-	-	-	-	-	-	4
p-hydroxybenzoic acid	370	-	-	-	-	-	-	-	-	-	2
Vanillic acid	20	3	2	2	-	-	-	-	-	-	-
Trans-cinnamic acid	100	5	-	6	4	2	-	-	-	-	-
Syringic acid	10	3	-	5	2	2	-	-	-	-	-
Ferulic acid	41	5	-	5	3	2	-	-	-	-	-
p-coumaric acid	41	-	-	3	-	-	-	-	-	-	-
Oxacillin		-	-	11	16	-	14	18	-	-	-
Norfloxacine		11	13	-	-	18	-	-	12	-	12

1. E. coli, 2. *K. pneumoniae*, 3. *B. cereus*, 4. *S. aureus*, 5. *Y. entereocolitica*, 6. *L. monocytogenes*, 7. *S. pyogenes*, 8. *P. mirabilis*, 9. *S. marcescens*, 10. *P. aeruginosa*

may form both ester and ether linkages owing to the bi-functional nature through the reactions involving their carboxylic and hydroxyl groups respectively. Phenolic acids are also found abundantly in cell walls and are linked to hemicelluloses in different forms such as 2-O-(5'-O-(E)-feruoyl-B-D-xylopyranosyl)- (1-4) D-xylopyranose (Shibuya 1982; Fincher and Stone 1986). Thus cross-linking of phenolic compounds may provide a physical barrier to invasive disease development and consumption by insects (Zupfer et al. 1998). This allows phenolic acids to form cross-links with cell wall macromolecules. The increased concentration of ferulates in the outer layers may be implicated in resistance to both insect and fungal pathogens.Grains with elevated levels of phenolic acids in caryopsis exhibit greater resistance to disease and insect (Pussayanawin et al. 1988; Arnason et al. 1992). Moreover, cross-linking of arabinoxylans with phenolic acids lower the arabinoxylan solubility and swelling in water, as well as reduce their microbial degradation capacity in the human colon (Hatfield et al. 1993).

The gram-positive bacteria are normally more susceptible, since they have only a outer peptidoglycan layer which is not an effective barrier for permeability (Scherrer and Gerhardt 1971). The difference in sensitivity between gram-positive and gram-negative bacteria is because of an outer phospholipidic membrane carrying the structural lipopolysaccharide components in gram-negative bacteria (Nikaido and Vaara 1985). Polyphenols are well documented to have microbicide activities against the large number of pathogenic bacteria (Scalbert 1991). Very clear differences were found between the effects of different phenolics in the extracts. Quercetin inhibited the growth of all the prokaryotic species studied (*Staphyococcus aureus*, *E. coli, Bacillus subtulis, Micrococcus luteus* and *P. aeruginosa*) (Rauha et al. 2000). Gallic acid showed higher antibacterial activity than vanillic acid and caffeic acid. Rodriguez

Vaquero et al. (2007) reported that gallic acid shows higher antibacterial activity than protocatechuic acid which could be due to the extra hydroxyl group present in gallic acid.

The mechanism of polyphenols toxicity against bacterial growth may be related to the inhibition of hydrolytic enzymes (proteases and carbohydrolases) or other interactions to inactivate microbial adhesions, cell envelope transport proteins, non-specific interactions with carbohydrates, etc (Damintoti Karou et al. 2005). Finger millet seed coat a rich source of polyphenols and also being edible material, may have a very high potential antimicrobial agent for food preservation.

Summary and conclusions

- The IC_{50} values for the antioxidant activity of the polyphenols extracted using acidic methanol determined in terms of 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radicals as well as hydroxyl and ABTS radicals scavenging activities were 62, 109 and 114 μg/ml, respectively.
- The crude polyphenols extract of acidic methanol exhibited inhibition of the proliferation of *E. coli, B. cereus, S. aureus, L. monocytogenes, S. pyogenes, P. mirabilis, P. aeruginosa, S. marcescens, K. pneumoniae and Y. enterocolitica* to some extent but different phenolic acids exhibited inhibition on selective microflora. However, quercetin inhibited the growth of all the gram positive and gram negative pathogenic bacteria tested.
- Finger millet polyphenols could be considered as natural source of antioxidants and may be useful for minimizing the risk of diseases arising from oxidative deterioration and also as antimicrobial agents against a good number of food borne microflora.

These observations revealed that, the millet polyphenols may serve as natural source of antioxidants as well as antimicrobial agents and may be useful as a nutraceutical for reducing the intensity of some of the diseases arising out of oxidative deterioration in humans, if not preventing them, and also for enhancing the safety of foods from microbial spoilage.

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