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Finger millet polyphenols: Optimization of extraction and the effect of pH on their stability

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Abstract

Finger millet, one of the minor cereals, is known for several health benefits and some of the health benefits are attributed to its polyphenol contents. Investigations of suitable solvents for extraction of polyphenols and their stability, during changes of pH and temperature, were carried out. Histochemical examination of the millet kernels, and also analysis of the seed coat and the endosperm fractions of the millet for the polyphenol contents, revealed that nearly 90% of the polyphenols were concentrated in the seed coat tissue. In view of that, the polyphenol contents of the seed coat fraction of the millet were extracted with different polar and non-polar solvents, and it was observed that 1% HCl–methanol was very effective for extraction of the millet polyphenols. Accordingly, the polyphenols were extracted with acidic methanol and the polyphenols obtained were examined for pH and temperature stability. The phenolic contents ($6.4 \pm 1.0\%$) of the extract remained constant at highly acidic to near neutral pH (6.5) but decreased gradually to $2.5 \pm 0.3\%$ as the alkalinity increased to pH 10. The increase in pH resulted in precipitation of some of the extracted matter, and this increased from $4 \pm 0.5\%$ to $40 \pm 3\%$ of the extracted matter, as the pH increased from 1 to 10. But, the polyphenol contents of the extract were stable to the changes in the temperature of the extract. Fractionation of the polyphenols extracted by high performance liquid chromatography (HPLC) showed that the analytes were derivatives of benzoic acid (gallic acid, proto-catechuic acid, and *p*-hydroxy benzoic acid) and cinnamic acid (*p*-coumaric acid, syringic acid, ferulic acid and *trans*-cinnamic acid). However, in a highly alkaline condition (pH 10) of the extract, only gallic acid and proto-catechuic acid were detected.

Keywords: Finger millet; Polyphenols; Histochemistry; pH stability; Fractionation by HPLC

1. Introduction

Finger millet (*Eleusine coracana*) is one of the important minor cereals and constitutes a staple food for a large segment of the population in the Indian subcontinent as well as of many in the African countries. It is a naked caryopsis with a brick red-coloured seed coat and is generally used in the form of whole meal for preparation of traditional foods, such as *roti* (unleavened breads or pancake), *mudde* (dumpling) and *ambali* (thin porridge). The seed coat of the millet is an edible component of the kernel and is a rich source of phytochemicals, such as dietary fibre and polyphenols, and is also a very good source of minerals, especially calcium (Hadimani & Malleshi, 1993). It contains 0.2–3.0% of polyphenols (Ramachandra, Virupaksha, & Shadaksharaswamy, 1977) but information on the nature of its polyphenols is scanty. Polyphenols exhibit a defence mechanism against animal as well as bird predators and also microbes for plants. Besides they are reported to improve aortic fragility and elasticity by attenuating elevation of blood pressure and they increase vasorelaxation (Mizutani, Ikeda, Kawai, & Yamori, 1999). Cereal polyphenols are important phytochemicals with one or more aromatic rings, with hydroxyl groups in different patterns (Towo, Svanberg, & Ndossi, 2003). They exhibit significant antioxidant activity (Sripriya, Chandrashekaran, Murty, &

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Chandra, 1996) and many other potential health beneficial properties, such as anti-inflammatory, antiviral, anticancer and platelet aggregation inhibitory activity. Limited information on the health benefits of wheat, barley, rye and oat polyphenols has been reported (Malleshi, Rati Rao, & Srinivas, 2003; Zielinski & Kozlowski, 2000).

Regular consumption of finger millet is known to reduce the risk of *diabetes mellitus* (Gopalan, 1981) and gastrointestinal tract disorders (Tovey, 1994) and these health beneficiary aspects of the millet could be attributed to its polyphenol contents. The utilization of wholemeal cereals and also their seed coat matter in food formulations is increasing world wide, since they are rich sources of phytochemicals and dietary fibre, which offer several health benefits. Nowadays, the millet seed coat is readily available as a by-product in the millet malting (Malleshi, Chakravarthy, & Kumar Sourav, 1995) and milling industries (Malleshi, 2003). Hence, information on the nature of polyphenols of millet could be useful for understanding the health benefits associated with millet and also for augmenting its utilization as a source of nutraceuticals. Accordingly, studies were conducted to isolate and to examine some of the properties of the millet polyphenols.

2. Materials and methods

2.1. Millet

A popularly cultivated variety of finger millet was purchased from the local market, cleaned, pulverized ('250 μm' mesh size) in a laboratory cyclone mill (Udy Company, Boulder, CO, USA) and used for the studies.

2.2. Materials

Phenolic standards: gallic acid, vanillic acid, ferulic acid, *p*-coumaric acid, 4-hydroxy benzoic acid, syringic acid, *trans*-cinnamic acid, *p*-catechuic acid, quercitin, kaempferol and tri-fluoroacetic acid were purchased from Sigma Chemical Co., St. Louis, MO. Ultra pure water was generated by the Milli Q system (Millipore, Bedford, MA). The other reagents and solvents used were of analytical or HPLC grade.

Wholemeal (1 g) millet, defatted using petroleum ether (60–80 °C), was suspended separately in water, acetone, propanol, ethanol and methanol, and stirred using a magnetic stirrer for about 3 h under ambient conditions (25–30 °C) to extract the polyphenols. Parallelly, the extraction was carried out by refluxing for about 3 h in these solvents. In a separate set of experiments, the solvents were acidified with 1% HCl and the extraction was carried in the cold (25–30 °C) and also, parallelly, by refluxing with the same set of solvents for 3 h using a water bath. The extracts from each of the solvents and each of the experiments were separated from the residues by centrifugation and the polyphenol content of the extracts was assayed (Singleton, Orthofer, & Rosa, 1995). Guided by the outcome of these experiments, the millet polyphenols were extracted by refluxing the millet meal with 1% HCl–methanol.

2.3. Varietal variations

A few popular 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 and pulverised in a cyclone mill (Udy Company, Boulder, CO, USA) fitted with a screen of 0.5 mm opening to prepare wholemeals. The samples (5 g) were refluxed with 100 ml of 1% HCl–methanol solvent to extract the polyphenols and the polyphenol contents of the extracts were assayed (Singleton et al., 1995). Subsequently, GPU 28 variety was used for the studies as it contained fairly high levels (2.3 ± 0.2) of polyphenols.

2.4. Localisation of the millet polyphenols

Sections (5–8 μm thick) were obtained from the millet, using a microtome (American Opticals, USA), and were soaked in 25% propylene glycol (1,2 propanediol) for about an hour, then mounted on glass slides and mixed with a drop of glycerine. Cover slips were placed over the sections and viewed under a fluorescence microscope (Olympus, USA) and the fluorescing locations were photographed.

2.5. Milling of the millet

The millet was sprayed with 5% additional water, tempered for about 10 min and pulverized in a comminuting mill (Apex comminuting mill, Apex constructions Ltd., England). Soon after pulverizing, the meal was sifted through a sieve (180 μm) (Kurien & Deshikachar, 1962) and the tailings ('+180 μm' fraction) were again pulverized immediately and sieved through the same sieve, and the process was repeated for a third time. The flours ('−180 μm' fraction) from 1, 2 and 3 passes were pooled and designated as refined flour fraction (RFF), whereas, the tailings was termed as the seed coat fraction (SCF). The polyphenol contents of the seed coat and the refined flour fractions were assayed and it was found that the SCF contained significantly higher levels of polyphenols, than did the RFF. To concentrate the polyphenol content of the SCF, it was washed with water to free it from adhering starch. The wash water and the residue were dried. The residue was micropulverized and sieved (180 μm) and the overtails ('+180 μm') and the throughs ('−180 μm') were separately collected. The water extract, and the 'over tails' and the 'throughs' were analyzed for their polyphenol contents. It was observed that the '+180 μm' fraction contained a considerably higher proportion of polyphenols than did other fractions and hence, it was termed as the polyphenol rich seed coat fraction (PRSCF) and used for the subsequent studies.

About 100 g of the PRSCF was refluxed with about 500 ml of 1% HCl–methanol, filtered and the residue

reextracted with fresh solvent; the process of extraction was repeated until the residue tested (with FC reagent) negative for polyphenols. The extracts were pooled and used for temperature and pH stability studies and also for fractionation of the polyphenols.

2.6. Temperature and pH stability of the polyphenols in the extract

The aliquots of the extract, placed in conical flasks fitted with water condensers, were left in a water bath maintained at 30–90 °C with a 10 °C increment for 48 h, and the polyphenol content was assayed. A predetermined quantity of NaOH was added to about 200 ml of the extract, taken in 12 beakers, separately, to raise the pH from 1 to 12, and the contents after about 30 min were centrifuged to separate the precipitate and the supernatant. The polyphenol content of the supernatant, as well as the precipitated matter from each of the samples, was assayed. The λ_{max} of the supernatants in the UV–visible range was also recorded, and the colours ($L^*a^*b^*$ values) were recorded with a Lab Scan XE (Hunter Lab Instruments, USA) with CIE standard observer (10° view angle) with illuminant D_{65}. In a separate experiment, about 2 l of the extract were neutralized to pH 7 and the supernatant and the precipitate formed were used for the proximate analysis. The proximate composition (AACC, 2000) and some of the mineral contents were determined using atomic absorption spectroscopy (Model "AA-670F", Shimadzu, Singapore).

To elucidate the possible similarities between the polyphenols of the extract and some of the pure phenolics commonly present in cereals, the absorption spectra of 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 1% HCl–methanol solvent system and the pH, adjusted to 3, 7 and 10 with NaOH, were determined. The molar extinction coefficients (ϵ) of the samples were determined, following equation $\epsilon = A/dc$; where A, absorbance or optical density; $d = 1$ cm, the thickness of the UV cell; and c, molar concentration of the test compound (Freifelder, 1982).

2.7. Fractionation of the polyphenols

The acidic methanol extract was neutralized to pH 3, 7 and 10, and the supernatant separated from the precipitate by centrifugation was concentrated at 40 °C under low pressure. The polyphenols were fractionated into the constituent phenolics using a reverse-phase analytical HPLC [Shimadzu LC-10A liquid chromatograph fitted with a 250 mm × 4.6 mm ODS 2 C_{18} column and equipped with a 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 [water acidified with 0.1% trifluoroacetic acid (solvent A) and 100% methanol (solvent B)], maintained at a flow rate of 1.0 ml/min. The gradient programme, initiated with 80% eluent A and 20% eluent B, was ramped linearly to 60% solvent A and 40% solvent B within 40 min. This proportion (60:40) was maintained for the next 10 min and, subsequently, the solvent gradient was reverted to the initial conditions (80:20) within the next 5 min (total run time was 55 min). A few phenolic standards normally present in cereals were also similarly run. The effluent was read at 295 nm and, based on the elution time, some of the phenolics were identified and also quantified.

2.8. Data analysis

Each experiment was performed, at least in duplicate or triplicate, and the results were expressed as the mean values ± standard deviation using the Microsoft excel programme.

3. Results and discussion

3.1. Identification of a suitable solvent for extraction of polyphenols

The polyphenol contents of the extracts, using water, acetone, propanol, ethanol and methanol in the cold (30 ± 2 °C) and after refluxing, presented in Table 1, indicate that, only 7.4%, 13.1%, 10.0%, 13.1% and 19.6% of the polyphenol contents of the PRSCF were extracted by water, acetone, propanol, ethanol and methanol in the cold and 10.9%, 39.0%, 37.4%, 44.3% and 53.9% after refluxing, respectively. On the other hand, the acidified solvents extracted 14.8%, 21.8%, 25.2%, 31.3% and 39.5% of the polyphenol contents of PRSCF in the cold condition and 19.6%, 45.2%, 53.5%, 60.0% and 100% on refluxing, respectively. This indicates that pure solvents at ambient temperature were very poor extractants of the millet polyphenols,

Table 1
Finger millet polyphenols (g%) extracted by different solvents under ambient conditions and also after refluxing

Solvents	Extraction under 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 independent determinations. Values in parentheses indicate percentages of total assayable polyphenols.

but the acidification of the solvents enhances the extractability considerably; further to that, refluxing seems to be very effective for extraction of the millet polyphenols. Among the different methods and different solvents tried, refluxing the meal with the 1% HCl–methanol solvent was very effective. Raising the temperature of the extractant might soften the tissues and weaken the phenol–protein and phenol–polysaccharide linkages, leading to migration of the polyphenols into the solvent.

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, methanol, ethanol, propanol, acetone, ethyl acetate, dimethylformamide and their combinations has been generally used for extraction of cereal polyphenols (Naczk & Shahidi, 2006). But, for finger millet, use of 1% HCl–methanol has been consistently reported (Ramachandra et al., 1977; Sripriya et al., 1996).

Most of the methods for 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 is non-specific for any particular phenolics and the colour developed depends on hydroxyl groups and their position in the polyphenol molecule. In view of this, FC reagent was used for assay of the millet polyphenols 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. 1, reveal 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} prominently at 220 and 280 nm. This shows that acidified solvents extract more phenolic constituents than do the pure solvents.

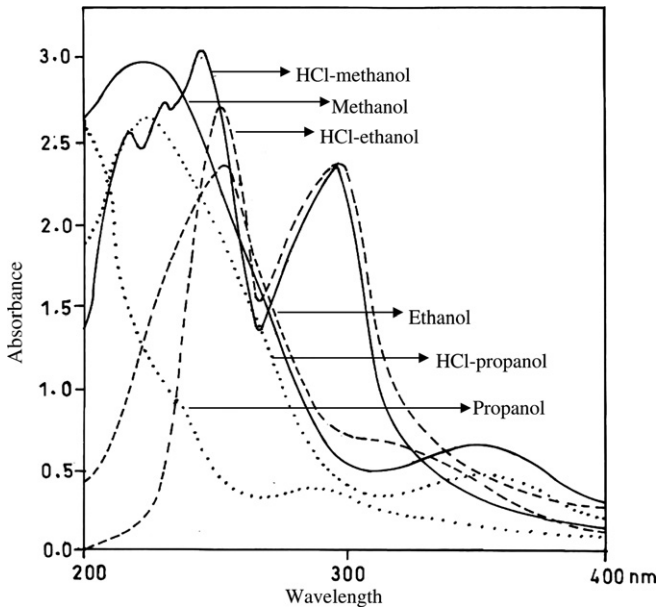

Fig. 1. Absorption spectra of the polyphenols extracted with pure and acidified (1 ml of conc. HCl to 100 ml of respective solvents) solvent.

3.2. Varietal variations in polyphenol contents

Considerable differences, with respect to the polyphenol contents of the varieties, were observed and it was found that the brown varieties contained (1.2–2.3%) higher proportions of polyphenols than did white (0.3–0.5%) varieties (Table 2). The noticeable difference between white and brown varieties could be due to the presence of the red pigments, such as anthocyanins, which are generally polymerized phenolics present in brown cultivars. The polyphenol contents 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. GPU 28, GPU 26, PR 202 and Indaf 9 showed λ_{max} at four, HR 911 at three and Indaf 5 and Indaf 7 at two different wavelengths (Table 2). Large varietal differences, with respect to the polyphenol contents 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). Likewise, among the 85 Indian finger millet varieties, considerable differences (0.19–3.37%) in the total polyphenol contents (as catechin equivalents) has been reported (Shankara, 1991).

Table 2
Polyphenol contents of a few finger millet varieties (g%) and their λ_{max}

Variety	Polyphenols[b]	Wavelength (nm)	Absorbance
Indaf 7[a]	0.3 ± 0.1	282	2.98
		241	3.1
Indaf 5[a]	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 28	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 26	2.3 ± 0.2	532	0.6
		460	0.5
		284	3.9
		246	3.99

[a] White seeds.
[b] Gallic acid equivalents.

3.3. Distribution of polyphenols in the millet kernel

The microscopic examination of the sections of the millet kernel under UV light showed strong fluorescence in the seed coat and feeble fluorescence in the endosperm portions, indicating that, the polyphenols of the millet are concentrated in the seed coat. The assay of the polyphenol contents of the seed coat and the endosperm fractions, of the millet prepared by milling, also confirmed that the polyphenol content of the seed coat (6.2%), was several-fold higher than that of the flour fraction (0.8%) (Table 3). This substantiates the histochemical observations and also corroborates earlier reports, on cereal polyphenols, that testa contains the bulk of the polyphenols (Hahn, Rooney, & Earp, 1984). In the case of sorghum and barley, a blue autofluorescence has also been reported in the cell walls, revealing the presence of ferulic and di-ferulic acids (Earp, Doherty, & Rooney, 1983).

3.4. Nutrient composition of the milling fractions

The seed coat fraction contained 13.1% protein, 3.2% fat, 5.6% ash and 43.8% dietary fibre and these values were significantly higher than those for the wholemeal millet (Table 3). It also contained 1.25% calcium, indicating that about 50% of the calcium content of the whole grain is concentrated in the seed coat. These values are in agreement with Kurien, Joseph, Swaminathan, and Subramanyan (1959), who reported the distribution of major nutrients in the millet kernel.

The yield of the seed coat fraction, prepared by milling the millet, was 20% of the kernel; however, the seed coat content of the millet accounts for only 12 ± 2% of the kernel. The higher yield was mainly due to the endosperm matter adhering the seed coat and this was washed away with excess water. Further, micro-pulverising the washed seed coat matter and removal of the finer fraction by sieving ('180 μm' sieve), enhanced the polyphenol content of the coarser ('+180 μm') fraction (Table 4). This method of preparation of polyphenol-rich fraction from the millet appears to be advantageous over the chemical extraction method, because a large quantity of solvent was required (about 2 l of acidic methanol for 100 g of seed coat) to extract the polyphenols which had to be evaporated to prepare the polyphenol rich material.

Table 4
Polyphenol contents of seed coat milling fractions (g%)

Sample		Polyphenols[a]
a.	Finger millet (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 μm' fraction	12.8 ± 1.3
f.	'−180 μm' fraction	2.6 ± 0.4

[a] Gallic acid equivalents, values on dry weight basis.

3.5. Properties of polyphenols in the extract

The assayable phenolics in the extract remained constant, even after 20 days of storage under ambient (30 ± 2 °C) conditions, and also on heating to 90 °C. This showed that extraction of the millet polyphenols by refluxing with acidic-methanol is feasible. The anthocyanins of wheat are also reported to be stable up to 80 °C (El-Sayed, Abdel-Aal, & Pierre, 2003). On the other hand, the polyphenols extracted exhibited a very high degree of sensitivity to changes in pH, as even a slight increase in pH, by addition of NaOH, resulted in formation of precipitate, and the quantity of precipitate formed increased concurrently with increase in pH (Fig. 2). While, the amount of precipitate formed was about 4.0% of the extracted material at pH 1, it was about 44% at pH 10. The precipitation was found to be reversible, as it dissolved completely when acidity was

Table 3
Proximate composition and polyphenol contents of wholemeal and the milling fractions of the millet (g%)

	Wholemeal	Refined flour ('−180 μm' fraction)	Seed coat ('+180 μm' fraction)
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[a]	*2.3*	*0.8*	*6.2*

ND, not detected. Averages of two determinants.
[a] Gallic acid equivalents.

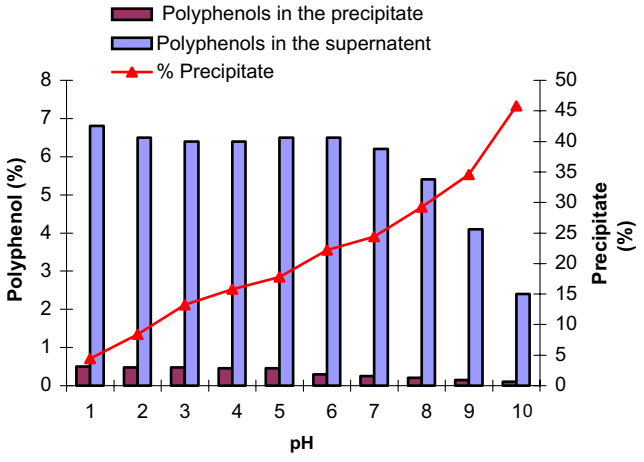

Fig. 2. Polyphenol content of the supernatant and the precipitate formed at different pH values.

increased equal to the pH of the 1% HCl–methanol solvent used for extraction.

It was observed that the assayable polyphenol contents in the extract decreased as the pH increased toward alkalinity and the absorption pattern for the phenolics in the supernatant also changed (Fig. 3). Peaks at 243 and 283 nm were prominent at pH 1 but, at alkaline pH, only the peak at 214 nm was prominent and the second peak at 280 nm was negligible (Table 5). Phenols usually show absorption maxima between 200 and 360 nm and the band at shorter wavelength is known as the B-band and that at longer wavelength as the C-band (Dearden & Forbes, 1959). Generally, both bands get displaced to longer wavelengths, depending on the nature of solvents, electron withdrawing and electron donating substituents in the benzene ring(s) (Mendel & Jurgens, 2000). Cabrita, Fossen, and Anderson (1999) also reported that the absorptivities were higher at pH 1 for all anthocyanidin 3-glucosides and they decreased as pH increased to 5.

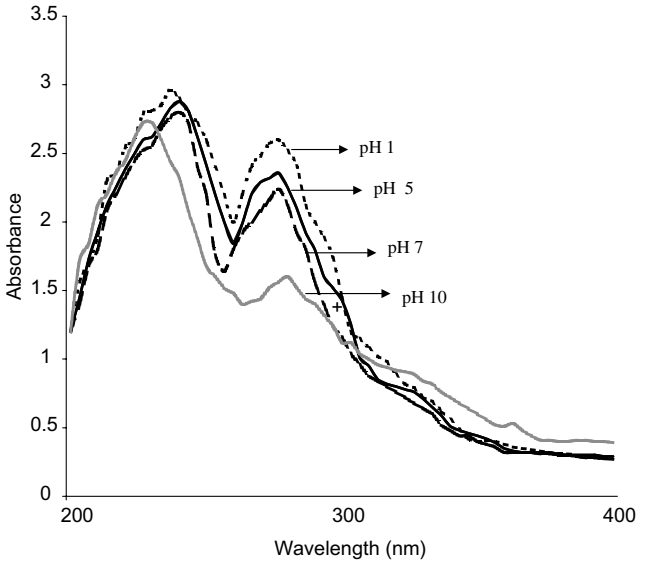

Fig. 3. Effect of pH on the absorption spectra of the millet polyphenols extracted with 1% HCl–methanol.

Table 5
Absorption maxima and corresponding phenolics of the polyphenolic extract at different pH values

Sample	λ_{max}		Corresponding standards
	Wavelength (nm)	Absorbance	
Control	533	0.77	Not identified
	461	0.69	Not identified
	393	0.31	Kaempferol
	282	3.31	Gallic acid, caffeic acid
	242	3.96	*p*-Hydroxy benzoic acid
pH 1	527	0.51	Not identified
	457	0.56	Not identified
	283	3.21	Gallic acid, caffeic acid
	243	3.99	*p*-Hydroxy benzoic acid
pH 3	282	3.21	Gallic acid, caffeic acid
	243	3.99	*p*-Hydroxy benzoic acid
pH 5	281	2.68	Gallic acid, caffeic acid
	240	3.99	*p*-Hydroxy benzoic acid
pH 7	282	2.65	Gallic acid, caffeic acid
	240	3.99	*p*-Hydroxy benzoic acid
pH 9	241	3.99	*p*-Hydroxy benzoic acid
pH 11	214	3.31	Not identified

The colour values of the supernatant at pH 1–10 recorded in terms of $L^*a^*b^*$, showed synergestic effects expressed as 'ΔE', $[\Delta E^* = (\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$, which represents the magnitude of the 'total colour difference' or deviation from standard white (Table 6). The apparent colour 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 colour or increase in lightness of pink colour) was concomitant with the increase in pH. However, slight deviations at pH 4 ($\Delta E = 31.8$) and pH 6 ($\Delta E = 35.4$) were noticed, which were lower than that at pH 7 ($\Delta E = 37.5$). The change in the colour of the extract with change in pH could be due to the precipitation of the coloured pigments or alteration in their chromophoric characteristics under alkaline conditions. The deviations observed at pH 4 and pH 6 may be due to selective solubility characteristics of some of the pigments and polyphenols at these pH values. In the case of red wine also, the pigment malvidin 3-glucoside is red at pH 1, colourless at pH 6 and 7 and changes to yellow at pH 8 (Lapidot, Harel, Akiri, Granit, & Kanner, 1999). The trend in change in the colour as a result of changes 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 polyphenol extract after adjusting to pH 7, presented in Table 7, shows that the precipitate contained 86% minerals but it does not contain protein or fat at measurable levels. The other matter could be pigments and some of the unidentified seed matter

Table 6
Colour measurement of the polyphenolic extract at different pH values

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 values indicate green.
b^*, (yellow–blue) axis indicates positive values; they are yellow when positive; negative value represent blue and '0' is neutral.
ΔE, magnitude of the 'total colour difference'.

Table 7
Proximate analysis (g%) and some of the minerals (mg%) of the supernatant extract and the precipitate, at neutral pH, of the extract

	Supernatant	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
Phophorus	0.05	0.4
Manganese	5.4	8.0
Calcium	290	590
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. Averages of two determinations.

extracted by acidic methanol. It also contained small amounts of gallic acid (0.37%) and *p*-catechuic acid (0.4%). The possibility of precipitation of some of the minerals as hydroxides, on addition of NaOH, explains the high proportion of minerals in the precipitate. Calcium content of the precipitate was highest compared to other minerals; besides, it contained 12.6% total dietary fibre, which could be the non-starchy polysaccharide extracted form the millet by the acidic methanol.

3.6. Fractionation of phenolics

The LC–DAD chromatograms of the polyphenols from the extract and that of the precipitated matter at pH 3, 7 and 10 are presented in Figs. 4 and 5, respectively. The mobile phase of the HPLC eluent was optimized to achieve good resolution, 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. The phenolics identified in the extract, at pH 3, were gallic acid, proto-catechuic acid, *p*-hydroxy benzoic acid, and also *p*-coumaric acid, syringic acid, ferulic acid and *trans*-cinnamic acid. But, at pH 7.0, gallic, syringic, ferulic and *trans*-cinnamic acid and, at pH 10.0, only gallic and syringic acid, were detected. However, a good number of peaks remained unidentified, which could be flavanols anthocyanins and anthocyanidins. But, in the precipitated matter, only benzoic acid derivatives, namely gallic and proto-catechuic acid were identified. From this, it may be inferred that the millet phenolics are more stable under

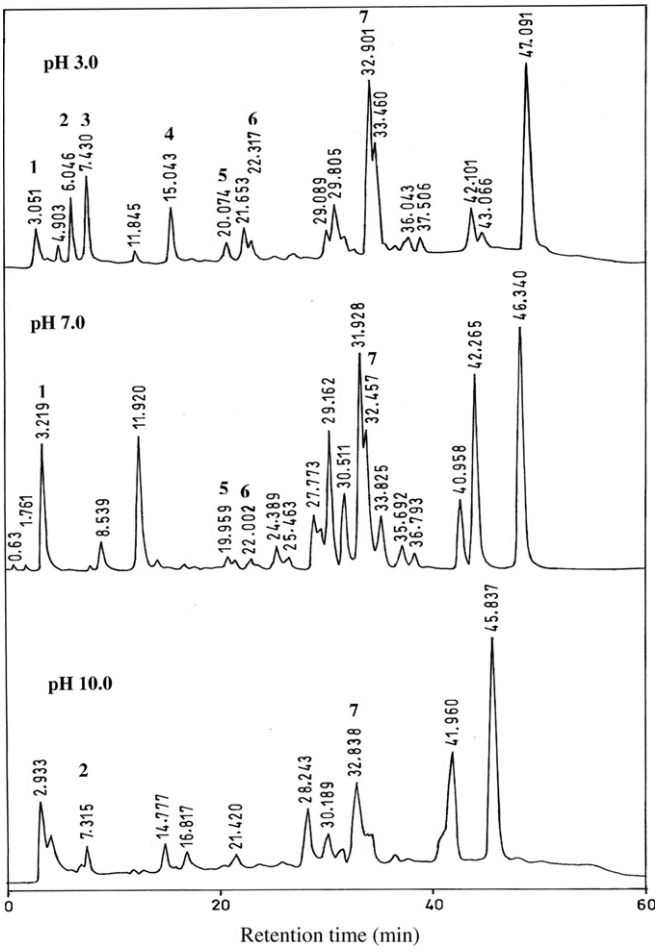

Fig. 4. Fractionation of the phenolics extracted at different pH values by HPLC at 295 nm. Peak numbers: (1) gallic acid; (2) proto-catechuic acid; (3) *p*-hydroxy benzoic acid; (4) *p*-coumaric acid; (5) syringic acid; (6) ferullic acid; and (7) *trans*-cinnamic acid.

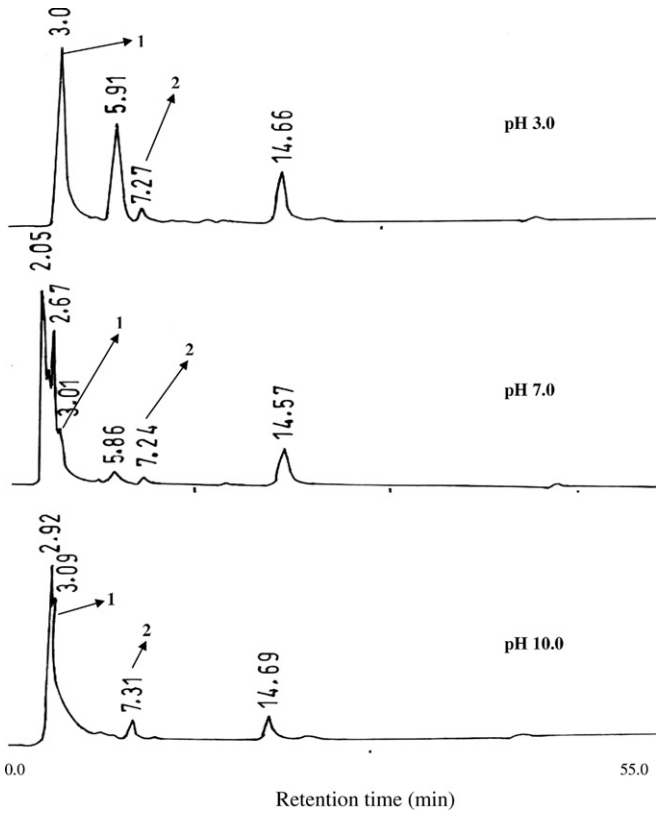

Fig. 5. Fractionation of the phenolics of the precipitate formed at different pH values by HPLC at 295 nm. Peak numbers: (1) gallic acid; (2) proto-catechuic acid.

Table 8
Relative percentage of phenolics calculated, based on total peak area in the extract at different pH values

Standard phenolic acids	Polyphenols (%)		
	pH 3	pH 7	pH 10
Supernatant			
Gallic acid	6.8	6.4	5.7
Proto-catechuic acid	2.24	2.13	1.9
p-Hydroxy benzoic acid	3.71	3.1	2.83
p-Coumaric acid	3.2	2.87	2.65
Syringic acid	1.56	1.05	0.89
Ferulic acid	9.0	8.62	6.7
trans-Cinnamic acid	1.9	1.86	1.43
Precipitate			
Gallic acid	0.15	0.15	0.37
Proto-catechuic acid	0.15	0.15	0.4

acidic conditions and are feeble or labile under alkaline conditions, as they may lose their identity by oxidation or be removed by chelating with metal ions. The retention time, area and the standard peaks, and also the percentage of the phenolics fractionated by HPLC, are presented in Table 8. The HPLC chromatogram for the supernatant at neutral pH exhibited 11 major and eight minor peak but, among those, only the phenolics, representing seven peaks, were identified. These accounted for 27.2% of the phenolics extracted but the rest of them remain to be identified. Efforts are underway to identify these phenolics, also, by LC-MS/MS and NMR analysis.

Limited reports on the polyphenols of a few cereals indicate that barley contains measurable amounts of catechins and some di- and trimer procyanidins, whereas sorghum, contains benzoic and cinnamic acid derivatives (Hahn, Faubion, & Rooney, 1983). In cereal grains, the phenolic acids are generally in the form of free acids, soluble and insoluble esters, and are concentrated in the outer layer (pericarp, testa and aleurone) of the kernel. Only insoluble

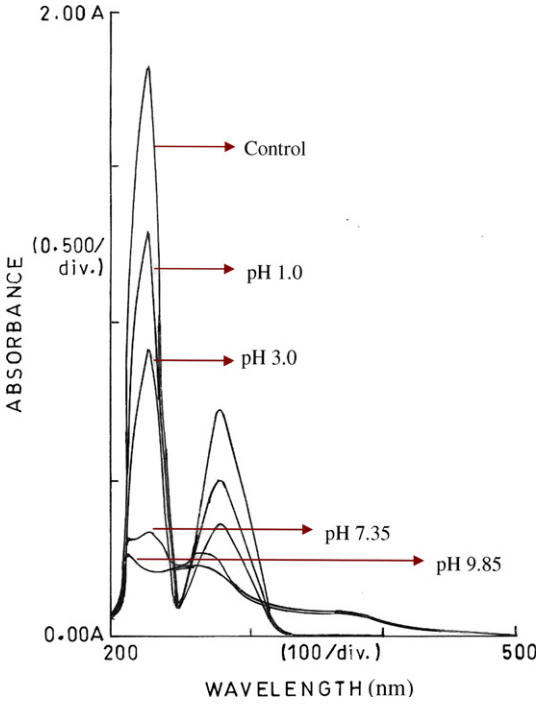

Fig. 6. UV–visible spectra of 5.8×10^{-6} M gallic acid at different pH values.

and tightly-bound phenols are present in the endosperm. The bound or insoluble phenolic acid esters appear to be associated with the cell walls of the grain. In the case of sorghum the major bound phenolic acid is ferulic acid (3-methoxy-4-hydroxy-cinnamic acid) and is generally associated with the cell walls (Hahn et al., 1984).

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

Table 9
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	113,758	326	720,000	276	252,166
	218	289,655	244	408,035	217	171,000
			218	539,285		
1.0	274	82,413	326	585,000	276	563,928
	218	213,793	242	325,714	217	178,333
3.0	256	42,206	326	490,357	276	632,142
	220	63,965	242	273,750	217	202,333
7.35	262	47,586	322	455,357	276	273,166
	214	50,000	218	42,500	217	188,333
9.89	258	20,344	318	322,142	275	268,000
	212	36,551	290	32,1428	216	19,8166

extract (Fig. 6). But, in the case of chlorogenic acid, the λ_{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 9). In the case of *trans*-cinnamic acid, the λ_{max}values were at 276 nm and 217 nm and there was a substantial change in the λ_{max} 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.

4. Conclusion

The polyphenol contents of finger millet are concentrated in the seed coat and the acidic methanol solvent happens to be an effective extractant for the millet polyphenols. The polyphenols of the millet are heat-stable but pH-sensitive and are largely unstable under alkaline conditions. The HPLC of the polyphenols of the millet indicated nearly 30 prominent constituent phenolics but only about 30% of them could be identified.

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