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Full Length Research Paper

Finger millet (*Eleusine coracana*) polyphenols: Investigation of their antioxidant capacity and antimicrobial activity

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Finger millet (*Eleusine coracana*) or ragi, one of the important minor cereals is a rich source of several phytochemicals and among them polyphenols are the most important because of their nutraceutical potentials. The seed coat-rich fraction (SCF) of the millet contains over 70% of polyphenols of the millet. Treated with different polar and non-polar solvents as such and after acidification with HCl for the extraction, it was observed that the polar solvents extracted more (1.7 to 7.4%) than the non-polar solvents (0.28 to 1.54%) while the 1% HCl–methanol extracted the highest proportion of polyphenols (13%). Fractionation of extracted polyphenols by HPLC revealed the presence of a large number of constituent phenolics. The inhibitory concentration (IC_{50}) values for the antioxidant activity of the polyphenols determined in terms of 1, 1-diphenyl-2-picrylhydrazyl (DPPH), hydroxyl and 2, 2'- azinobis – 3 – ethylbenzothiazoline – 6 – sulfonate (ABTS) radicals scavenging activities of acidified methanolic extract, were 63, 109, and 114 µg/mL phenolic, respectively. The polyphenols extracted from finger millet seed coat using 1% HCl –methanol was fractionated by reverse phase– high performance liquid chromatography (RP-HPLC) and the constituent phenolics were characterized by electrospray ionization mass spectrometry (ESI-MS) and NMR. The phenolics identified were the benzoic acid derivatives (gallic, protocatechuic, *p*-hydroxybenzoic, vanillic, and ferulic acids) and cinnamic acid derivatives (syringic, trans-cinnamic and *p*-coumaric acids); and also a flavonoid compound namely, quercetin. The millet polyphenols showed proliferation inhibitory activities on *Escherichia coli, Bacillus cereus*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Streptococcus pyogenes*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Serratia marcescens*, *Klebsiella pneumonia*, and *Yersinia enterocolitica*. Quercetin exhibited substantial inhibition of the growth of these pathogenic bacteria revealing the potential nutraceutical properties of the millet polyphenols. From the observation, it could be inferred that finger millet polyphenols could be used as natural source of antioxidants especially for minimizing the risk of diseases arising from oxidative deterioration.

Key words: Finger millet, polyphenols, antioxidant activity, 1, 1-diphenyl-2-picrylhydrazyl (DPPH), radical scavenging activity.

INTRODUCTION

Antioxidants are defined as molecules that can delay or prevent oxidation of an oxidizable substrate (Halliwell and Whiteman, 2004) and has multiple functions in biological systems, including defence against oxidative damage and exerting beneficial effects in the major signalling 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

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(H_2O_2), the superoxide anion ($O_2^{\bullet-}$), and free radicals, such as the hydroxyl radical ($O^{\bullet}H$). ROS are endogenous intermediates constantly produced in the human body. These are unstable and highly reactive component, generated continuously in signalling the cascade involved in cellular functions such as cellular proliferation and inflammation. 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). Imbalance of cellular oxido/redox status, results in pathological manifestation (Frlich and Riederer, 1995). Hence, for proper functioning of various biochemical and physiological activities of the human body, formation and denaturing the ROS has to be balanced.

The anti-microbial agents normally used for preservation of foods are mostly synthetic chemicals and there is greater interest in natural products with anti-microbial properties for food preservation. In view, identifying edible anti-microbial agents is receiving attention worldwide. Polyphenols, the group of highly hydroxylated phenolic compounds present in plants and products of plant origin are potential inhibitors on the growth of microorganisms. They are derived from phenylalanine and tyrosine and generally occur as glycosylated derivatives with antioxidant activity and inhibit the oxidation of low – density lipoproteins as well as reduce thrombotic tendencies (Hertog et al., 1995).

Dietary polyphenols exert beneficial biochemical properties such as free radical scavenging, metal chelation and inhibition of lipid peroxidation. Naturally, edible compounds with anti-microbial activity may have nutraceutical value and serve as a low cost pharmaconutrients and also as components of drugs (Middleton and Kandaswami, 1994). Polyphenols also play a role on inhibition of tooth decay, amelioration of allergy and prevention of gout (Sakanaka et al., 1989).

Millets are crops of food security and have unique position among cereals due to their nutritional specialty and health benefits (Hahn et al., 1984). Finger millet (*Eleusine coracana*) commonly known as 'Ragi', one of the important minor millets of Indian subcontinent and some of the African countries, is a rich source of several phytochemicals, dietary fiber and minerals especially calcium (Hadimani and Malleshi, 1993) and offers several health benefits to its consumers.

Polyphenols are the most important phytochemicals of the millet because of their nutraceutical potentials such as antioxidant activity, anti-inflammatory, anti-carcinogenic, antimicrobial, anti-diarrheal, antiulcer, and anti-cardiovascular properties (Sripriya et al., 1996). Besides, the polyphenols are also useful in management of several physiological disorders such as diabetes mellitus, hypertension, vascular fragility, hypercholesterolemia, prevention of oxidation of low-density lipoproteins (LDLs) and also improvement of the health of gastrointestinal tract (Scalbert et al., 2005). In a recent study, over 50 phenolic compounds has been identified in several whole millet grains like kodo, finger, foxtail, proso, little and pearl using HPLC and ESI-MS^n and also their antioxidant and antiradical activity was estimated (Chandrasekara and Shahidi, 2011). Soluble and bound phenolics are rich source of phenolic compounds with an antioxidant, metal chelating and reducing power (Chandrasekara and Shahidi, 2010).

The millet contains 0.5 of 3.0% polyphenols and there are no reports about any adverse effect even though wholemeal millet is used for food (Malleshi, 2004). Hence, investigations have been initiated on finger millet polyphenols towards health beneficial effects and the present communication deals with extraction and characterization of the millet polyphenols and their nutraceutical potentials.

MATERIALS AND METHODS

The millet

A high yielding popular finger millet variety (GPU 28) procured from the University of Agricultural Sciences; Bangalore, India was used for the studies.

Chemicals

Ferulic, gallic, *p*-coumaric, *p*-catechuic, *trans*-cinnamic, vanillc, and syringic acid; quercetin; 1,1-diphenyl-2-picrylhydrazyl (DPPH); butylated hydroxyanisole (BHA); phenazine methosulphate (PMS); hydrogen peroxide (H_2O_2); thiobarbituric acid (TBA); ethylenediaminetetraacetic acid (EDTA); deoxyribose; ammonium persulphate (APS); 2,2'- azinobis – 3 – ethylbenzothiazoline – 6 – sulfonate (ABTS) 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 and Folin and Ciocalteu's phenol reagents were from Sisco Research Laboratories Pvt. Ltd., Mumbai, India and anhydrous sodium carbonate, potassium ferrricyanide, trichloroacetic acid (TCA); sodium phosphate and ammonium molybdate were from Ranbaxy Laboratories Ltd., New Delhi, India.

Preparation of seed coat rich millet fraction

The millet was milled to prepare the seed coat rich fraction (SCF) following the protocol developed at CFTRI, Mysore (Chethan and Malleshi, 2007) and the SCF was stored at 8°C in closed air tight container and used for further studies. It was defatted using petroleum ether (60 to 80°C) in a Soxhlet apparatus and the defatted material was used for extraction of polyphenols.

Extraction of polyphenols

The defatted SCF (1 g) was suspended in 100 ml of different polar (methanol, ethanol, propanol, water and acetone) and non – polar (ethyl acetate, chloroform, benzene and diethyl ether) to extract the polyphenols. In another set of experiment, the polar solvents were acidified with 1% HCl and the extraction was carried out by refluxing for about 60 min using a water bath. The individual extracts were

centrifuged (Research centrifuge, R 24, Remi Instruments, India) at 5000 rpm for 20 minutes and the clear supernatant was collected. The residue was re-extracted with 50 ml of fresh solvent and the process was repeated till the residues tested negative (with Folin – Ciocalteu's phenol reagent) for polyphenols. The extracts were pooled, concentrated under reduced pressure using a rotary flash evaporator, freeze-dried and used for the studies.

Determination of total phenolics content

The total phenolic contents of the extracted material was assayed following Folin - Ciocalteu's (FC) phenol reagent (Singleton et al., 1995) and expressed as gallic acid equivalent. To an aliquot of the extract, 15 ml of 20 % sodium carbonate solution was added, mixed well, and after 15 min, 5 ml of FC reagent [2 (N) Folin-Ciocalteu's phenol was diluted with distilled water in the ratio 1: 2 (v/v)] was added and the reaction mixture incubated for 30 min at room temperature. The contents were diluted to 100 ml with distilled water and the absorbance was measured at 760 nm using a UV – Visible Spectrophotometer (UV - 1601, Shimadzu Corporation, Kyoto, Japan).

To elucidate the possible similarities between the polyphenols of the different extracts and some of the pure phenolics commonly present in cereals, the absorption spectra were determined. Different phenolic standards were scanned in a range of 200 to 900 nm wavelengths using a UV – Visible Spectrophotometer (UV - 1601, Shimadzu Corporation, Kyoto, Japan) and the λ_{max} of each of the phenolics were recorded. The extracts from different solvents were also scanned similarly and the λ_{max} were matched with the corresponding phenolic standards.

Identification and quantification of phenolic compounds

The lyophilized polyphenols extract dissolved in water was membrane (0.45 µ) filtered and an aliquot (20 µL) of the filtrate was fractionated in a reverse phase HPLC system [Shimadzu (Kyoto, Japan)], LC-8A integrated system controller, a Spherisorb C-18 reverse-phase column (250 × 4.6 mm; ODS 2; 5 µm particle size), Waters corp., Massachusetts, USA and a Helwlett Packard 1040 UV diode array detector with an attached HP analysis computer and data storage system] in to the constituent phenolics. The gradient elution schedule was standardized based on the resolution of the sample, with 50 min run of 15% methanol and 1% of acetic acid in water followed by a linear gradient to 40% methanol over 40 min at a flow rate of 1 $mL \cdot min^{-1}$. Elutes were detected by a Waters 2487 dual wavelength detector at 295 nm and the peaks were recorded. Scanning was performed from 200 to 600 nm. The constituent phenolic compounds were identified by comparing the retention times and also the UV–visible spectra of the pure standards to indicate the preparations of standards and the range of calibration curves. The analyses were replicated (n = 3) and the contents given as mean values, plus or minus the standard deviation. The results were expressed as milligrams of each compound per 100 g of dry weight.

Assay of antioxidant activities

Reducing power

The reducing power was determined according to Yen and Duh (1993). An aliquot of the extracts containing 1 mg/mL polyphenols from different solvents were mixed with 5.0 mL phosphate buffer (2.0 M, pH 6.6) and 5 mL 1% potassium ferricyanide (1 %) 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 colour developed was measured at 700 nm.

Total antioxidant potentiality

According to Prieto et al. (1999), to 20 µL of the extract 1.23 mL of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) was added and the contents 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 (M / 100 g of extract) equivalent.

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 to 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;

$$\text{Radical scavenging activity (\%)} = \{(Abs_{control} - Abs_{sample}) / Abs_{control}\} \times 100$$

Where, $Abs_{control}$ = absorbance of DPPH radical + methanol; Abs_{sample} = absorbance of DPPH radical + sample extract.

The scavenging activity was expressed as µmol DPPH radical scavenged /g 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 concentrations, 2.5 mM phosphate buffer (pH 7.4), 31.7 µL of freshly prepared H_2O_2 (88.3 mM), 37.2 µL of EDTA (2.68 mM), 6.5 µL of $FeCl_3$ (6.152 mM), 17.6 µL of ascorbic acid (5.68 mM) and 75 µL of deoxyribose (74.65 mM) were added and incubated at 37°C under dark for 90 min. To the mixture 1 mL of TBA (49 mM) and 1 mL of TCA (153 mM) were mixed and left in boiling water bath for 10 min, cooled rapidly to room temperature, the absorbance was measured at 532 nm and the hydroxyl radical scavenging activity was calculated as follows:

$$\text{Scavenging activity (\%)} = [A_0 - (A_1 - A_2)/ A_0] \times 100$$

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 radical scavenging assay

Radical scavenging capacity of the polyphenols was evaluated against the ABTS according to the protocol of Auddy et al. (2003). The ABTS radical was prepared by oxidizing 10 ml of 7 mM aqueous solution of ABTS [2, 2'- azinobis (3 – ethylbenzothiazoline – 6 – sulfonate)] with 10 ml of 2.45 mM ammonium persulphate [APS], mixed well and incubated at 23°C in dark for 16 h. Then 680 µl of ABTS radical solution (3.5 mM) was diluted to 10 ml with 10 mM phosphate buffer saline [PBS] at pH 7.4, so as to obtain an

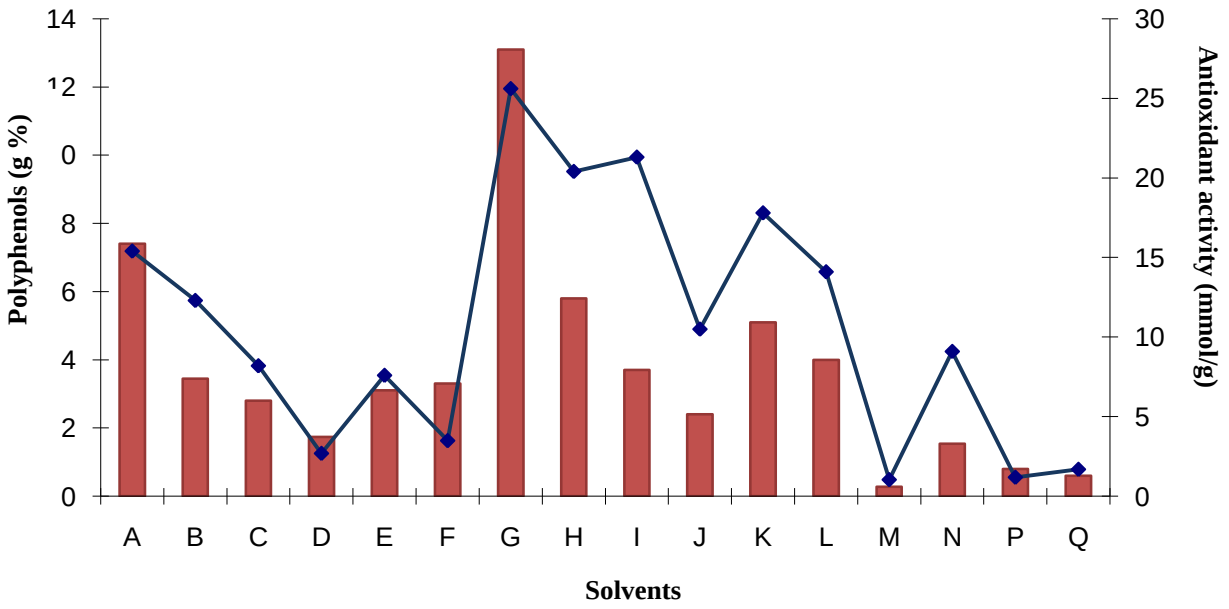


Figure 1. Total polyphenols (g %) content extracted from different solvents and its total antioxidant activity. A, methanol; B, ethanol; C, propanol; D, butanol; E, acetone; F, water; G, HCl – methanol; (H) HCl – ethanol; (I) HCl – propanol; (J) HCl – butanol; (K) HCl – acetone; L, HCl – water; M, diethyl ether; N, ethyl acetate; P, benzene; Q, chloroform.

absorbance of 0.700 ± 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 of 10 mM PBS (pH 7.4) 230 µl ABTS radical solution (0.238 mM) was added and mixed vigorously and after 6 min 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:

Inhibition (%) = (Absorbance $_{(control)}$ – Absorbance $_{(sample)}$ / Absorbance $_{(control)}$) × 100

Antibacterial activity

The agar well diffusion assay was used to investigate antibacterial effects of phenolic compounds. The assay was carried out according to the method of Murray et al., (1995) with slight modification using Mueller Hinton agar. Following organisms used for antibacterial assay; *B. cereus, Pseudomonas aeruginosa, Y. enterocolitica, Proteus vulgaris, Listeria monocytogen, Staphylococcus aureus, Streptococcus pyogenes, Escherichia coli, Klebsiella pneumoniae, Shigella sonnei* and the cultures were maintained on Nutrient agar. A loopful of organisms was pre-cultured in 10 mL nutrient broth for 6 hours. The turbidity of the culture was adjusted to 0.5 McFarland optical density. From the seed culture 0.1 mL of bacterial suspension was inoculated on Mueller Hinton agar plates by spread plated method. Using sterile cork borer wells were made (6 to 9 mm) on agar and an aliquot (50 µg/mL) of individual isolated fractions of phenolics were placed into wells and the plates were incubated for 24 h at 37°C. The test was carried out in triplicate and anti-bacterial activity was measured as clear zone of diameter (mm) formed due to inhibition of growth. Oxacillin (20 µg/mL) and norfloxacin (200 µg/mL) were used as positive controls for gram-positive and gram-negative bacteria respectively. Methanol was included in every experiment as solvent control.

Minimum inhibitory concentration determination

Minimum inhibitory concentration of the phenolic fractions against food borne pathogens was determined according to the method of Vairappan (2003), with slight modification. The fractions were diluted in 200 µL Methanol to different concentration and the diluted fractions were mixed with the desired bacterial strains cultured in 9.8 mL of nutrient broths (10^4 Colony forming unit). 200 µL of Methanol was used as solvent control. Bacterial growth was monitored turbidometrically at 4, 8, 12, 16, 20 and 24 h and quantified as colony forming unit (CFU) by plate count method.

Statistical analysis

All the analyses were performed in triplicate and the data calculated as mean ± standard deviations. The mean of all the parameters were examined for significance by analysis of variance (ANOVA) and in case of significance, mean separation and comparison was accomplished by Duncan's multiple range test (DMRT) using STATISTICA software.
P values (< 0.05 and 0.01) were regarded as significant and very significant, respectively. Analysis of variance was adopted to test the differences in the antioxidant activity by various assays.

RESULTS AND DISCUSSION

Extraction of polyphenols

The yield of the extracted matter from the millet seed coat in different polar solvents differed considerably among the solvents (Figure 1). The yield of polyphenols extracted in methanol was highest (7.4 ± 1.1 g %), followed by ethanol (3.45 ± 0.4 g %), acetone (3.1 ± 1.1 g

Table 1. Anti-microbial activity of the phenolics against some of the pathogenic bacteria.

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

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

%) and butanol (1.74 ± 0.2 g %). On the other hand, among the different non – polar solvents, the yield of the phenolics extracted with ethyl acetate was highest (1.54 ± 0.3 g %) and with diethyl ether least (0.28 ± 0.02 g %) among the different solvents tried. The acidified methanol extract contained 13.1 ± 0.02 g % polyphenols followed by the extract of the acidified ethanol (5.8 ± 1.4 g %). This indicates that, the extraction of the millet polyphenols in acidified polar solvents is more effective than the pure solvents. This could be due to cleaving of inter-molecular bonds, because of the acidic conditions that enables better extraction. Polar solvents due to their polarity are more useful for extracting polyphenols from the protein matrices, since they degrade the polyphenols–protein complexes. Variations in the yields and their phenolic contents of various extracts are attributed to the polarities of the phenolics present in the seeds. Extraction of phenolics from any of the natural material depends on the solubility of their polyphenols (Naczk and Shahidi, 2006). Such differences have been reported for other cereals also (Ragaee et al., 2006).

Spectrophotometric analysis of phenolics in different extracts

Most of the polyphenols exhibited two major absorption maxima; one in the range of 240 to 285 nm (band II – due to the absorption of benzoyl system in ring A) and the other in the range of 300 to 400 nm (band I – the result of absorption due to cinnamoyl system of ring B).

The absorption maximum for the simple phenolics lies between 220 and 280 nm, and it is largely influenced by the pH of solvent employed. The UV and visible spectroscopic techniques are often used for identification of extracted phenolic compounds, particularly flavonoids as well as other groups of predominant phenolic compounds. However, the possibility of interference by UV absorbing substances such as proteins, nucleic acids and amino acid also exists.

The λ_{max} and the absorption (O.D) of the polyphenols extracted in different solvents presented in Table 1 reveals that the λ_{max} of the phenolics in pure solvents was only around 220 nm or even lesser, but in acidified solvents, the phenolics exhibited λ_{max} in the range of 220 to 280 nm. Among the different solvents used for extraction, HCl – methanol extract shows the highest number of peaks in range of 200 to 600 nm indicating the complex number of constituent phenolics than the other solvent extracts, and the extracts from acidified polar solvent contained higher number of constituent phenolics than the pure polar as well as the non-polar solvents.

The different λ_{max} could be attributed to the number, position and substitution of hydroxy phenolic groups as well as intermolecular hydrogen bonding and steric interactions. The phenolic group in a specific compound shows different types of resonance interactions in the exited state of the molecule because of the replacement of the phenoxide or the phenolate (only in basic media) ion that reflected in the maximal wavelength (Dearden et al., 1959). It may be noted that, the acidified methanolic extract contained higher number of constituent phenolics.

Composition of phenolics

Normally, the profile of the phenolics is species-specific and typical for most plant material (Maillard and Berset 1995). This was true for the millet also as the profiles of the phenolics in the extract did not differ significantly between different solvents. The baseline resolution of these compounds was achieved under the

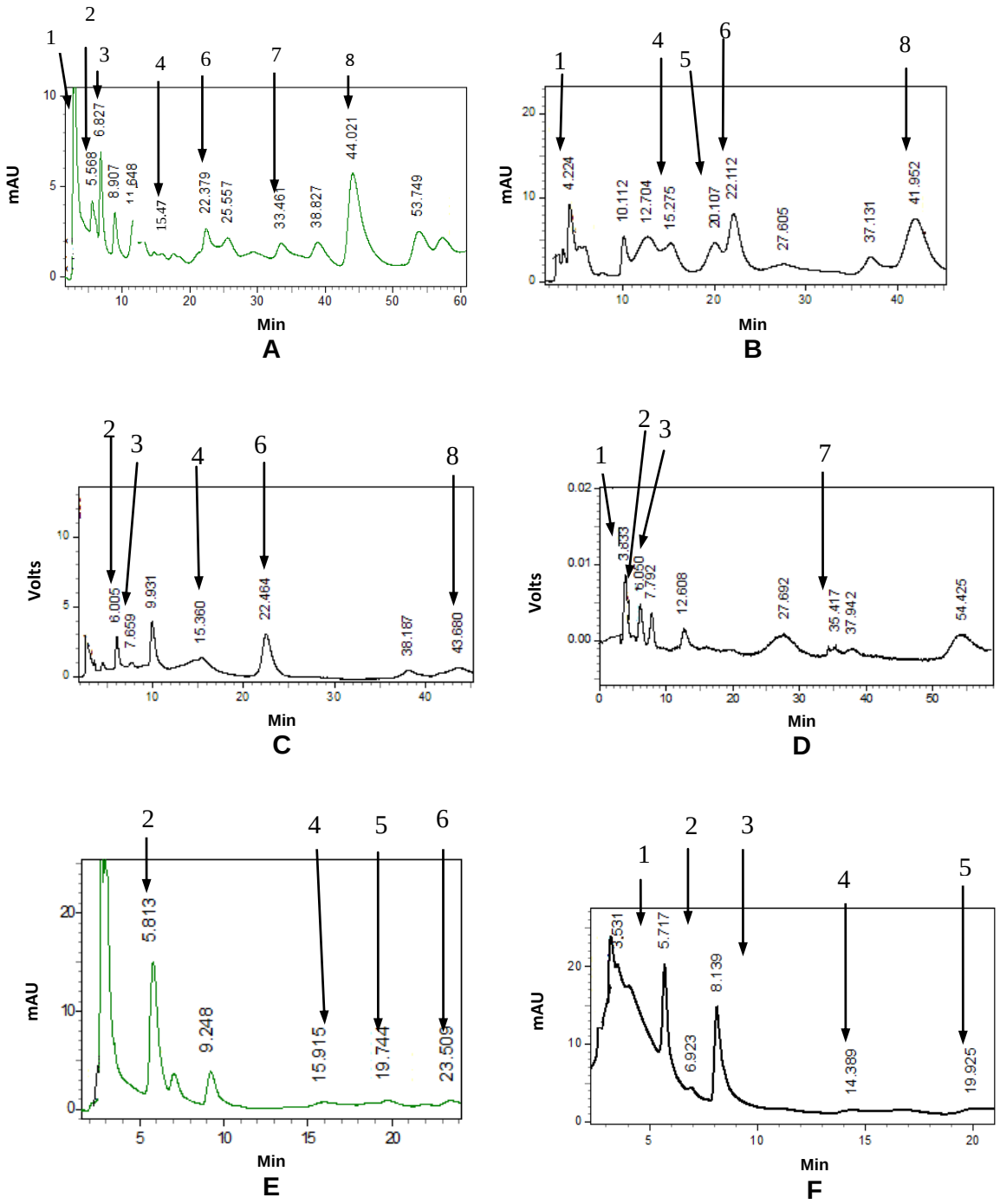
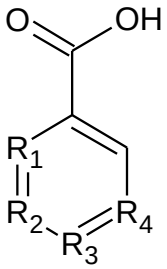


Figure 2. Fractionation of the millet polyphenols extracted in different solvents. Peak numbers: 1, gallic acid; 2, proto-catechuic acid; 3, p-hydroxy benzoic acid; 4, p-coumaric acid; 5, syringic acid; 6, ferullic acid; 7, trans-cinnamic acid; 8, quercetin. A, HCl – methanol; B, HCl – ethanol; C, HCl – propanol; D, HCl – acetone; E, HCl – water; F, ethyl – acetate.

chromatographic conditions described and the identities of the different peaks were confirmed from retention data for authentic standards.

The constituent phenolics extracted with different solvents varied significantly. The phenolic compounds identified were the hydroxybenzoic acid derivatives namely, gallic, protocatechuic acid, *p*-hydroxy benzoic and syringic acids and the hydroxycinnamic acid derivatives such as ferulic, chlorogenic and coumaric acids (Figure 2). The phenolic structures identified are



Hydroxybenzoic acid

Gallic acid: R_1=H; R_2=R_3=R_4=OH

Gentisic acid: R_1=R_2=R_3=R_4=OH

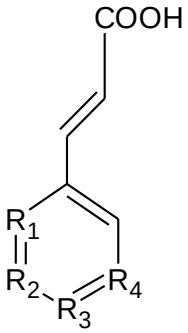
p-hydroxy benzoic acid: R_1=R_2=R_4=H; R_3=OH

Salicylic acid: R_1=OH; R_2=R_3=R_4=H.

Protocatechuic acid: R_1=R_4=H; R_2=R_3=OH

Syringic acid: R_1=H; R_2=R_4=OCH_3; R_3=OH

Vanillic acid: R_1=R_4=H; R_3=OH; R_2=OCH_3



Hydroxycinnamic acid

p – coumaric acid: R_1=R_2=R_4=H; R_3=OH

Ferulic acid: R_1=R_4=H; R_2= OCH_3; R_3=OH

Sinapic acid: R_1=H; R_2=R_4= OCH_3 ; R_3=OH

Figure 3. Structures of some important phenolic acid derivatives found in millet polyphenol extract.

presented in Figure 3. It was found that coumaric acid and ferulic acid were the most common phenolics present in almost all the extracts. In case of acidified ethanolic extract, gallic acid was present along with *p*-coumaric, syringic, ferulic acids and quercetin. Proto-catechuic, *p*-coumaric, syringic and ferulic acids were the major components in acidified propanol and water but their quantity varied respectively. Ethyl acetate extracted maximum phenolics among non-polar solvents, and among them gallic and proto-catechuic acid were prominent. From this, it may be inferred that, polar solvents extract both hydroxybenzoic and hydroxycinnamic acid derivatives along with the flavonoids but the non-polar solvents extracted mainly hydroxybenzoic acid derivatives. However, a good number of peaks remained unidentified, which could be flavonoids, anthocyanins and also anthocyanidins besides phenolics.

According to McDonough and Rooney (2000), ferulic, *p*-coumaric and cinnamic acids are the major phenolics in finger millet. According to Subba and Muralikrishna (2002); nearly 70% of finger millet phenolic acids are free and 30% in bound form and ferulic acid (18.60 mg/100g) is the major bound, whereas protocatechuic acid (45.0 mg/100g) is the major free phenolic acids. According to Chandrasekara and Shahidi (2011) hydroxycinnamic acids and their derivatives constitute the insoluble bound fractions whereas flavonoids are present in free phenolics. Kodo millet had the highest total phenolic content, whereas proso millet contains the least. Ferulic and p-coumaric acids present in higher amount in the bound fractions compare to the soluble phenolics (Chandrasekara and Shahidi, 2010).

Antioxidant activity

Reducing power activity

It is reported that the antioxidant activity of reductones 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 (Gordon, 1990). 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 inves-tigated 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, 1997).

The reducing powers of millet polyphenols extracts from different solvents assayed following potassium ferricyanide method at various concentrations are presented in Table 2. From that, it could be seen that, at any given concentration (between 1.25 to 10 mg) the

Table 2. Reducing power assay of the polyphenols at different concentrations

Solvent	Concentration (mg/ml phenolics)				
	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

Absorbance at 700 nm. Mean values ± standard deviations (n = 3).

activity was highest in the methanolic extract followed by acetone and ethanol extracts among the polar solvents and it was highest in ethyl acetate and least in diethyl ether among the non-polar solvents.

Total antioxidant activity

Among the various extract the total antioxidant activity of the methanolic extract was higher (25.6 mmol/g) than the extracts from ethanol (21.3 mmol/g) and acetone (20.4 mmol/g) (Figure 1). The millet extract in acidified polar solvents showed higher antioxidant activities (between 14.1 to 25.6 mmol/g seed coat) compared to non – acidified polar solvents (between 3.5 to 15.4 mmol/g). 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 differences in their phenolic contents and, it has also been reported that the solvents used for extraction 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 acid and their derivatives.

DPPH radical scavenging activity

The DPPH radical-scavenging activity ($P < 0.05$) of the millet polyphenols extracts using different solvent system. Acidified methanolic extract at all the concentrations was higher than other extracts. 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 and 200 µg/mL. The IC_{50} values for acidified methanolic, ethanolic and acetone extracts were 63, 92 and 112 µg/mL, respectively. Very less activity was observed in case of non-polar solvent extracts and among them diethyl ether was the 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 industry. This indicates that, the millet polyphenols posse's 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 ($O^{\bullet}H$) or its phenoxide anion ($O_2^{\bullet -}$) to $DPPH^{\bullet}$

(i) $OH + DPPH\ radical \rightarrow O^{\bullet} + DPPH$

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

The contribution of one or the other pathway depends on the nature of the solvent 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 is predominant, but in polar solvents such as methanol or ethanol the electron transfer mechanism becomes important as it forms strong hydrogen bonds with the phenol 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. 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). 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).

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 in DPPH reaction. Among the polar solvents, the degree of inhibition was highest from the methanol extract with IC_{50} value of 190 µg/mL and least in butanol with IC_{50} value of 1234 µg/mL. The extract from the acidified methanol showed higher antioxidant properties (109 µg/mL, IC_{50} value) compared to the extract from pure methanol (190 µg/mL, IC_{50} value). This could be due to the breaking of the intermolecular bonds of the complexes and facilitated enhancement of extraction of phenolics. The non-polar solvents showed very little free radical scavenging activity compared to polar solvents. The ability of the millet polyphenols extracts to quench reactive hydroxyl radical species has potential application to extend shelf-life of food products (Yuan et al., 2005).

ABTS scavenging activity

The ABTS scavenging activity of millet seed coat polyphenols extract in different solvents indicates that the acidified methanolic extract showed the highest scavenging capacity (114 ± 1.8 µg/mL) towards quenching of $ABTS^{\cdot+}$ followed by ethanol (170 ± 1.5 µg/mL) and acetone (205 ± 2.6 µg/mL). The $ABTS^{\cdot+}$ scavenging activity of extracts of the non-polar solvents were less compared to polar solvents (4408 ± 3.6 to 20329 ± 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 cations under these systems. The results suggest that phenolic compounds in the millet may be able to inhibit the excessive formation of free radicals 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 effect of the concentration of antioxidant compounds on the DPPH radical as well as the deoxyribose scavenging activity and the ABTS radical scavenging potentials was observed even at a very low concentration (10 to 200 µg/mL). The free radical scavenging activity (IC_{50} values) increased with increasing concentration of extract in all the experimental parameters indicating the concentration dependency of the polyphenols for the antioxidant activity (Figures 4a to c). However, the methanolic extracts exert less radical scavenging activity compared to standard antioxidant (BHA). This present finding corroborates well with earlier reports on other higher plants (Kumaran and Karunakaran, 2007) and their enzymatic extracts (Park et al., 2004).

The *in vitro* antioxidant capacity of the millet polyphenols is mainly due to the presence of hydroxycinnamic and benzoic acid derivatives, as well as other flavonoids and such other compounds. These are largely present as soluble esters and also a small proportion of free acids; hence are easily extracted in acidic polar solvents (Andreasen et al., 2001a).

According to Adom et al. (2002), the antioxidant activity of any of the compounds is normally dose-dependent. 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., 2001b) and both the ester - linked and the free soluble phenolic acids may exert some antioxidant effect in the luminal side of the intestinal tract.

The antioxidant activity of polyphenols depends on the structure and substitution pattern of hydroxyl group. 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 structure and it increases the radical scavenging activity.

Reducing power of the millet polyphenols was concentration dependent and increased with increasing concentration between 1.25 to 10 mg/mL, in all the samples. Same trend has also been reported by Kumaran and Karunakaran (2007), in methanolic extracts

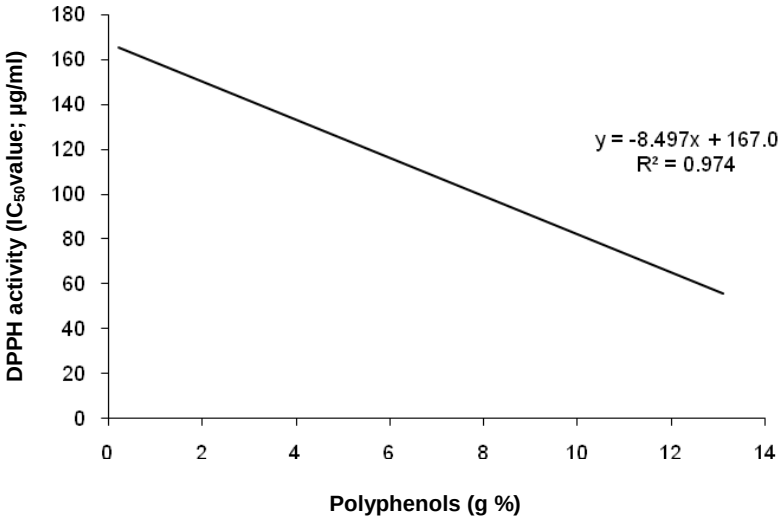


Figure 4a. Comparison of polyphenol content and DPPH activity.

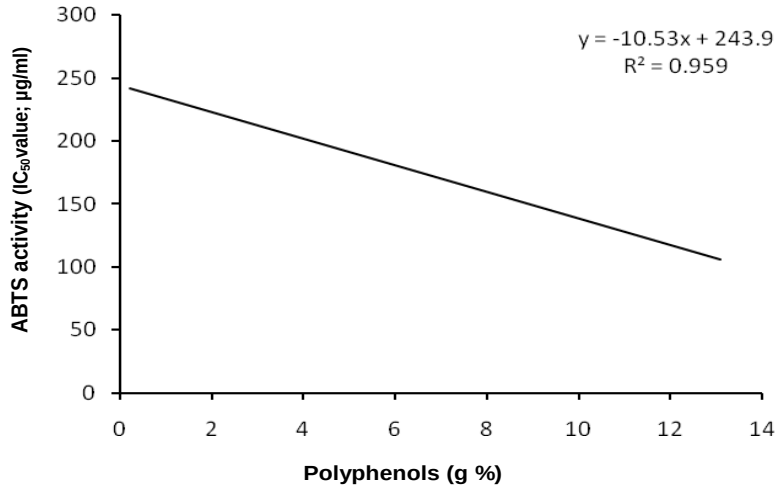


Figure 4b. ABTS radical scavenging activity and polyphenol concentration.

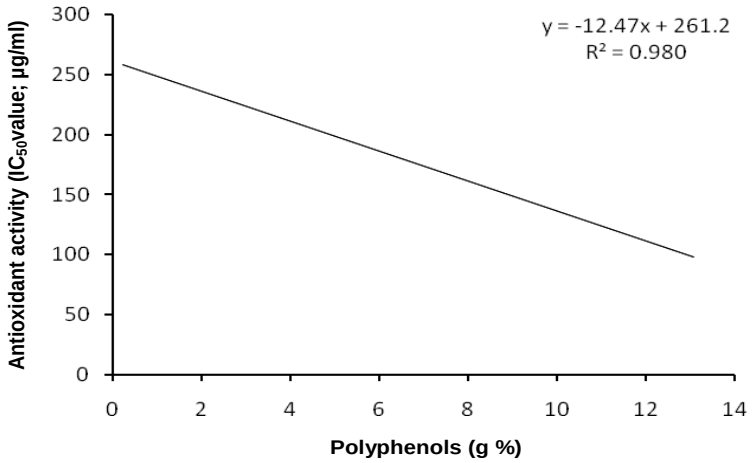


Figure 4c. Radical scavenging activity and polyphenol content.

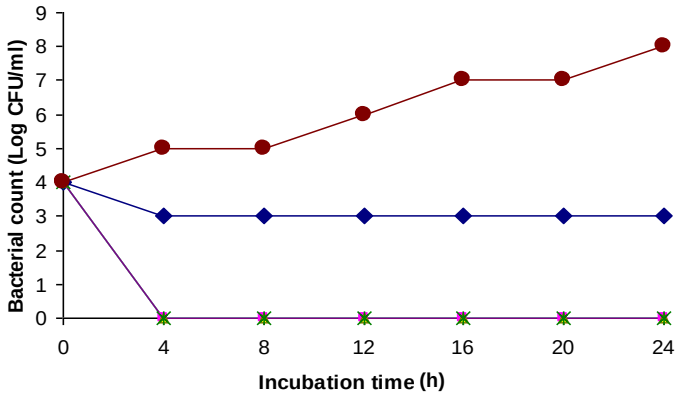


Figure 5a. Viability of *B. cereus* as a function of time in various concentration of crude phenolic extract [Control (●), 25 µg/ml (◆), 50 µg/ml (■), 100 µg/ml (▲), 200 µg/ml (X), 400µg/ml (Ж)].

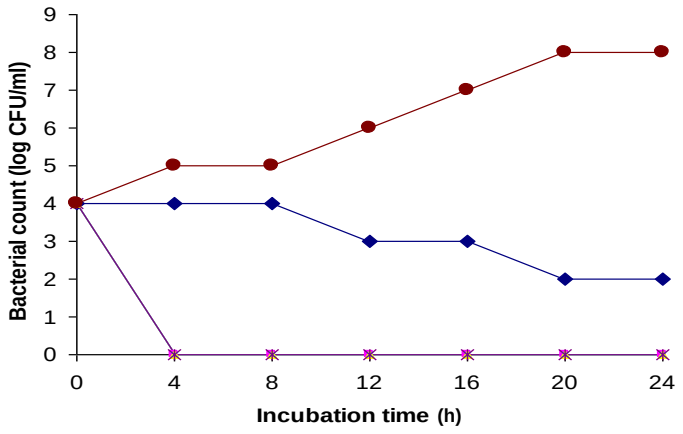


Figure 5b. Viability of *Y. enterocolitica* as a function of time in various concentration of gallic acid fraction [Control (●), 25 µg/ml (◆), 50 µg/ml (■), 100 µg/ml (▲), 200 µg/ml (X), 400 µg/ml (Ж)].

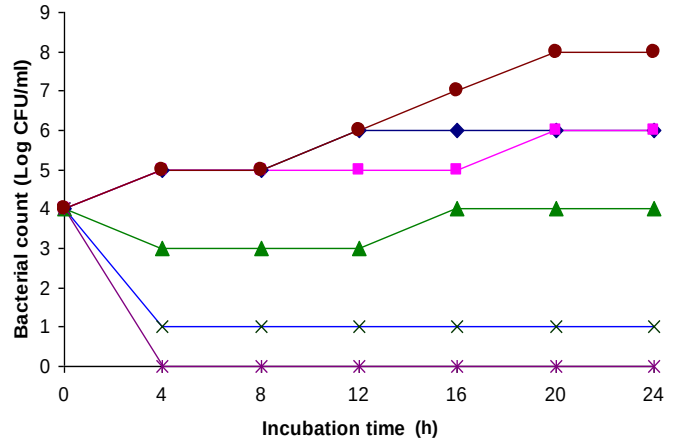


Figure 5c. Viability of *E. coli* as a function of time in various concentration of quercetin fraction [Control (●), 25 µg/ml (◆), 50 µg/ml (■), 100 µg/ml (▲), 200 µg/ml (X), 400 µg/ml (Ж)].

of higher plants. Also, it was observed that at any given concentration acidified methanolic extracts of millet phenolics had higher reducing power than other solvents. This property is associated with the presence of reductones that are reported to be terminators of free radical chain reaction. These observations reveal the potent antioxidant activity of finger millet polyphenols. Hence, the information may be useful in understanding the health benefits of the millet.

Antibacterial activity of fractionated phenolics

The agar diffusion bioassay of crude and fractionated phenolics showed considerable activity against all gram-positive bacteria compared to gram-negative bacteria. The screening of the isolated compounds for their antibacterial activity of gallic acid, protocatechuic, *p*-hydroxybenzoic acid, vanillic acid, *p*-coumaric acid, syringic acid, ferulic acid, trans-cinnamic acid and quercetin indicated the different level of inhibition at different concentrations (Table 1). Quercetin inhibited the growth of all the bacteria strains used whereas gallic, ferulic, proto-catechuic, *p*-hydroxy benzoic acids confined activity only to a few bacterial strains, namely *E. coli*, *B. cereus*, *S. aureus*, *Y. enterocolitica* and *L. monocytogenes.*

Investigation was further carried out to determine minimum inhibitory concentration (MIC) of ployphenolic fractions. Viability test was conducted on five food borne pathogens: *E. coli*, *B. cereus*, *S. aureus*, *Y. enterocolitica* and *L. monocytogenes* using three polypenolic fractions (Crude, Gallic acid, Quercetin) which showed highest inhibition zones in agar well diffusion studies. All three polyphenolic fractions showed minimum inhibitory concentration in the following tendency; *B. cereus*, *L. monocytogenes* and *Y. enterocolitica* (50 µg/mL) < *S. aureus* (200 µg/mL) < *E. coli* (400 µg/mL). Figures 5a to c show the results of time course experiments carried out to determine MIC value for pigment against five food borne pathogenic organisms. The results showed that as the concentration of the fractions increases the viability of the bacterial strains decreases gradually. Organisms like, *L. monocytogenes*, *Y. enterocolitica*, *B. cereus* shown drastic decrease in viability at lesser concentration of fractions (50 µg/mL) where as in *S. aureus, E. coli* decrease in viability was found at higher concentration of fractions (200 and 400 µg/mL, respectively).

It has been reported earlier that, the composition of finger millet comprises flavonoids such as orientin, isoorientin, vitexin, isovitexin, saponarin, violanthin, lucenin-1, and tricin (Hilu et al., 1978). McDonough et al. (1986), identified ferulic (405 µg/g), coumaric (67 µg/g), gentisic (53 µg/g), cinnamic (35 µg/g), caffeic (15 µg/g), vanillic (15 µg/g), protocatechuic (14 µg/g), p-hydroxy benzoic (09 µg/g), syringic (07 µg/g) and sinapic acids (04 µg/g). Similarly, Sripriya et al. (1996), reported catechin as the major phenolic constituent, whereas,

Subba and Muralikrishna (2002), identified gallic, vanillic, coumaric and ferulic acids as free phenolics, and ferulic, caffeic and coumaric acids as bound phenolic acids. Phenolic acids may form both ester and ether linkages owing to their bi-functional nature through their carboxylic and hydroxyl groups, respectively. Bound phenolics may be released by alkali, acid, or enzymatic treatment of samples prior to extraction (Sosulski et al., 1982). Phenolic acids are mainly located in the outer layers (husk, pericarp and aleurone) of the grain and ferulic acid is the predominant free phenolic acid in barley seeds (Nordvisk et al., 1984). Besides the phenolic acids are also found abundantly in cell wall 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). Phenolic acids and flavonoids are generally present in cereals in the free and conjugated forms. Although, highest concentration of phenolic acids and flavonoids is in thealuerone layer of cereal grains, these compounds are also found in substantial amount in the embryo and seed coat of grains (Shirley, 1998). Thus cross-linking of phenolic compounds may provide physical barrier to invasive disease development and consumption by insects (Zupfer et al., 1998). In barley, most of the phenolic acids exist in the bound form with other grain components such as starch, cellulose, β-glucan and pentosans (Yu et al., 2001). The increased concentration of ferulates in the outer layers may be to offer resistance to both insect and fungal pathogens. On the other hand, p-hydroxybenzoic acid is the major bound phenolic acid detected in barley extracts obtained by sequential treatment of grain with acid, α-amylase and cellulase (Yu et al., 2001).

Phenolic acids are known to contribute to the anti-oxidative potential of grains (Maillard and Berset, 1995; Adom et al., 2003) and may also be used for the extension of shelf-life of cereal products (Pussayanawin et al., 1998). Grains with elevated levels of phenolic acids in caryopsis exhibit greater resistance to disease and insect (Arnason et al., 1992) but exhibit reduced extractability of endosperm (Pussayanawin et al., 1998). 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, 1993). Significant amounts of alk(en)yl resorcinols containing non-isoprenoid side chain (15 to 25 carbons in length) attached to the hydroxybenzene ring have also been detected in some cereals (barley, rye, wheat) (Ross et al., 2003). These compounds are also located in the outer layers of grains and displayed antioxidant as well as membrane modulating effects (Rauha et al., 2000).

Very clear differences with respect to the inhibitory activities were found among the phenolic constituents in the extracts. Quercetin inhibited the growth of all the prokaryotic species studied (*S. aureus, E. coli, B. subtulis, M. luteus and P. aeruginosa*) (Rauha et al., 2000). Gallic acid was shown to exhibit higher antibacterial activity than vanillic and caffeic acids (Yildirim et al., 2001).

Rodriguez et al. (2007) reported that gallic acid shows higher inhibitory activity than protocatechuic acid which could be due to the extra hydroxyl group present in gallic acid. The site(s) and number of hydroxyl groups on the phenol group are thought to be related to their relative toxicity to microorganisms, with evidence that increased hydroxylation results in increased toxicity (Geissman, 1963). The mechanisms thought to be responsible for phenolic toxicity to microorganisms include enzyme inhibition by the oxidized compounds, possibly through reaction with sulfhydryl groups or through more non-specific interactions with the proteins (Mason and Wasserman, 1987). Flavonoid activity is probably due to their ability to complex with extracellular and soluble proteins, and to complex with bacterial cell walls; thus, inhibiting microbes.

Conclusion

The acidic methanol solvent happens to be an effective extractant of the finger millet polyphenols compared to many other polar and non–polar solvents. The antioxidants activities of the millet polyphenols indicated a strong positive association ($r > 0.9$, $p < 0.01$) with free radical scavenging activities, against ABTS and DPPH radicals and also on the total antioxidant activity and reducing power. The very low IC_{50} values for the millet phenolics for the antioxidant activity reveal the potentials of the millet as a source of natural antioxidant. Hence, the information generated under the present study useful to undertake further research work towards identification, isolation and characterization would be the specific bioactive compounds responsible for higher antioxidant activity and reveals that nutraceutical potentials of finger millet polyphenols.

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